Biochemical Characterisation of the Enzymatic Activity of Chloride Intracellular Ion Channel Proteins

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I declare that this dissertation has been written by me, and all information sources used are fully acknowledged within the text. This thesis is being submitted for the degree of Master of Science in the University of Technology Sydney. In addition, I certify that it has not been submitted for any other degree or examination at any other University. I acknowledge the Australian government for RTP scholarship "This research is supported by an Australian Government Research Training Program Scholarship".

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DEDICATION

I would like to dedicate this thesis to my family for all of their endless love and support

First and foremost I would like to sincerely thanks my parents, Mr. Mahdi Moghaddasi and Mrs. Saja Tayyeb for all their years of love and encouragement. I am greatly thankful for all they provided throughout my life to make me the person who I am today. Thank you both for giving me guidance, faith and strength to chase my dreams.

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List of Abbreviations

ΔG..... free energy of activation
ΔH..... standard enthalpies
λ ..... Wavelength
A..... Ascorbic acid
A9C..... Anthracene-9-carboxylic acid
ATP ..... adenosine triphosphate
AFR ..... ascorbate free radical
Ala ..... Alanine amino acid
Arg..... Arginine amino acid
Asp ..... Asparagine amino acid
BSA ..... Bovine serum albumin
ºC..... Degree celsius
$CaCl_2$ ..... Calcium chloride
CFTR ..... Cystic fibrosis transmembrane conductance regulator
CHO-K1..... Chinese hamster ovary cells
CIC ..... Chloride ion channel
Cl- ..... Chloride ion
CLIC1 (WT)..... Chloride intracellular ion channel protein (wild- type)
Cys..... Cysteine amino acid
DHA ..... Dehydroascorbate
DmCLIC..... Drosophila- melanogaster CLIC protein
DNA ..... Deoxyribonucleic acid
E-coli..... Escherichia coli
Eact..... active enzyme
Einact..... inactive enzyme
ES..... enzyme and substrate complex
ER ..... Endoplasmic reticulum
Ero1 ..... ER oxidoreductin 1
E ..... total enzyme concentration
G-site..... Glutathione binding site
Grx..... Glutaredoxin
Grx-1, 2 to 5 ..... Glutaredoxin-1, 2 to 5
GR..... Glutathione reductase

GSH ..... Reduced glutathione

GSSG ..... Oxidised glutathione

GST ..... Glutathione-S-transferase

GST-β ..... Glutathione-S-transferase beta class

GST-Ω ..... Glutathione-S-transferase omega class

GST-Ω1 ..... Glutathione-S-transferase omega group 1

H-site ..... Hydrophobic region

H_2O_2 ..... Hydrogen peroxide

HcTrx-5 ..... Thioredoxin-related protein in Haemonchus contortus

HEDS ..... 2-hydroxyethyl disulfide

HCl ..... Hydrochloric acid

HEPES ..... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

His ..... Histidine

IAA ..... Indanyloxyacetic acid

IUBMB ..... The International Union of Biochemistry and Molecular Biology

IPTG ..... Isopropyl-β-thiogalactopyranoside

K^+ ..... Potassium ion

KCl ..... Potassium cholride

K_m ..... Dissociation constant of the enzyme-substrate complex

K_d ..... equilibrium constant

k_{cat} ..... catalytic rate constant

KH_2PO_4 ..... Mono-potassium phosphate

$K2HPO_4$ ..... dipotassium phosphate

M ..... Molar

MW ..... molecular weight

mg ..... Milligram

min ..... Minute

mM ..... Millimolar

NaCl ..... Sodium chloride

NADH ..... Nicotinamide adenine dinucleotide (NAD) + hydrogen(H)

NaN_3 ..... Sodium Azide

Na_2SeO_3 ..... Sodium selenite

NCC27 ..... Nuclear chloride channel protein-27kDa

NTA ..... nitrilotriacetic acid

Ni^{2+} ..... Nickel ion

NTA ..... Nitrilotriacetic acid

N-domain ..... Amino terminal domain

nm ..... Nanometer

Ox ..... oxidized state

OD ..... Optical density

P64 ..... Bovine chloride channel protein -64kDa

PBS ..... Potassium buffered saline

Phe ..... Phenylalanine amino acid

Pro ..... Proline amino acid

P ..... product

pKa ..... measure of acid strength

PDI ..... protein disulfide isomerase

ROS ..... Reactive oxygen species

rPsGrx ..... Glutaredoxin protein from antarctic sea-Ice

S.E ..... Standard error

SEC ..... Size exclusion chromatography

$SeO(OH)_2$ ..... Selenite

$SeO_2(OH)_2$ ..... Selenate

Ser ..... Serine amino acid

S ..... substrate

SDS ..... Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TCEP Tris .....(2-carboxyethyl)phosphine

Triton-X100 ..... Octylphenyl-nonaoxyethylene

$Trx(SH)_2$ ..... reduced thioredoxin

Trx-1 ..... Thioredoxin reductase

TrxR ..... Thioredoxin reductase

Trxs ..... Thioredoxins

Tyr ..... Tyrosine amino acid

µg ..... Microgram

µM ..... Micromolar

UV ..... Ultraviolet

V_0 ..... reaction rate

V_{max} ..... maximal reaction rate

WT ..... WT

X ..... Any amino acid

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ABSTRACT

The chloride intracellular ion channel (CLIC) family, are the group of unusual proteins that exist either in monomeric soluble or integral membrane form. This family is comprised of six protein members in humans, CLIC1-CLIC6. CLIC1 is classified as a "metamorphic" protein, which means it can exist in two stable tertiary conformations. The CLIC proteins can spontaneously insert into phospholipid membranes from their soluble state where they act as ion channel proteins. In addition, the CLIC proteins have structural similarities with both the Glutathione-S-Transferases and glutaredoxin enzyme families. Recently it has been demonstrated by *in vitro* assay systems that the monomeric CLIC proteins have similar "oxidoreductase" activity to the Glutaredoxin family. Therefore, following on from these new discoveries, further detailed characterization of their enzymatic activity was required, which was the main purpose of this research project.

The first objective of this thesis was to determine the important structural regions of CLICs that could be sensitive to the various environmental conditions. In that regards, a comparative study was conducted on both "Histidine tagged" and "non-tagged" CLIC proteins in order to check whether the "6 Polyhistidine tag" and "imidazole" compound that are routinely used in the preparation and purification of the recombinant CLIC proteins, interferes with the functional activity of CLIC1 and CLIC3 proteins. Indeed, the results indicate that the His tag altered the enzymatic function by lowering its catalytic activity. In addition, the imidazole compound was also found to interfere with CLIC's catalytic activity by acting as a second substrate that led to inaccurate assay measurements. As such, it is recommended that removal of both the "His-tag" and "imidazole" be done in order to avoid any interference in subsequent enzyme characterization studies.

The project then proceeded to determine the optimal conditions for the enzymatic activity of the CLIC1 and 3 enzymes, across a range of pH and temperatures. CLIC3 was found to be heat resistant compared to CLIC1, which demonstrated heat sensitivity at higher temperatures. CLIC1 was seen to decrease in it activity and stability at increased temperature. The optimal thermal activity of both proteins obtained was 37°C in the HEDS enzyme assay. However, these studies revealed that the optimal catalytic activity of CLIC3 is

obtained under acidic conditions (around pH 6), in contrast to CLIC1 that was optimally active at under more physiological conditions (pH 7).

Furthermore, this study focused on exploring alternate new substrates for CLIC enzymes. It was found that soluble CLIC3 and CLIC1, demonstrate an affinity to a number of substrates including Imidazole, DHA and sodium selenite. The inhibitory effect of the ion channel blocker, drug IAA94 on the oxidoreductase activity of these two proteins was also examined, which showed that it inhibited the enzymatic activity of CLIC3 similar to CLIC1.

The current study also sought to define the kinetic profile of oxidoreductase catalytic activity of these CLIC proteins. Based on our characterization study, kinetic constants (Vmax, Km) for both CLIC1 and CLIC3 were determined. By comparing these catalytic efficiencies, it was evident that both CLIC1 and CLIC3 obey a Michaelis-Menten kinetics module with Vmax values of (2.026, 3.33 µM/min) and Km value of (2.503, 0.9941 mM) respectively. Also, this characteristic study revealed that both CLIC1 and CLIC3 enzymes are following first-order kinetic reactions with a Vmax value of (1.4mM/min) and (0.7mM/min), respectively.

These combined *in vitro* studies revealed the distinct enzymatic activity and profile of each protein member. Such information is critical in beginning to unravel the newly described enzymatic activity of these proteins and will allow the discrete roles within cells to be assigned to each of these proteins. Future studies determining intracellular function will need to take into account the role of pH and local environmental conditions, along with consideration of relevant substrates and likely protein targets for the enzymatic oxidoreductase activity of the CLIC proteins.

Chapter 1

Literature review

1.1 Introduction

Enzymes as biological macromolecules have been extensively studied and are considered vital for the survival of all living organisms. These catalyst proteins have a critical region known as the active site, which is often comprised of highly conserved and critical residues that perform various actions, of which, the catalytic activity is the most important. In addition, these residues can interact with specific substrate molecules via binding interactions that serve to position the substrate molecule in the vicinity of chemically active catalytic residues. They also act to decrease the activation energy of the transition state and consequently apply their catalytic action. The binding residues, in contrast to the catalytic residues, are less important to the enzyme catalytic function and are more prone to change under environmental evolutionary influences. Such changes provide opportunities for the enzyme to interact with new substrates. Thus studies of enzyme active sites and their vital residues, help us to better understand the relationship between the structural and functional properties of proteins, as well as designing novel enzyme and inhibitor drugs.

As such, the purpose of this project is to investigate and further characterise the newly discovered enzymatic activity of a family of proteins well known to act as ion channels in their membrane-associated state, but whose function as soluble proteins was previously overlooked. The focus of this Masters project is the chloride intracellular ion channel protein family, also known as the CLIC proteins. These proteins are unusual existing in both monomeric soluble and integral membrane-bound states. Moreover, the CLIC1 is classified as “metamorphic” protein which means it can exist in two stable tertiary conformations[1].

These proteins can spontaneously insert into phospholipid membranes from their aqueous soluble state, where they are capable of transporting both anionic and cationic species by acting as ion channel proteins [2]. In addition, the CLIC proteins have structural similarities to the Glutathione S-Transferase (GST) superfamily, especially the GST-Omega class [3, 4]. The CLICs also demonstrate structural homology to the Glutaredoxin (Grx) enzyme family by sharing a similar active site motif (Cys-X-X Cys/X) that is located in their N-terminal CLIC domain. This active site couples with the Glutathione peptide (GSH) that is used as a cofactor in the redox reactions catalyzed by members of the Grx family. As a result of this activity, the disulfide bonds in protein targets and other component substrates are

reversibly reduced, helping to maintain a “healthy” reduced state within cells’ intracellular environment.

To that end, the team in the School of Life Sciences, at UTS, led by A/Prof Valenzuela recently demonstrated by *in vitro* assay systems that members of the CLIC protein family have similar oxidoreductase activity to the Glutaredoxin family [5, 6]. This enzymatic function was demonstrated for the monomeric soluble state of the proteins and appears to be independent of their integral membrane, ion channel activity. The active site of the CLIC enzymes was confirmed, along with identification of certain critical residues. In addition, the enzymatic activity of these CLIC members was inhibited by the same drugs that block their ion channel activity. Therefore, following on from these new discoveries, further detailed biochemical characterization of their enzymatic activity is required, which is the main purpose of this research project.

1.1.1 What are enzymes and what are their functions?

The most multipurpose macromolecules in cells are proteins, which have diverse and vital roles in all biological systems. One of their functions is to act as catalysts. These biological protein catalysts are called “enzymes”. They are able to regulate forms of chemical transformations, as well as, intercede the conversion of different patterns of energy. For example, during photosynthesis, the light energy via an ionic gradient is converted to chemical-bond energy. Or the energy of adenosine triphosphate (ATP) can be converted to mechanical forms of energy for contracting muscles via the myosin enzyme [7].

However, enzymes are most famous and remarkable for their catalytic potency and their specificity. Their catalytic activity occurs within the specific region of the enzyme known as the “active site”. Enzymes are able to catalyse various chemical reactions as a result of their ability to bind with a vast range of substrate molecules through intermolecular forces. Subsequently, this connection achieves the ideal orientation, the enzyme and substrate complex (ES) reaches the transition state (the highest-energy level in the reaction) followed by a release of the product [7]. Although virtually all enzymes are catalytic proteins, it is now known that there are also a small group of catalytically active RNA molecules capable of catalysing specific biochemical reactions [7]. However, these are beyond the scope of this study and therefore will not be considered further.

1.1.2 The importance of enzymes as catalysts

In order for the various chemical reactions taking place within biological systems at a sufficiently fast rate, there is a need for catalysts to speed these along, which is the principal function of enzymes. Normally most chemical reactions proceed slowly in the absence of this enzymatic activity, which would be highly inefficient for a cell or an entire organism's normal metabolic processes. Hence enzymes increase the rate of such reactions by a factor of more than a million fold in less than second (figure 1.1)[8].

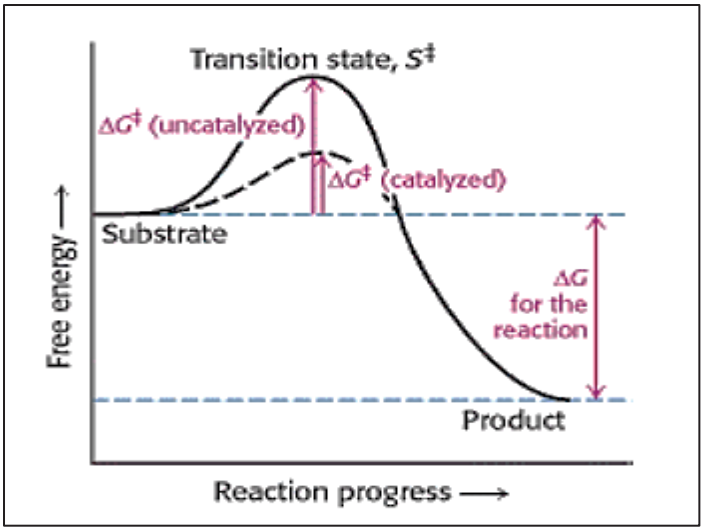


Figure 1.1: **Enzymes reduce the activation energy.** This diagram indicates both catalysed and non-catalyzed reactions. In the catalyzed reaction, the activation energy is decreased by enzyme catalytic activity through the reduction in ΔG, the free energy of activation, and thereby leads to speeding up of the reaction process and production of the product. As such, the non-catalysed reaction will take place much slower compare to the catalysed one [7].

Enzymatic or catalytic activity is characterized by two important steps: First, the chemical reaction rate can be increased by enzyme catalytic activity, without the enzyme itself being consumed or altered during this action. In the second stage, the enzyme can enhance the reaction rates without altering the chemical equilibrium among reactants and products [8].

1.1.3 The relationship between the enzyme catalytic activity and its active site

It was Linus Pauling [9] who first introduced the following concept with respect to protein structure; *'...The only reasonable picture of the catalytic activity of enzymes is that which*

involves an active region on the surface of the enzyme which is closely complementary in structure not to the substrate molecule itself in its normal configuration, but rather to the substrate molecule in a strained conformation corresponding to the 'activated complex' for the reaction catalysed by the enzyme'.

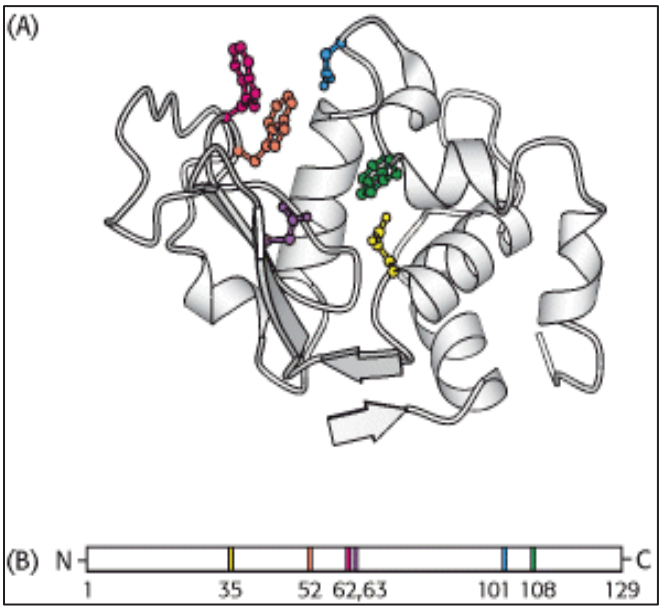


Figure1.2: Enzymes active sites may consist of different residues. (A) This ribbon diagram indicates the active site residues (shown in colour) in the enzyme lysozyme that cluster together when the protein folds into its tertiary structure, resulting in the formation of a slot or groove, wherein substrate would bind; (B) This schematic is of lysozyme's primary structure which shows that the residues in the active site can have distal locations on the polypeptide chain [7].

Today we would describe this enzyme-transition-state as complementarity [10]. Accordingly, this interaction (the substrate interaction with the enzyme's active site) is very specific. In fact, the enzyme active site typically consists of amino acid residues that are located on or near the enzyme's surface which forms a type of slot or groove as is the case for the enzyme lysozyme (figure 1.2). This allows the substrate to interact with the enzyme via multiple non-covalent attractive forces, resulting in tight binding between substrate and enzyme to form the enzyme-substrate (ES) complex. Subsequently, by mechanisms, which are substrate and enzyme dependant the ES complex will be converted to the product (P), with the release of the enzyme, which is not consumed in the reaction. By this interaction, the energy of activation for the forward reaction is lowered and hence the transition state would be formed (figure 1.3)[7, 8].

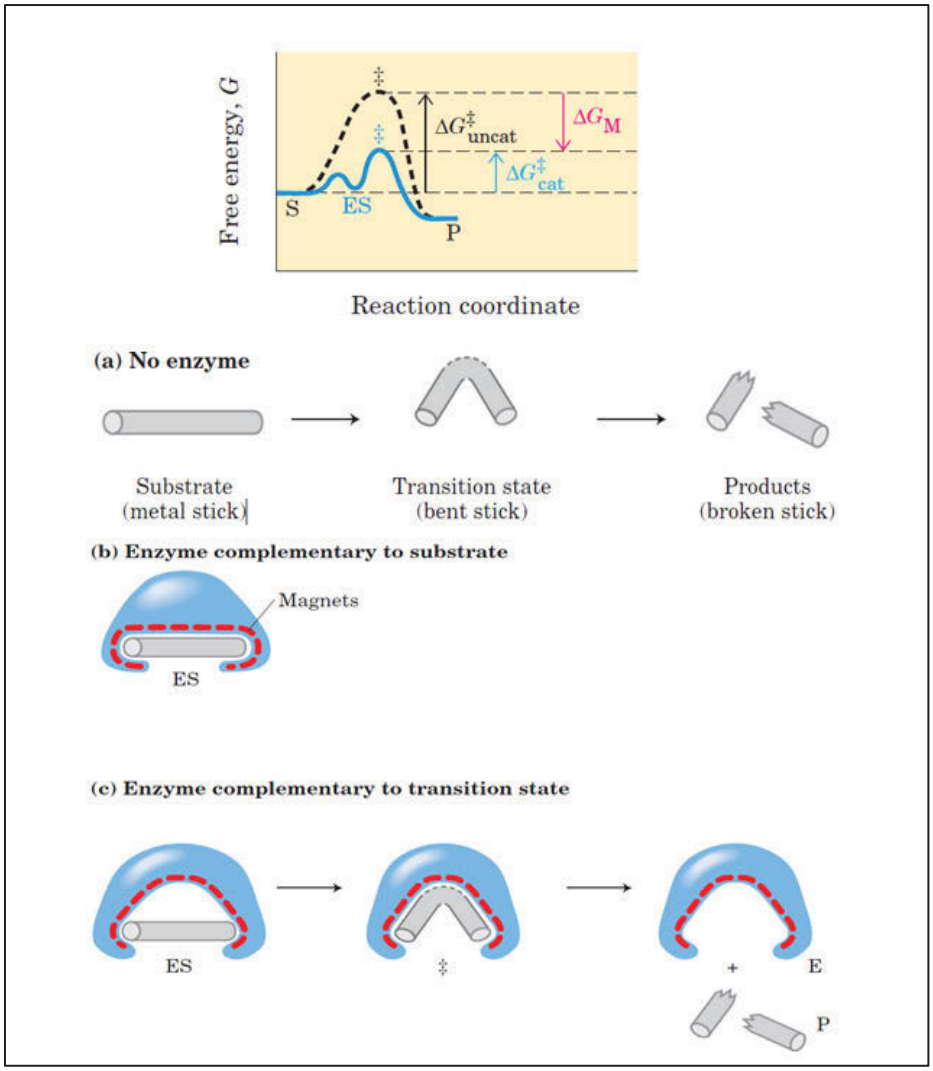


Figure1.3: Optimal enzyme catalytic activity is complementary to the transition state. (a) Before the substrate is converted to the final products, it passes through a transition state. (b) This model indicates that the active site of the enzyme is complementary to its substrate. (c) This model indicates that the active site of the enzyme is complementary in shape to the substrate, only after they reach the transition state [11].

1.1.4 Enzyme kinetics

Principally enzyme function has to be compatible with the needs of the organism. As such, the enzyme needs to act as catalyst in order to increase the rate of biochemical reactions and thus derives its main function. Referring to the reaction below, an enzyme catalytic pathway is described [12]:

$$E + S \underset{\text{(Binding)}}{\overset{K_d}{\rightleftharpoons}} ES \xrightarrow[\text{(Catalysis)}]{k_{cat}} E + P \quad (1)$$

At the beginning of this reaction, the enzyme (E) attracts and combines with the substrate (S), resulting in the formation of an enzyme substrate or (ES) complex. There are two possible destinies for the ES complex: 1) to dissociate back to their original state as E and S (ES → E+S) by equilibrium constant K_d or in the other case 2) be activated via catalytic activity (ES→ (ES–EP)) and subsequently proceed in the formation of product (P) and free enzyme by rate constant $k_{cat.}$ Therefore, when the steady state approximation is achieved, the rate of the enzyme activity can be expressed by the Michaelis–Menten equation:

$$V_0 = V_{max} [S/ K_м +S] \quad (2)$$

In this equation, $V_{max} = k_{cat}[E]$ is the maximal reaction rate when the substrate concentration on the enzyme catalytic site is saturated. Therefore, as mentioned above, k_{cat} is the catalytic rate constant, while [E] represents the total enzyme concentration. Where, K_M in this equation, which is known as the Michaelis constant, represents the concentration of the substrate at which the reaction rate is half its maximum value (V_{max}). However, in the Michaelis–Menten kinetics reaction (1) if the catalytic activity was slower than the dissociation of ES to (E+S); in this case, K_M converts to $K_{d.}$

1.1.5 The influence of temperature and pH on enzyme kinetics

The importance of combined studies of an enzyme's kinetics is that such evaluations provide valuable information about an enzyme's active site structures and functions. For example, by analysing the effect of pH on enzyme kinetics such as V_{max} and $K_{m,}$ the enzyme's ionisable groups within the active site that are involved in the catalytic activity, can be identified. Moreover, based on this analysis the pK_a parameter can also be determined. Accordingly, it has been previously shown that the pK_a value of the active site (involving the critical catalytic cysteine residue) is one of the important factors that effect the reactivity of the mammalian Glutaredoxin-2 (Grx2) protein [13].

In addition, biochemical studies on several Glutaredoxin proteins from *Chlamydomonas reinhardtii* have revealed that these proteins show remarkable differences in their catalytic activities as well as in the pK_a value of their N-terminal catalytic cysteine. For instance, as a result of lower pKa value of active site cysteine in GRX1, this protein catalyzes deglutathionylation more efficiently than GRX2[14]. Importantly, temperature conditions

also affect changes that influence the pK_a value, and due to this, the standard enthalpies (ΔH) of ionization groups can be obtained. This subsequently leads to recognition of the ionizable groups [12] such as the case for the Urease enzyme, which was assigned pKa values to the ionizable groups in its the active site through a combined temperature-pH study[15]. Furthermore, the enzyme kinetic properties are highly temperature sensitive. The velocities of enzymatic reactions can be generally accelerated by increasing in temperature, such that, for many reactions with each $10^{o}C$ increase in temperature the reaction velocity could roughly double, commonly under substrate saturating concentrations (maximal velocity (V_{max})), however, this should be considered that this is not literally applied to all enzymatic reaction [16].

Similarly, the interactions between the enzyme and its ligands are highly temperature dependent, so the effect of temperature on ligand binding also influences enzyme metabolic function and catalytic rate [16]. For instance, the biochemical characterization of the Glutaredoxin protein from antarctic sea-Ice (rPsGrx) has indicated that this protein was a cold-active protein and maintained low thermostability [17]. The consequence of the type of analysis as described above is that it helps us to understand the feasible catalytic activity of the enzyme residue active site, which aids to provide a better insight into enzyme actions. In addition, through such understanding, one can speculate and make predictions regarding the affinity of enzymes for various ligands (or inhibitors) which is essential for structure-based ligand design.

1.1.6 Effect and role of cofactors on enzyme activity

Enzymes usually require a molecule, which assists them in their catalytic activity. This "helper molecule" is a non-protein chemical group or metallic ion, known as a cofactor[18]. Cofactors are either organic compounds like flavin and heme or inorganic compounds such as metal ions and iron-sulfur clusters [11]. Accordingly, cofactors promote various functional activities and are utilized by enzymes in an enormous variety of catalytic reactions. These enhanced functionalities that cofactors give to enzymes include: redox capabilities, electrophilic centrs, sites for coordination of substrates as well as new functional groups[18].

Cofactors also demonstrate functional roles in catalytic reactions, which are classified based on the overall chemical changes that impact on the enzyme's substrate(s). These functional roles include moiety transferrer, redox, mobility, activation, bond cleavage/formation, polymerisation and rearrangements of atoms in substrate[19].

In addition, cofactors have the ability to boost enzymatic activity by performing hydride shuttling functions, which is an important missing functionality amongst the oxidoreductase class of enzymes that allows the redox chemistry to occur efficiently[19]. In this regard , several studies have been investigated on the relationship between the specific cofactors such as Glutathione peptide (GSH) and redox enzymes. For instance, it has been indicated that the glutaredoxin proteins are able to use the glutathione as a cofactor in their catalytic system through which they can protect the protein against oxidative damage [20]. Besides, the study on thiol–disulfide redox activity of glutaredoxin was revealed that this enzyme utilize the GSH as a cofactor in order to reduce protein disulfides [21]. In view of that, in the following section the detailed information about the enzyme classifications, redox enzymes, their catalytic system that involves GSH as their cofactor and how these information's are related to the catalytic activity of CLIC protein will be explained.

1.2 Enzyme classifications

The International Union of Biochemistry and Molecular Biology (IUBMB) established an International Commission on Enzymes in 1956 with the aim of addressing enzyme classification and nomenclature. Therefore, the enzyme classification system was formulated and developed by this committee [22] and was further expanded in the second edition of Enzymes in 1964 [23].

According to this Enzyme Commission, enzymes categorized into six major classes based on the type of reactions they catalyse, as shown in Figure 1.4. These categories include oxidoreductases, transferases, hydrolases, lyases, isomerases and ligase enzymes. The oxidoreductase class of enzymes catalyses oxidoreduction reactions by which the substrate is oxidized as a result of hydrogen ions or electron transfer. In contrast, enzymes of the transferase class are acting as transformers by transporting a specific group between two different compounds. The hydrolase enzyme families, on the other hand, are able to

catalyse the hydrolysis of several bonds. Whereas, the enzymes in the lyase family are capable of cleaving bonds such as, C-C, C-O or C-N in a way that is different from oxidation or hydrolysis. The isomerase enzymes can apply structural changes onto a molecule by their catalytic function, while the catalysing role of ligase enzymes would accelerate the connection between two molecules that are joined by hydrolysis of a pyrophosphate linkage [7, 24, 25].

CLASS	NAME	REACTION CATALYSED
1.	Oxidoreductases	AH2 + B = A + BH2
		Or AH2 + B+ = A + BH + H+
2.	Transferases	AX + B = A + BX
3.	Hydrolases	A - B + H 2O = AH + BOH
4.	Lyases	A = B + X - Y = A - B | | X Y
5.	Isomerases	A = B
6.	Ligases	A + B + NTP = A – B + NDP + P
		Or A + B + NTP = A – B + NMP + PP

Figure 1.4: Enzyme classification. Six major classes of the IUBMB enzyme list is adopted from [26].

1.2.1 The oxidoreductase enzyme classes

1.2.1.1 The Thioredoxin superfamily as oxidoreductases

Oxidation-reduction reactions are those in which electrons are removed from an electron donor and then transferred to an electron acceptor; as a consequence, the electron donor is oxidized and the electron acceptor is reduced. Such reactions perform a vital function within the cell environment; examples of such functions include the production of ATP in the mitochondrial organelle[27], folding and processing of oxidised proteins in the endoplasmic reticulum[28], regulating signaling molecules [29] and transcription factor activities[30].

Accordingly, the oxidoreductase enzymes, such as thioredoxins intercede oxidation-reduction reactions[31]. In these types of reactions, where oxygen molecules are to be the final electron acceptors, then reactive oxygen products (ROS) will be produced[32].

The oxidoreductase enzymes structurally have a catalytic site that includes a pair of cysteine residues. This catalytic site has the ability to mediate the oxidation-reduction of specific target substrates. The Thioredoxin (Trx) superfamily is an example of such enzymes and they have been identified is key protein members within the oxidoreductase enzyme class, which have a preserved structural entity known as the Trx-fold [33]. The first description of this fold was from the eponymous enzyme Trx [34]. Structurally the Trx-fold is comprised of four beta-strands interspersed by three alpha- helices (Figure 1.5)[33]. In fact, an extra N – terminal beta- strand and an alpha-helix, in addition to the Trx-fold, exists in almost all mammalian enzymes of the thioredoxin superfamily.

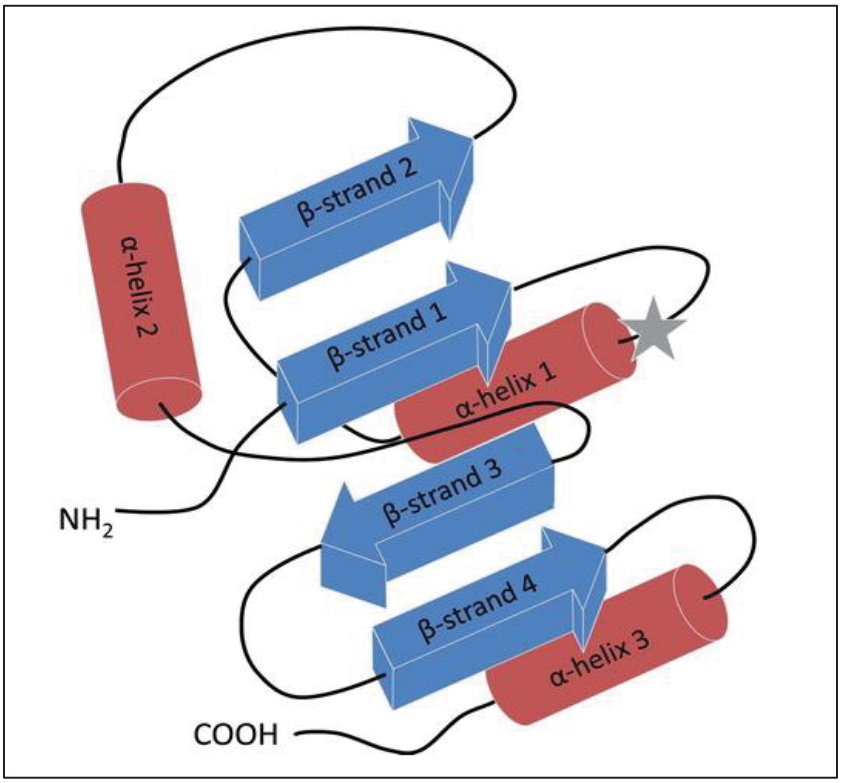


Figure1.5: The Trx-fold structure: The beta-strands are shown in blue where the alpha-helix strands are shown in red. The grey star represents the catalytic cysteine residues of the oxidoreductases, in the loop which connects beta-strand 1 and alpha-helix 1. This figure is adapted from [35].

Indeed, the catalytic site in thioredoxin, like most oxidoreductase enzymes, is comprised of a pair of cysteine residues that are segregated from each other by two distinct amino acids, which leads to the formation of the consensus motif Cys-X-X-Cys. The two divider amino acid residues in this motif vary between individual thioredoxin family members [36, 37]. In addition, this ubiquitous antioxidant enzyme has been found to be present in all species from mammals to Archebacteria, and plays a crucial role in numerous biochemical processes such as protecting proteins from oxidative damage and inactivation [31, 38] cell protection against environmental stresses like reactive oxygen species (ROS), peroxynitrite and arsenate [39, 40], also in redox conditions it can act as a reductase [31].

Thioredoxin enzyme (Trx) was first discovered in *Escherichia coli* as an electron donor for ribonucleotide reductase, an essential enzyme in the process of DNA synthesis [41, 42]. Structural studies of the *Escherichia coli* bacterial protein indicate that the Trx protein is comprised of 108 amino acid residues [43], which contains an active site motif Cys32-Gly-Pro-Cys35 and exists in two different forms in nature, the oxidized thioredoxin form (Trx-S 2) and the reduced thioredoxin form (Trx(SH)2)[31]. In this regard, the study that was investigated on functional behavior of this enzyme has been proposed that the Trx would be involved in the nucleophilic attack by use of its redox-related functions. This occurs via its Cys32 residue acting on a disulfide-containing substrate resulting in the production of a mixed disulfide intermediate molecule, which in turn is reduced by the thiolate of Cys35 and subsequently resulting in realise of the free oxidized thioredoxin [44].

Moreover, in mammalian species, two forms of Trx isoenzymes exist the Trx1 with cytosolic/nuclear localization and the Trx2 with mitochondrial localization (Table 1)[45]. In view of that, as the Trx1 protein is known as a strong reductase, able to reduce various protein targets. Through its catalytic reaction activity, both of its cysteine residues in its active site are oxidized, which then need to subsequently be reduced again. This final reduction and hence regeneration of the enzyme occurs via a Trx recycling system to allow future reduction activity by the enzyme. In fact, the recycling system is accomplished by the use of a dimeric flavoenzyme, thioredoxin reductase (TrxR). This TrxR is classified as pyridine nucleotide-disulfide oxidoreductase, which reduces the Trx by using nicotinamide adenine dinucleotide phosphate (NADPH), which is the main electron donor for this system (Figure

1.6A)[45]. Three forms of these isoenzymes can be found in mammalian cells: TrxR1 and TrxR2, as well as the Trx glutathione (GSH) reductase (TGR)[46, 47].

Name	Catalytic site	Cellular location	Recycling system
Trx1	Cys-Gly-Pro-Cys	Cytosol, nucleus and secreted	NADPH + TrxR
Trx2	Cys-Gly-Pro-Cys	Mitochondrial matrix	NADPH + TrxR
Grx1	Cys-Pro-Tyr-Cys	Cytosol, mitochondrial intermembrane space, (nucleus and secreted)	NADPH + GR + GSH
Grx2	Cys-Ser-Tyr-Cys	Mitochondrial matrix and nucleus	NADPH + GR + GSH
Grx3	Cys-Gly-Phe-Ser	Cytosol	NADPH + GR + GSH
Grx5	Cys-Gly-Phe-Ser	Mitochondrial matrix	NADPH + GR + GSH
PDI	Cys-Gly-His-Cys	Endoplasmic reticulum, (cytosol, nucleus) and secreted	O_2 + Ero1 for the oxidizing activity of PDI GSH or TrxR1 for the reducing activity of PDI

Table 1: Demonstrates specific features of the Trx1 and 2, Grx1, 2, 3 and 5 as well as PDI oxidoreductases [35].

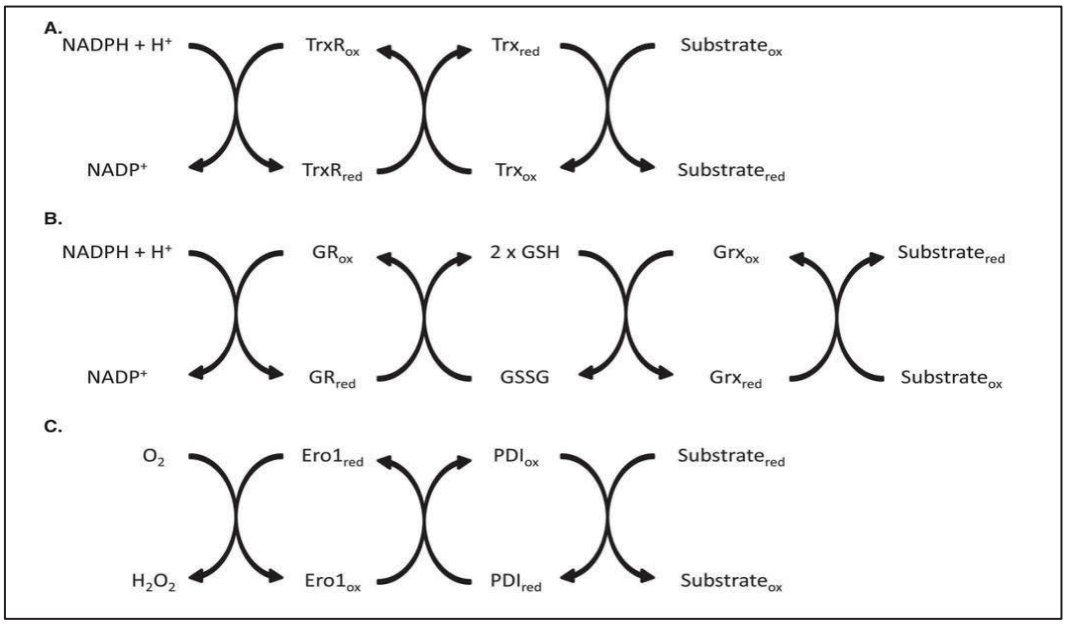


Figure 1.6: **This figure indicates the oxidation-reduction reactions by oxidoreductase enzymes**. (A) Demonstrates the Trx recycling system that leads to reducing the disulfide bonds of protein

substrates through an NADPH dependent manner. B) This cycle demonstrates the Grx recycling system by which the disulfide bonds of protein substrate would be reduced by NADPH as an electron donor. (C) This cycle shows that the free thiol groups are oxidized in protein substrates, via oxygen dependent Ero1/PDI system. Ox represents the oxidized state, where Red represents reduced state [35].

1.2.1.2 The Glutaredoxin family of oxidoreductases

Glutaredoxin proteins structurally belong to the thioredoxin superfamily of enzymes. These proteins are classified into monothiol Grxs, which contains only one cysteine residue in their active site motif (C-X-X-S), or dithiol Grxs, which have two cysteine residues in their catalytic active site motif (C-X-X-C). The monothiol Grxs are also classified as both single- and multi-domain proteins [48, 49]. In addition, several redox-active enzymes such as protein disulfide isomerase (PDI) [50], T4 glutaredoxin [51] and NrdH redoxin [52] have been identified, that structurally contain similar active sites (the CXXC motif) to the thioredoxin and glutaredoxin enzymes. To date, various Grxs such as Grx1, Grx2, Grx3, Grx4, and Grx5 have been identified.

The protein Glutaredoxin 1 (Grx1), was first discovered in *Escherichia coli* bacteria as a hydrogen donor for ribonucleotide reductase in thioredoxin mutants [53]. This protein was subsequently identified and isolated from a diverse range of species including *Chlamydomonas reinhardtii*, *Synechocystis* PCC 6803, *Oryza sativa*, *Populus trichocarpa*, *Homo sapiens* as well as yeast *Saccharomyces cerevisiae* [54-57]. The Grx enzymes in mammalian cells have two different forms, both dithiol, and monothiol isoenzymes. The Grx1 is a dithiol protein with molecular weight of 12KDa, which can localize to different regions in the cell such as cytosol (main location), nucleus, and mitochondria (intermembrane space) or even found extracellularly [58-61]. The active site of this dithiol isoenzyme (Grx1) consists of Cys-Pro-Tyr- Cys residues (Table 1). Whereas, the 14KDa Grx2 isoenzyme has mitochondrial, cytosolic and nuclear localization, in mouse and human cells, and contains Cys-Ser-Tyr-Cys as its active site (table 1) [62, 63].

The mammalian Grx3 and Grx5 are monothiol isoenzymes, which have Cys-Gly-Phe-Ser motif in their catalytic site. The Grx3 localizes in the cytoplasm, whereas the Grx5 exists in the mitochondrial matrix (Table 1). The monothiol Grxs have diverse roles in the cell, for instance, they can de-glutathionylate substrate cysteine residues. However, in comparison

with dithiol Grx, their de-glutathionylation activity is inferior. They also have the ability to regulate iron homeostasis [48, 64]. Moreover, the Grxs have been demonstrated to act as dehydroascorbate reductases [65, 66]. Furthermore, a certain kind of peroxiredoxin can be reduced by this protein [67, 68]. It can also function as a redox control for transcription factors and signal transduction in mammals [69].

Essentially in all organisms that contain glutathione (GSH), several Grx isoforms can also be found. Glutathione (L-ɣ-glutamyl-L-cysteinyl-glycine, GSH) as a tripeptide molecule which is one of the most crucial components in cellular antioxidant systems, which is maintained at a low millimolar concentration in the cytoplasm of eukaryotic cells [70, 71]. GSH in its reduced form can control redox homeostasis by its ability to scavenge reactive oxidative species. In its oxidized form, this molecule exists as GSSG by forming a dimeric disulfide bond and can be reduced again by Glutathione reductase (GR) rapidly, with 90-99% of the reduced form of GSH maintained within the intracellular space. Consequently, as a result of the production of reactive oxygen species (ROS) from cellular metabolic processes, GSH serves as a strong antioxidant and defends the cell from oxidative damage [71].

Additionally, this peptide is vital for the preservation of the reducing potential of the cell's cytoplasm. Accordingly, the Grx proteins participate in crucial and variable roles in the cell such as cell defence from oxidative stress, with studies showing that in the presence of oxidants, their genes are induced [72]. Therefore, reduction of a substrate by Grx leads to its own oxidation, requiring it to reduce again via the Grx recycling system (Figure 1.6 B). In this system, there is a need for an electron donor, which performs by NADPH. It does this by transferring electrons to glutathione reductase (GR), after which the electrons transfer through GR to glutathione (GSH), subsequently, the GSH will transfer the electrons to the dithiol Grx protein, in its function as a thiol-disulfide oxidoreductase [73, 74]. Consequently, the Grx will reduce the disulfide bonds of its protein targets and other compound substrates, via a dithiol mechanism or glutathionylation mechanism [21].

1.2.1.3 Glutaredoxin isoform classifications

Several isoforms of the glutaredoxin family exist in different living organisms and are diverse in their structural properties as well as their catalytic activities. The Glutaredoxin enzymes

can be classified into three different classes based on their structures and catalytic functions [75].

The first category is comprised of classical glutaredoxin proteins, which structurally have the thioredoxin/glutaredoxin fold (Figure 1.7). The proteins in this class have a molecular weight ranging from 9- to 12-kDa and contain in their active site the sequence C-X-X-C (CPYC) motif (Figure 1.8). For example, different isoforms such as Grx1 and Grx3 of *E. coli* bacteria, glutaredoxins 1 and 2 of yeast, and human and phage T4 glutaredoxins [76] belong to this category.

The second category is structurally defined based on having a monothiol residue active site C-X-X-Ser (usually a CGFS motif). The proteins from this category have been discovered in different organisms including humans (Protein Kinase C-interacting with Thioredoxin fold (PICOT)) and yeast (yGrx3, yGrx4, and yGrx5) [77, 78].

The third class of Grxs family is exemplified by *E. coli* Grx2, which has a molecular weight of 24.3 kDa. The three-dimensional structure of Grx2 (Figure 1.7.B) reveals that this protein shares high structural homology with the GST family, even though there is not a high level of sequence homology between these proteins [79]. In addition to this example, other proteins that are likely classified in this category are the human GST theta class, which includes the human protein GST omega1 (GSTO1), the mouse GST theta-like class (p28), and the human chloride intracellular channel protein-1 (CLIC1)[4, 80-82]. These proteins are known as stress response or detoxifying proteins, which have a single active site cysteine and do not share significant amino acid residue homology.

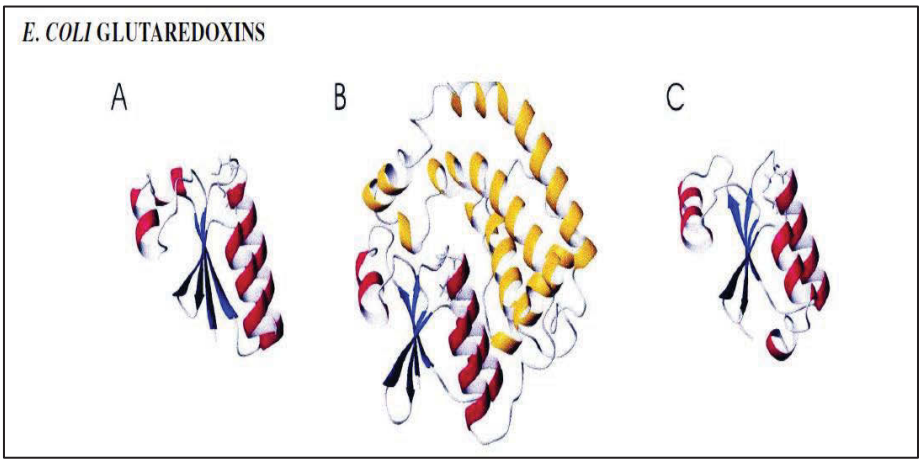


Figure 1.7: The figures (A) and (C) are representing the oxidized form of E.coli Grx1 and Grx3, respectively [83, 84]. Where figure (B) is representing the reduced form of E.coli Grx2 [79]. (The beta-strands are shown in blue whereas, the alpha-helix strands are shown in red for (A) and (C). The alpha-helix strands of the C-terminal domain in Grx2 are shown in yellow.

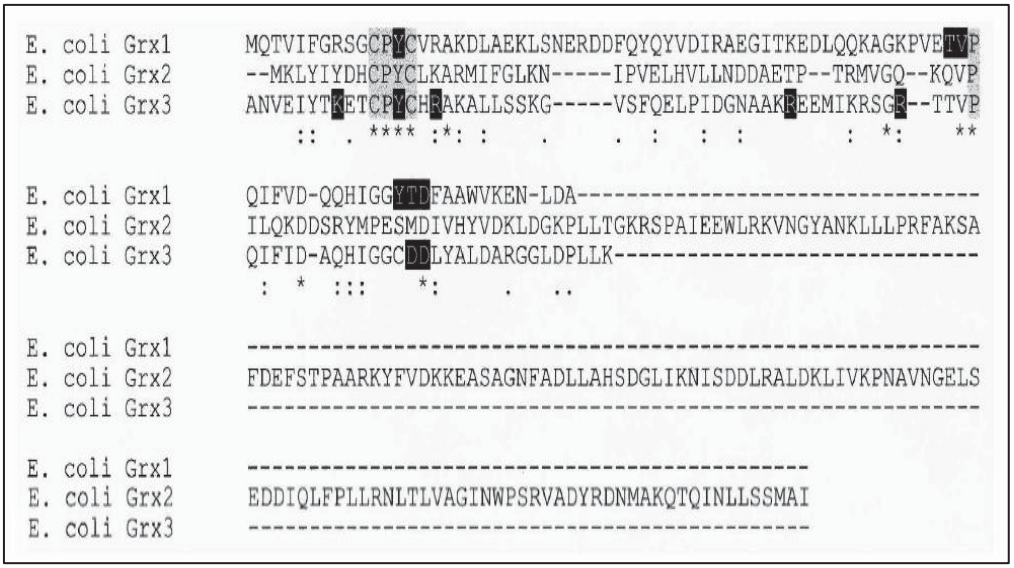


Figure 1.8: This figure is an amino acid sequence alignment of the E. coli glutaredoxins. The grey boxes highlight the active site motif, as well as the consensus proline amino acid residues. The black boxes highlight the amino acids that participate in binding of GSH molecules in E.coli Grx1 and Grx3.

1.2.1.4 Protein disulfide isomerase (PDI) as an oxidoreductase enzyme

The protein disulfide isomerase (PDI) which has a molecular weight of around 55 kDa is another member of the thioredoxin superfamily (Table1) that is classified as an oxidoreductase enzyme. This enzyme structurally consists of four Trx-folds; one pair functions as the catalytic activity, whereas the other two pairs function in the protein substrate interactions [85].

PDI contains a Cys-Gly-His-Cys motif in its catalytic site and is mainly distributed in the endoplasmic reticulum (ER) in low millimolar range concentration. However, this enzyme is also distributed within the cytosol, nucleus and located at the cell surface [86]. The ER environment is very oxidizing compare to the cytoplasm in eukaryotic cells. Thus, this condition promotes a good opportunity for PDI enzyme to play its role in oxidative protein folding [87].

However, the PDI oxidizing system is reliant on an enzyme is known as ER oxidoreductin 1 (Ero1). In this system, PDI is reversibly oxidized by the Ero1 enzyme in ER environments and subsequently, electrons are transferred to oxygen molecules, which leads to the production of hydrogen peroxide (H_2O_2) (Figure 1.6C) [88]. Despite the fact that PDI activity that occurs in the endoplasmic reticulum is principally oxidation or formation of disulfide bonds in newly produced proteins, surface-associated PDI can also act as a reductase to reduce disulfide bonds and isomerize them. This reduction occurs by GSH or TrxR1 donating electrons to PDI [86].

To conclude, the Trx and its family member's known as oxidoreductase enzymes, have the capability to control numerous biological processes that are principally involved in activities that help to maintain cellular redox homeostasis. They can establish a complex cycle system through which electrons can transfer to diverse target substrates. Moreover, these enzymes could be considered for potential therapeutic applications, such as in viral entry and virus infection which are related to oxidative stress [35].

1.3 Glutathione S-Transferase superfamily (GSTs)

The Glutathione-S-transferase proteins are a family of multifunctional isozymes that are mainly found as dimers in the cellular cytoplasmic environment [89]. Structurally the dimeric GSTs are comprised of two domains, the thioredoxin folded N- terminal domain and an all alpha helical C- terminal domain [90]. The hydrophobic domain interface functions to keep GST proteins stable in the cytoplasmic environment [91].

It has been shown that the GSTs domain 1 topologically resembles structures found in some glutathione – binding proteins such as E.coli thioredoxin and T4 glutaredoxin, despite the fact that they do not share any appreciable level of sequence homology [92]. These proteins have a molecular weight of around 24-25 kDa and exist across most living biological kingdoms including animals, plants, fungi, and bacteria [93].

They have been classified into various classes, including alpha, mu, pi, theta, sigma, beta, chi, omega and zeta based on differences in their structure, kinetics and their amino acid sequence homology [93]. Each member of the GST superfamily plays a critical role in cellular mechanisms such as cell defence [94], prostaglandin D synthesis [95] intracellular binding

and transporting hormones [89]. Furthermore, GSTs as antioxidant proteins have the capability of catalysing the conjugation of glutathione (GSH) to harmful electrophilic xenobiotics and their metabolites [96], [94] these enzymes are also able to detoxify the cell of ROS products and consequently protect the cell from oxidative stress effects [97].

In addition, it has been shown that the GST isoenzymes contribute to the effects of therapeutic drugs and can enhance chemical carcinogen resistance by utilizing glutathione [98].

The Omega class of GST proteins was found in both mammals and *Caenorhabditis elegans.* The human protein omega-1 class, which is expressed in most tissue in the body, has diverse activities such as thiol transformation and dehydroascorbate (DHA) reduction that are typical to the glutaredoxins, and are glutathione-dependent mechanisms. GST omega 1 protein is different from other GSTs, in that it contains unique active cysteine residues that promote the formation of a disulfide bridge with glutathione. The enzymatic activity of the mammalian GST-omega class is distinct to other GST classifications and resembles the glutaredoxin proteins [80] along with similar structure to small redox thioredoxin proteins. It also has the ability to maintain a healthy redox via a deglutathionylation reaction which contributes to decreasing disulfide linkages within the cellular environment [99],[38, 100]. This detoxifying reaction occurs within its monothiol GSH binding sites (or G-site), that contains a single cysteine residue (Cys-Gly-Phe-Ser) and exists within the conserved thioredoxin domain.

1.4 The CLIC family of intracellular chloride ion channel proteins

The chloride intracellular ion channel (CLIC) proteins are the most recently discovered family of chloride ion channel proteins. The CLIC proteins are comprised of various orthologue members that are highly conserved in both vertebrate and invertebrates. So far seven CLIC members in humans have been identified: CLIC1 [101], CLIC2 [102], CLIC3 [103], CLIC4 [104, 105],CLIC5 [105, 106], CLIC5B (p64 in bovine) and CLIC6 [107], with all containing around ~230 amino acid residues except CLIC5B and CLIC6 which contain 437 and 408 amino acid residues respectively. These proteins have high sequence homology between them, ranging from 46 – 76% [108].

In this regards, the first protein member from vertebrates to be identified was p64 (CLIC5B), which was isolated from microsomes of bovine kidney tissues and trachea by affinity purification using the chloride channel inhibitor, drug IAA-94 [109-111], and has a sequence identity of 60% with CLIC1 protein. P64 was found to function as a chloride-selective channel in lipid bilayer membranes of kidney cells and likely plays an important role in kidney functions, like acidification of secretory vesicles [112]. Whereas, p62, the avian homologue of this protein was found to have an important role in the function of osteoclast cells [113].

The CLIC protein family members have an enigmatic ability to exist in two different forms, globular soluble and an integral membrane form. The soluble form is generally located in the cytoplasm and nucleoplasm but, they can also insert into the lipid membranes where they act as anion-selective channels [2, 104, 114, 115]. Surprisingly, it appears that the CLIC proteins contain only a single putative transmembrane domain [106, 116, 117] which does suggest an ability to function as ion channels.

Morphologically, the CLIC proteins in the soluble form are members of the glutathione S-transferase (GST) superfamily. However, the GST family compared with soluble CLIC proteins do not have the ability of membrane auto-insertion [3, 4]. The CLIC proteins can serve in a diverse range of vital physiological roles within the cell due to their distinct intracellular locations within the cell environment as exemplified in Table 2. For example, in the course of the G2/M phase of cellular mitosis, the endogenous CLIC1 can relocate to the plasma membrane and subsequently enhance chloride conductance of the cells. Thus, this action indicates a function of CLIC1 in cell cycle and cell volume regulation [118].

In addition, CLIC2 was shown to function in intracellular calcium homeostasis by its catalytic activity with glutathione transferase substrates and also acts as a strong inhibitor of cardiac ryanodine receptor channels [3, 119]. These proteins are notable for their involvement in critical functions, such as regulation of processes including cellular growth, cellular division and apoptosis [118, 120]. In addition, they are involved in the acidification of intracellular organelles [121, 122], the formation of stereocilia [123] and the development of the organ of Corti [112, 124].

Furthermore, members of the CLIC protein family have a crucial role in development of skeletal muscle and brain [125], bone resorption [113] and kidney function [126]. Amongst the several CLIC protein family members, CLIC4 protein (also known as mtCLIC, p64H1, RS43) has been studied extensively. This protein is currently being explored for its use in anti-cancer therapies [127]. However, other members such as CLIC1 which is overexpressed in human gastric carcinoma [128], colorectal cancer [129] and gallbladder carcinoma [130] could also be a potential candidate for improving the influence of drugs on diseases; as a result of its behaviour which allows this protein to co-localized into the plasma membrane in response to changes in the redox state of the cells. Furthermore, this protein could even potentially assist in the treatment of Alzheimer's disease by acting as an antioxidant during oxidative stress [131].

On the other hand, CLIC proteins have been identified in mammals, birds, fish, and amphibians while CLIC-like proteins were also discovered in insects, sea squirts and nematodes as invertebrates classes [132]. For example, CLIC-like proteins such as the EXL-1 and EXC-4 proteins were identified in the nematode *Caenorhabditis elegans* (*C.elegans*) [126]. These proteins are similar to human CLIC1 with sequence similarity of 42% and 43% respectively [108, 133].

Accordingly, it has been shown that the vertebrate CLIC proteins and invertebrates CLIC-like proteins share ∼35% sequence identity[134]. For instance, the *Drosophila melanogaster* fruit fly which contains a single CLIC1-like gene (*Dm*CLIC) which has a sequence identity of 20% with human CLIC1 [135]. Moreover, it has been demonstrated that all known invertebrate CLIC homologs have the ability to function In a homologous manner to EXC-4 protein, and, N-terminal domain region is the essential factor in this functional specificity in these proteins[136].

Besides, it has been indicated that both vertebrate and invertebrate CLIC proteins act differently when they are expressed in *C. elegans.* Indeed, it appears that invertebrate CLIC proteins possess an evolutionarily conserved activity, due to the ability of EXC-4, C.elegans EXL-1 and Drosophila DmCLIC in targeting to the luminal membrane and providing rescuing function, while vertebrate CLICs could not demonstrate this ability[136].

In addition, CLIC-like proteins have also been identified in plants such as *Arabidopsis thaliana.* Four genes of this plant share sequence homology of 61% and sequence identity of 26% with human CLIC1[137]. Where, in the chordate *Ciona intestinalis* (sea squirts) a single CLIC-like protein has been identified which shares 45% similarities with the human CLIC family [134].

In vertebrates, the CLIC proteins are highly conserved, and usually, possess six distinct paralogues (CLIC1–CLIC6). However, there is some distinction between CLIC family paralogues within species, where the Teleostei or bony ray-finned fishes contain a duplicate copy of CLIC5 (CLIC5L), while in birds and lizards CLIC1 is missing. Furthermore, the CLIC proteins such as CLIC2, CLIC5, and CLIC6 have maintained variant connections. For example, an additional N-terminal domain, which is frequently repetitive sequences, exists in both CLIC5B and CLIC6 as weakly conserved domains in size and sequence [134].

All in all, members of the CLIC family demonstrate a great sequence homology between 47% and 76% [134, 138]. Whereas, a large difference in their sequence similarity exists among the vertebrate class of this family. Although the CLIC family mainly shares a high sequence similarity (60-75%) between each other, they differ in their localization within the cell [139].

CLIC Proteins	Molecular Mass	Tissue Localization and expression	References
CLIC1	26.9 kDa	Localization is dependent on the cell type or the different phases of the cell cycle. For instance, CLIC1 distributed on the Nuclear envelope and the plasma membrane, cytoplasm, apical domain, endoplasmic reticulum and membrane vesicles.	[101, 140-143]
CLIC2	28.2 kDa	Widely expressed in human tissues including heart and skeletal muscle. Whereas, has no expression in human brain.	[119]
CLIC3	26.7 kDa	Predominantly expressed in Human Placenta and Fetal Membranes. Also is localized in the intracellular nucleus	[103, 144]
CLIC4	27 kDa	Localized in the cytoplasm, mitochondria, cell cortex, intracellular membranes as well as the nucleus. Highly expressed in brain, vascular endothelial cells and in the liver. Also expressed in lung alveolar septae, pancreatic acini, spermatogonia, renal proximal tubules, cardiomyocytes, endoplasmic reticulum and thymic epithelial cells.	[143, 145-147]
CLIC5	46 kDa	Highly expressed in Hair Cell Stereocilia as well as in human skeletal muscle. Also is expressed in the endoplasmic reticulum (but with lesser extend to CLIC1 and CLIC4).	[143, 148-151]
CLIC6	71 kDa	Localized to the cytoplasm and apical membrane. Also is expressed in brain tissue	[107, 152]

Table2: Summary of molecular mass, tissue expression and cellular localization of the 6 CLIC protein members.

1.4.1 History, molecular structure and cellular localization of CLIC1

As previously mentioned, the human protein CLIC1 (originally known as NCC27) is one of the members of the highly conserved chloride intracellular ion channel proteins and contains 241 amino acids in mammalian cells with a molecular weight of 26.9 kDa and a pI of 4.85 [4, 101, 153]. Likewise in both plant tissues [137] and invertebrates [135] homologues of this protein have been discovered.

CLIC1 was first discovered by Valenzuela et al., in 1997 from the monocytic cell line which was differentiated by retinoic acid, followed by activation with phorbol 12-myristate 13-acetate (PMA)[101]. In 2001 the crystal structure of the soluble globular form of CLIC1 was resolved by Harrop et al., [4] (Figure1.9).

Structurally, the CLIC1 protein contains two distinct domains, the N- terminal and the C-terminal domains, which connected by a proline-rich region between Cysteine 89 and Asparagine 100. It is postulated that this region plays a critical role in the translocation of the soluble protein into its membrane form, by conversion of cis to trans conformation of these two states by using proline 91 [4].

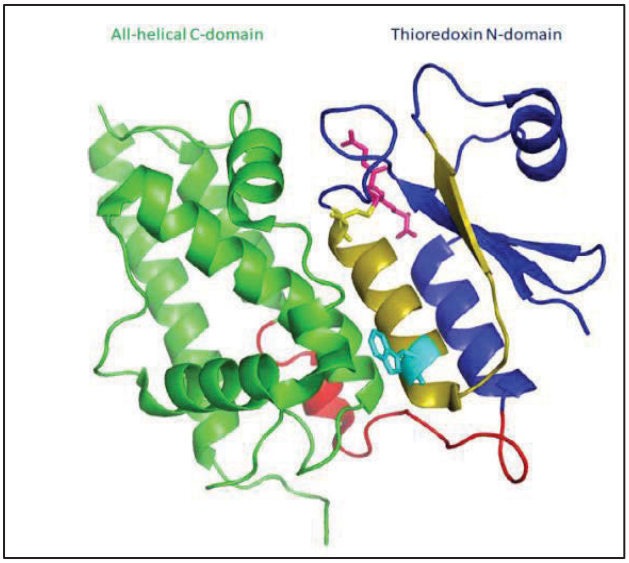


Figure 1.9: Crystal structure of the globular soluble CLIC1 protein. (This figure represents the crystal structure of globular soluble CLIC1 protein. The blue ribbon demonstrates the thioredoxin N-terminal domain which contains the catalytic cysteine active site (Cys24) that is shown in yellow. This active site is connected to both glutathione (pink) and the transmembrane region [124] via Trp35. The green ribbon represents the all alpha helical C-terminal domain. The two domains are connected via a proline-rich loop (in red). The program PyMol v0.99 [154] and CLIC1 pdb file 1K0N were used to generate this figure [4].

However, the crystal structure of the membrane-inserted form remains unsolved. The relationship between CLIC1 and the lipid membrane explained in a model by Litter et al., in 2004. This model proposes structural changes in monomeric CLIC1 as a result of oxidation. Due to these changes, hydrophobic residues located in the N-terminal region that is also structurally similar to glutaredoxin, become exposed, which stimulates dimerization, thus minimising their contact with the cytoplasmic aqueous environment. In fact, it estimated that two-thirds of the protein converts into the dimer structure because of an increased concentration of pro-oxidant molecules within the cell. On the other hand, this hydrophobic region when situated in proximity to cell membranes assumed to facilitate membrane insertion of CLIC1 by acting as a membrane-docking region. These two distinct shapes of CLIC1 mainly distinguished by the loss of beta-sheet secondary structures when the protein is oxidised. The dimer structure for CLIC1, is stabilised by the formation of an intermediate intramolecular disulfide bridge between Cysteine 24 and Cysteine 59 upon oxidation[138].

As such, it appears that oxidation is a critical stimulus that controls the conversion of the monomeric soluble form into an all alpha helical structure, that is capable of forming dimers [138] (figure 1.10) [155]. This structural rearrangement has also been termed as metamorphic, which mean that the protein in its native condition is able to interconvert between different folded conformations [156].

In addition to redox, intracellular pH is an additional factor that affects membrane insertion. Some experiments have indicated that in both basic [2] and acidic pH [139], the probability of CLIC1 inserting into artificial lipid membranes would be increased. It has been also shown that the concentration and presence of cholesterol in lipid membrane is another critical factor which has a regulatory effect on CLIC1 ability in order to spontaneously be inserted into the membrane bilayer plus this factor would also influence on CLIC1 ion channel activity[157]. However, much still remains unknown regarding the process of this regulatory mechanism, which governs the protein membrane insertion.

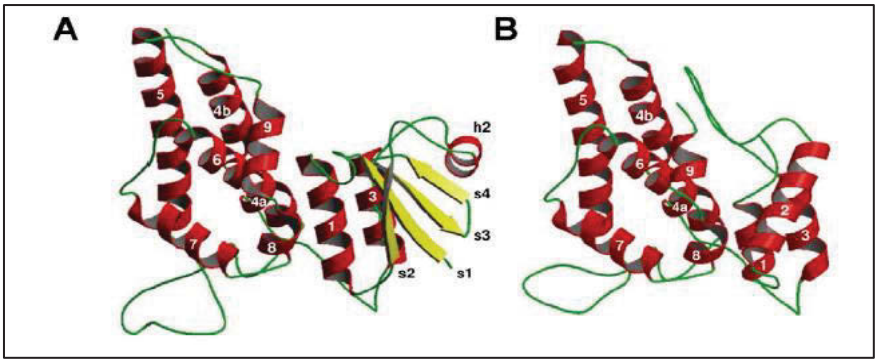


Figure 1.10: The structural transition of CLIC1 protein. The structural comparison of soluble monomer CLIC1 (A) with its half-dimer structures (B) that observed after oxidation condition [138].

It has been revealed that CLIC protein insertion into either the plasma membrane or nuclear membrane has the same electrophysiological properties such as Channel kinetics, mean open times, and open probabilities [114]. This is so, even though the intracellular localization of the CLIC1 protein varies in different cell types, such as polarized and non-polarized cells. In non-polarized cells like basal epithelial cells of the upper gastrointestinal tract, skeletal muscle, as well as cultured Panc1 cells, the expression of the CLIC1 protein is in a non-polarized distribution within the cytoplasmic environment. Whereases, in T84 cells, distribution of CLIC1 is quite different from non-polarized Panc1 cells. Indeed, The T84 cells express the CLIC1 protein in a polarized distribution with a large membrane-associated distribution [140].

1.5 Structural similarities between CLIC proteins and the Glutathione-S-Transferase, Thioredoxin and Glutaredoxin enzyme families

1.5.1 CLIC1 protein is members of the Glutathione -S- Transferase superfamily

The monomeric chloride intracellular ion channel 1 protein has structural homology to the canonical cytosolic GST fold family, most notably it bears the closest similarity with the GST omega class [4], [3] (figure 1.11). Structurally, monomeric CLIC1 is composed of two distinct domains: an all-helical C-terminal domain that is most similar to the GST-omega class, as well as the N-terminal domain (residues 1–90), which is analogous to the thioredoxin fold and consists of four beta-strands along with three alpha helices.

The N-terminal domain also holds similarity to the glutaredoxin family (which are distinct members of the GST superfamily) as it contains the monothiol motif Cys-Pro-Phe-Ser [4]. It has been indicated that the monothiol active site of CLIC1, with cysteine 24 residue (Cys-X-X-Ser), can covalently bind to Glutathione (GSH) in a manner analogous to the GST-omega class which also contains a monothiol G-site, Cys-Pro-Phe-Ala [80].

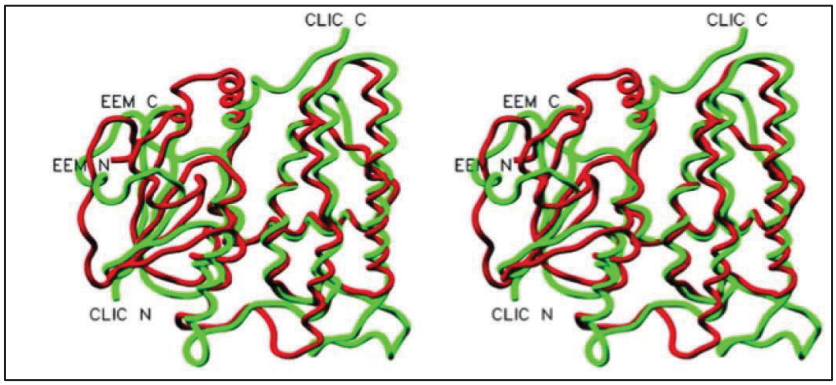


Figure 1.11: This figure demonstrates a stereogram of the structural homology between CLIC1 and GST omega class of protein. The CLIC1 structural superposition is shown in green where the red ribbon represents the GST- omega structure [4].

1.6 Function of the CLIC proteins:

1.6.1 Evaluation of the CLIC protein structures as novel enzymes

Like the Glutaredoxin (Grx) protein family, all six of the human CLIC family members contain what appears to be a redox active site motif (Cys-Pro-Phe-(Ser/Cys)) (figure 1.12 A) that is located in the N-terminal domain (specifically at the amide terminal of alpha helix (h1)), which is shaped into the classical thioredoxin fold. As such, this region was proposed to be the active site of an enzyme, which uses GSH as the cofactor for its activity [4, 133, 135, 158-160]. In the GST-Omega class proteins, a similar site exists [80] which suggests that the CLIC's active site might have more features akin to the GST fold specifications. In this regard, in order to evaluate the possible function of the CLIC proteins as novel enzymes, further details regarding the structural similarities between the CLIC and the GST families has been reviewed in the following sections.

To begin with, as it was mentioned before, orthologue members of Intracellular chloride ion channel proteins exist in both vertebrate and invertebrate species, which are subdivided

into different clades (figure1.13 A)[134]. Accordingly, the cysteine active site region was found in various clades by using representative crystal structures and in doing so, the results showed structural superposition with the CLIC structures. The clades can be subdivided into two categories based on the structural configuration of the motif (figure1.13 A)[134].

In addition, the plant dehydroascorbate reductase (DHAR) proteins are the most closely linked clade to the human CLIC proteins [161]. For example, *Arabidopsis thaliana* DHARs, specifically the AtDHAR1 protein was shown to have similar structural features to CLIC proteins [137]. Besides, dehydroascorbate reductases are unique proteins in which, they exist as a plant-specific GST class with defined endogenous functions. In addition, these proteins and some of the GST's (GSTL1, GSTL2and GSTL3), which in many ways resemble the DHAR class, contains a conserved active site cysteine residue and GSH binding site. And as a result, they have the ability to catalyse a typical GST catalytic reaction and therefore act as an enzyme [161].

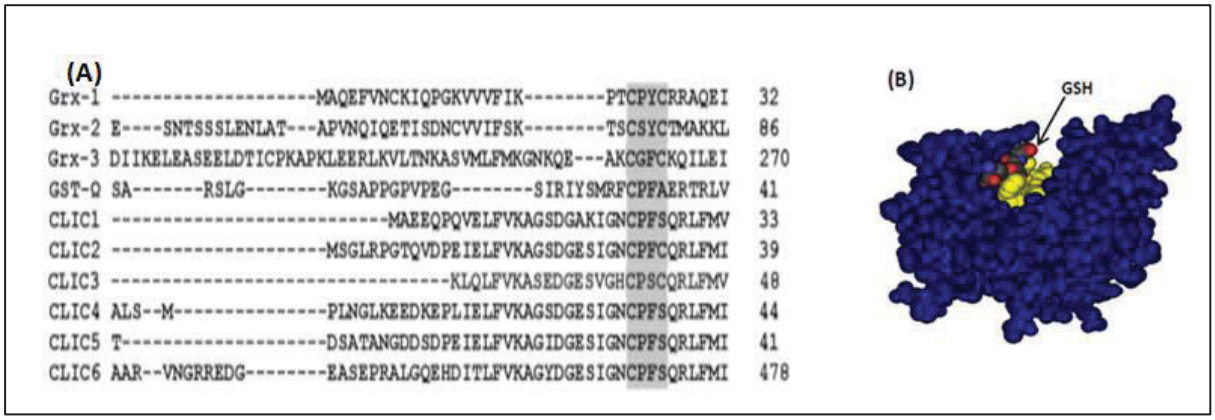


Figure 1.12: CLIC proteins and their GSH binding site; (A) show the amino acid sequence alignment of the human Grx1-3, human CLIC members and human GST omega. The amino acids marked in red are conserved glutaredoxin/thioredoxin-like motifs, which have a catalytic cysteine residue. (B) demonstrates the CLIC1-GSH complex. In this complex, the GSH binding site (G-site) is positioned nearby to another binding site (yellow) in CLIC1 structures [5, 162].

In fact, when the amino acid residues at the catalytic active site in both CLIC members and GSTs were compared to each other, this becomes evident that some GST proteins, which have cysteine residues at their catalytic site, are not matched with the CLIC active site motif. These proteins are namely the bacterial GSTs (GSTB), maleylacetoacetate isomerase (GSTZ), insect GSTs (GSTD) as well as plant GSTs (GSTF) (figure1.13 B)[134]. Where the remainder of

the clades members together with GST-omega class and CLICs shares a region that coincides with the common motif Cys-Pro-(Phe/Tyr/Trp)-(Ser/Cys/Ala/Val)-X-Arg (figure1.13 B).
Indeed, the functions of proteins in some of these clades have been determined that are include the bacterial SspA, bacterial Grx 2, plant DHARs [137, 161], plant GST lambda (In2) [161], GSTOs [80] and yeast Gto [163]. These proteins exist principally as monomers except for the GSTs which are typically dimers and contain a GSH site state that is positioned at their dimeric interface[92]. The GST proteins that have an active site motif (Cys-Pro-(Phe/Tyr/Trp)-(Ser/Cys/Ala/Val)-X-Arg) and are classified in some of the most common characterised clades such as omega class GSTs (GSTO), function as enzymes by coupling the Glutathione peptide with a redox reaction [134], where, the target substrate could be either a small molecule or a biological macromolecule [80].

Accordingly, it was demonstrated that CLIC1 has a structural homology to the GST superfamily with a glutathione (GSH) redox-active site, which resembles glutaredoxin [4]. However, the crystal structures of CLIC1 and GSH complex indicated that GSH group is poorly ordered in the active binding site with a few contacts with CLIC1 protein due to its location in this slot[4]. Therefore, it speculated that this slot might be a binding region for an extended macromolecular chain, which is most likely a polypeptide. Therefore, this substrate linkage may alter the GSH binding region and subsequently induce stronger binding by forming a mixed disulphide connection with GSH molecule (figure 1.12 B)[134].

In addition, crystal structural studies of CLIC2 [133, 160] and CLIC4 [158, 159] have been shown that an internal peptide loop exists in the neighbourhood of the GSH moiety which in theory would assert that it is capable of incorporating with such substrate as described above. As such, based on the structural and sequence homology data that has amassed regarding the similarities between the vertebrate CLICs and the GSTs, it was speculated that the CLIC proteins would also likely have enzymatic activity, and would use GSH as a cofactor [4, 108, 134].

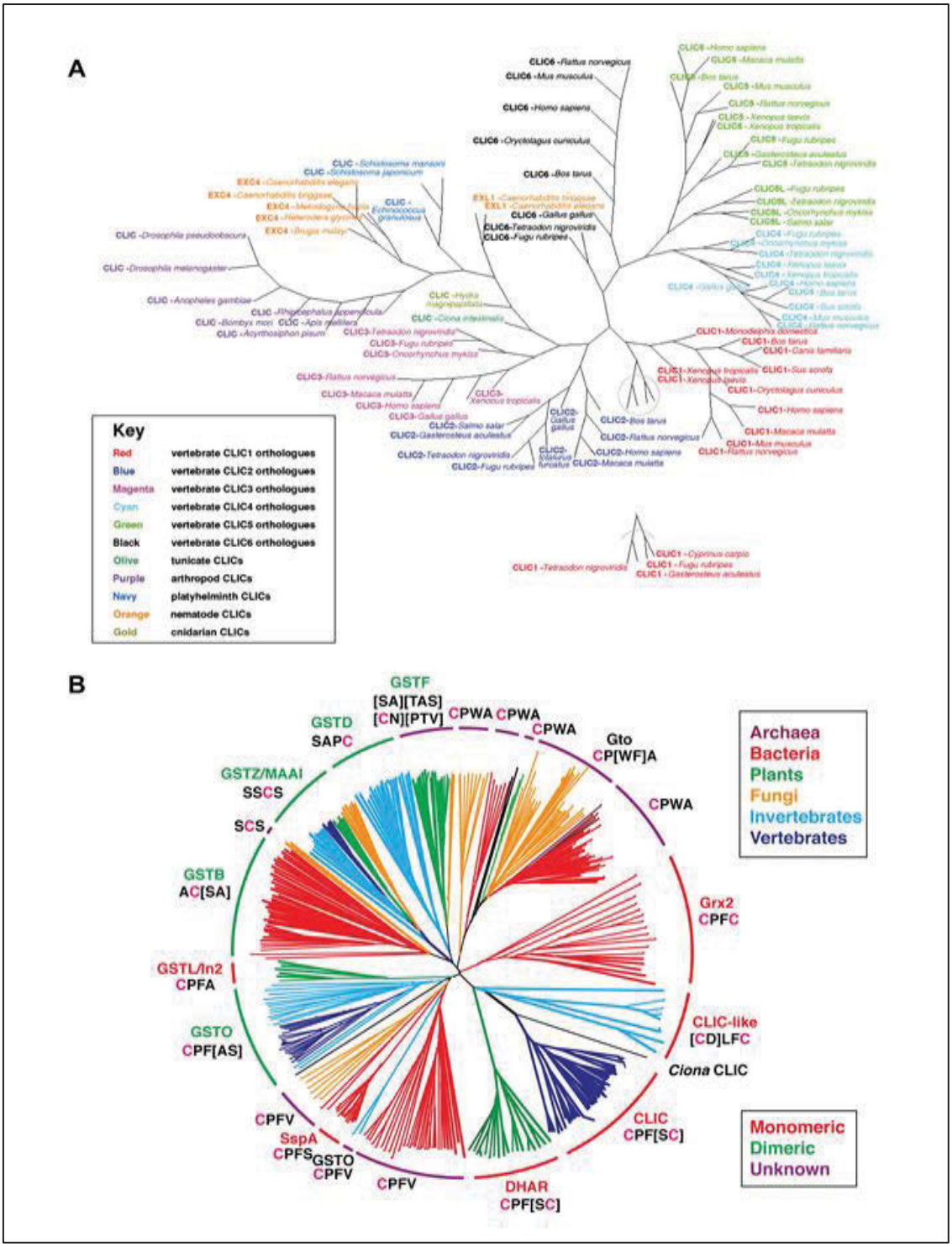


Figure 1.13: Phylogenetic analysis of Chloride Ion channel proteins (CLICs). (A) The representative of vertebrate CLIC and in vertebrate CLIC-like proteins in a phylogenetic tree (Clades classified with different colure). (B) The representative of proteins with GST fold and catalytic site cysteine in a phylogenetic tree (classification are demonstrated on the top right with different colure). For each clade, putative CLIC active site are specified with cysteines colored in magenta[134].

1.6.2 The putative active site of the invertebrate CLIC-like proteins and their enzymatic activity

As explained above all the members of the human CLIC protein family have active cysteine residue/s within their N-terminal domain that bears sequence similarity with the glutaredoxin family active site motif. The active site in human CLIC proteins has the sequence motif CP(F/Y/S)(A/S/C) which resembles the Grx sequence motif CP(F/Y)C [4, 164]. By comparing the invertebrate CLIC-like proteins with vertebrate CLICs, it appears that the invertebrate CLICs contain a distinct active site despite the fact that they cluster in the vicinity of the vertebrate CLIC clads.

In this regards, one of the explanations for this distinction is that the invertebrate CLIC-like proteins have (Cys/Asp)-Leu-Phe-Cys-Gln-Glu sequence[134]. On the one hand, it has been shown that both DmCLIC and EXC-4 are structurally more closely related to the mammalian CLICs compared to the GST-fold family members. For instant, the DmCLIC (drosophila CLIC) protein has two-cysteine residues in its motif CLFC, which is similar to the dithiol thioredoxin/glutaredoxin motif and located at the N-terminal domain of helix1. Also, the cysteine residues in this protein adopt a similar structural conformation with the same residues in the CLIC1 and CLIC4 proteins. However, the worm EXC-4 protein unlike DmCLIC possesses an aspartic acid residue in place of the amino cysteine residue and forms a D-X-X-C motif in its N-terminal of helix1. The crystal structure of EXC-4 revealed that all residues except the aspartic acid adopt a similar structural conformation to that of the CLIC proteins[135].

The second explanation is based on the crystal structural study, which also indicated that the invertebrate CLIC-like proteins (both EXC-4 and DmCLIC) could bind to metal ions, such as calcium or potassium ions, as part of their GSH binding site. Due to these two pointed features, one could assume that the invertebrate CLIC-like proteins would likely not exhibit enzymatic activity[135].

1.6.3 Enzymatic activity of CLIC proteins

Given all of the homology and structural evidence discussed above, it is not surprising that speculation arose that the soluble CLIC proteins would also have oxidoreductase enzymatic activity in addition to their membrane ion channel activity [4], [134]. Recently, our group at UTS provided for the first time, the experimental evidence in support of this hypothesis. These studies demonstrated the glutaredoxin-like enzymatic activity of soluble CLIC1, CLIC2 and CLIC4 [5] and more recently it was extended to include CLIC3[6], which showed that these four CLIC proteins exhibited similar enzymatic activity to the Glutaredoxin (Grx) proteins. The substrate, 2- hydroxyethyl disulphide (HEDS), which is the typical substrate used by the glutaredoxin proteins, could also be used by the CLIC proteins. Furthermore, this CLIC oxidoreductase enzymatic activity was also highly dependent on the presence of the glutathione molecule.

Given that CLIC1 and 4 have only one cysteine in their active site, they would likely perform a reaction similar to the GST-Omega protein, where via a Glutathionylation cycle they would form a mixed disulfide bond with the GSH molecule, followed by addition of GSH onto a target protein or onto low molecular weight thiol substrates. In so doing, they may function as detoxifying or stress response proteins within the cellular environment [165].

Moreover, as it has been shown that the human glutathione transferase Omega 1 (GSTO-1) also has the ability to catalyze the deglutathionylation of protein thiols *in vitro* and in cell culture, this suggested that the deglutathionylation activity of GSTO-1 would possibly influence the function of multiple proteins in signaling pathways. So in this regards, based on the CLICs structural similarities to this protein, this would suggest that the CLIC proteins may also be involved in such deglutathionylation reactions and therefore, possess a similar effect of GSTO1-1[165].

Indeed, the Glutathionylation cycle is a major post-translational modification that regulates the functional properties of target proteins. By this mechanism proteins from the Grx and the GST-omega class, have the ability to protect the cellular sulfhydryl homeostasis. Thus, in this manner the cysteine residues would be protected from oxidative damage and also the cellular homeostasis would be maintained [165].

Besides, based on work by Starke et al.,[166] the human Grx protein is involved in glutathionylation reaction in the presence of glutathione-thiyl radicals. Also in this study, it was reported that this protein is likely to be involved in catalysing the deglutathionylation, which is the reverse reaction of glutathionylation. In this respect, the reaction occurs following a monothiol mechanism, by which, only one active site cysteine is required in order to reduce the mixed disulfide between the target protein and glutathione (figure1.14)[167].

However, it has been shown that a disulfide oxidoreductase CGFS-type GRX3 from Chlamydomonas reinhardtii, has the ability to catalyze deglutathionylation, likely via a dithiol mechanism but without the use of external GSH[56]. So it is speculated that by such Glutathionylation mechanisms the CLIC proteins may control their own ion channel activity or membrane insertion, as well as the other cellular functions [134, 168].

The CLIC1 protein can bind and localize to a particular target molecule by using its G site (GSH binding site)[4] and also has the ability to co-localize in plasma membrane where it can function as ion channel protein [155]. Besides, it has been suggested that the enzymatic activity of CLIC proteins could be important in cell defence by protecting the cell from oxidative damage [5].

In recent work on the enzymatic activity of CLIC1, this protein was assayed in various experiments to study its substrate affinity, enzymatic activity as well as inhibition of this activity, by using different reductases, substrates and, inhibitors [5]. In this research, it was demonstrated that CLIC1 oxidoreductase activity is also capable of reducing both sodium selenite and dehydroascorbate (DHA) as substrates [5]. This catalysing reaction occurs via cysteine 24 in CLIC1 that is previously identified as a catalytic cysteine residue by mutagenesis experiments [5]. Moreover, the CLIC1 enzymatic activity inhibited by known CLIC channel inhibitor drugs IAA-94 and A9C. Thus, this study hypothesised that the functional property of CLIC ion channels could also affected by the enzymatic activity of its soluble form.

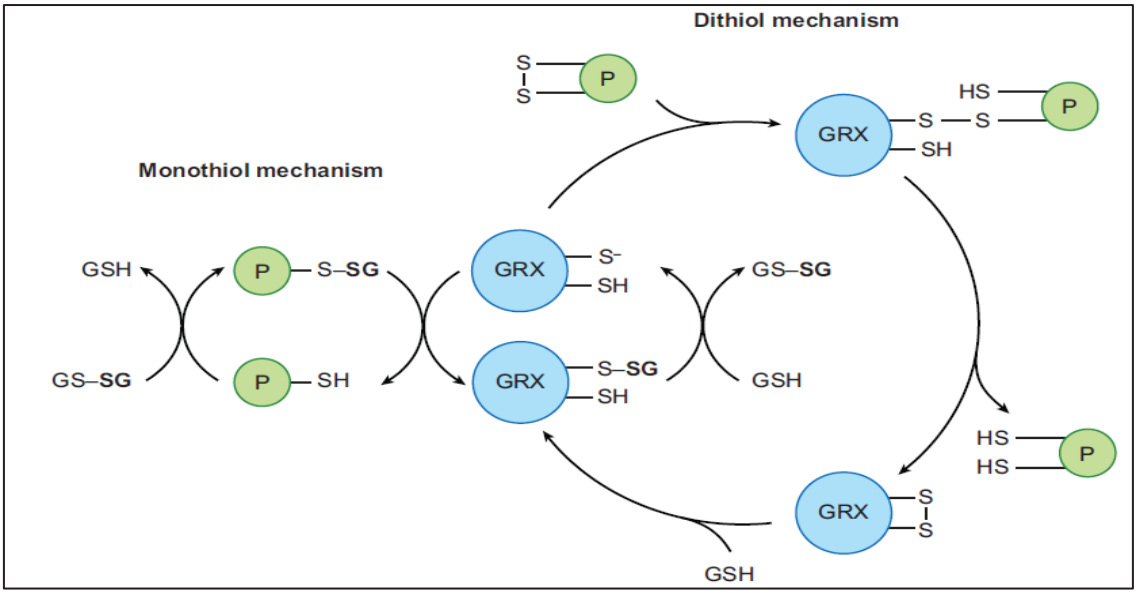


Figure 1.14: Glutaredoxin (GRX) catalytic mechanisms in both monothiol and dithiol forms. Grx proteins would reduce the disulfide bond or protein-glutathione adducts by use of glutathione molecules through two different mechanisms. For instance, two thiol groups of GRXs are needed in order to conduct the catalytic dithiol mechanism by which, an intramolecular disulfide bond would form between the two active site cysteines that is eventually reduced with reduced glutathione (GSH). Whereas, GRX in the monothiol mechanism which, used for deglutathionylation reactions, required only one active site cysteine in order to reduce the mixed disulfide bond between the protein and glutathione. Subsequently, this glutathionylated GRX would be regenerated by use of another Glutathione[169].

1.7 Aims of this research project

The novelty of the Chloride Intracellular Channel (CLIC) proteins arises from their existence in two distinct structural forms, which likely regulated by cell redox conditions that may also contribute to their different cellular localization, as both soluble globular and integral membrane proteins. Based on structural analysis, the CLICs structurally related to the glutathione S-transferases (GST) and glutaredoxin (Grxs) families by adopting similar canonical fold, which has a redox-active site region. Based on such analysis, our group at UTS recently demonstrated that members of the CLIC family in their soluble form have enzymatic activity similar to the Glutaredoxin proteins [5]. Therefore, the aims and objectives of this current Masters project are to:

- Further characterize the enzymatic function of members of the CLIC family (specifically: CLIC1, CLIC3, and mutant CLIC1-Cys24A). This characterisation will

include determining the optimal conditions for the enzymatic activity of the CLIC proteins, including pH and temperature. Such studies will also allow for the stability of the CLICs proteins' enzymatic activity to be assessed following storage of the proteins under different conditions. These studies will be undertaken using purified recombinant proteins in order to assess their function via such assays as the Glutaredoxin HEDS enzyme assay.

- Determine the enzyme kinetics of the various CLIC proteins in the HEDS assay under different conditions such as pH and temperature.
- Compare His-tagged versus non His-tagged recombinant CLIC proteins to determine the importance of the added tag as a region sensitive to environmental conditions such as redox, pH and temperature.
- Study of CLIC enzyme activity using various substrates and inhibitor drugs to determine if these optimal conditions are substrate dependent or not. This should provide insight into the possible physiological roles of the various CLIC proteins given differences in their cellular expression profiles and intracellular localisation.

Chapter 2

Study of the Oxidoreductase activity of His-tagged and Non His-tagged CLIC Proteins, Reveals Imidazole Can Act as a Substrate, while the “His-tag” acts on enzyme efficiency

2.1 Introduction

2.1.1 Expression of recombinant CLIC proteins

The recently discovered enzymatic activity of CLIC family members necessitates further characterisation of their activity. In order to do so, purification of recombinant CLIC proteins, include His-tagged and non His-tagged CLIC1, CLIC3 and mutant CLIC1 (cys24A), was essential. Recombinant proteins are derived by genetically engineering and manipulating cloned DNA which carries the encoding cDNA sequence of the protein of interest. The protein coding region (open reading frame) is cloned into a plasmid (vector DNA) that is designed to assist with the expression of the gene by the host cell.

Different expression systems can be used for the production of recombinant proteins that include: prokaryotic systems such as *Escherichia coli* (*E. coli*) and *Bacillus subtilis*, eukaryotic systems like yeast, insect cells, and mammalian cells or by using *in vitro* translation systems. Accordingly, in this project, the *E. coli* bacterial strain BL21 (DE3) in conjunction with the His-tagged pET28a vector (Novagen) (Figure2.1) were used in order to express recombinant CLIC (WT) proteins.

We chose *E. coli* because it has the advantage of easy manipulation, rapid growth as well as a low cost; as such it has become the preferred host for recombinant protein expression [170, 171] ever since its first use in 1972 by Paul Berg (Stanford University). His team together with a team led by Herbert Boyer (University of California, San Francisco), were able to transform *Escherichia coli* bacterial cells with a recombinant plasmid that later became established as 'Genetic Engineering Technology'. Since those first days, the technique has been developed and refined, leading to the expression and synthesis of the first human recombinant protein, recombinant human insulin (Humulin) in 1983 [172, 173].

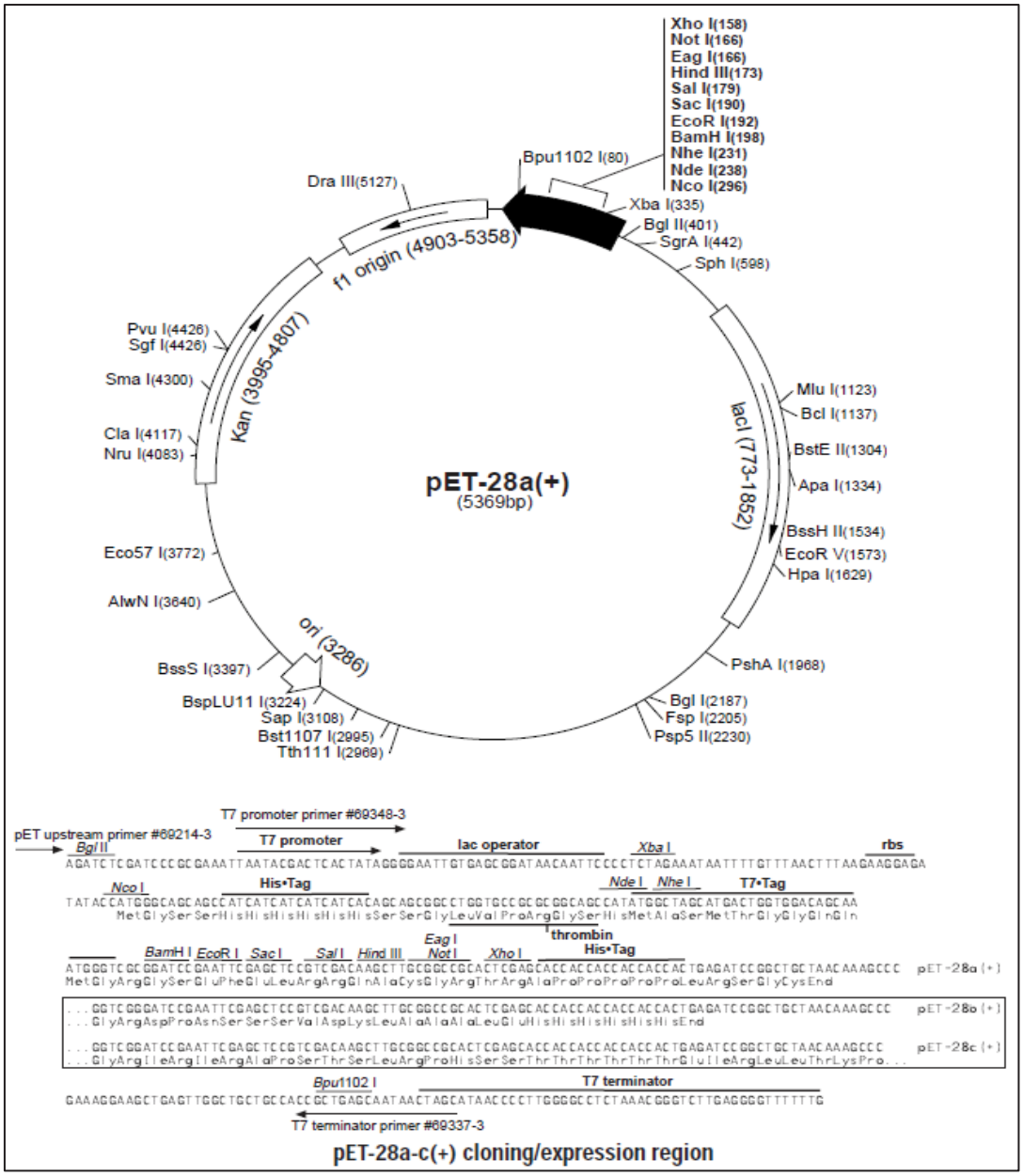


Figure 2.1: Map of the pET-28a (+) Vector The pET-28a (+) vector carries an N-terminal His-Tag configuration as well as an optional C-terminal His-Tag sequence (map is taken from Novagen company web page).

2.1.2 Affinity tags and affinity protein purification system

Protein expression systems modified and improved to provide additional functionality, including purification sequence tags that allow for subsequent localisation and visualisation of the protein of interest. Traditionally separation methods used for the purification of a recombinant protein from crude biological extracts based on protein physicochemical properties like its charge, size or hydrophobicity. These methods are often difficult and

time-consuming. For that reason, alternative simple methods developed, which known as affinity purification. These systems work based on a genetically engineered protein that is enriched by virtue of its particular property of an amino acid sequence that is appended to the protein as a 'tag'[174].

Several affinity tags now commonly used in chromatographic purification systems, which consist of protein domains, enzymes, or small polypeptide molecules and most of them bind to a variety of substrates with high specificity in order to permit quick and efficient purification of proteins. Examples of these affinity or epitope tags include:

The glutathione S-transferase or “GST tag” that is utilized in combination with glutathione–Sepharose beads [175]. The FLAG tag system [176] and the maltose-binding protein fusion system used with maltose beads [177]. The chitin-binding protein fusion system which is used together with chitin beads [178]. The epitope-tagging system that is utilized in combination with particular antibodies [179]-. The biotin tag fusion system used together with avidin–agarose [180], and finally the poly-histidine-tagging or “His-tag” that is used together with a metal chelate affinity chromatography system [181-188].

The advantage of the Histidine-tagging technique is that it can be applied under denaturating conditions, in contrast to other methods [189]. Although the affinity tags are designed to purify recombinant proteins, they are also used in diverse fields such as immunohistochemistry (IHC), immunoprecipitation (IP), western blot, protein localization and flow cytometry (FCM). However, all described tags have their own distinct benefits and drawbacks [190, 191], which should be considered dependent upon the application and downstream uses of the protein of interest.

2.1.3 An overview of poly-histidine-tag application in affinity chromatography system

The Histidine-tagging method, which is a polypeptide fusion system, was designed in order to assist the purification of recombinant proteins [192] through the use of a novel Metal Chelate Adsorbent method. This method is based on the selective interaction that occurs between a fused poly-histidine peptide ‘tag’ located on the target protein, and a Novel

Metal Chelate Adsorbent [183]. The immobilized metal chelate affinity chromatography (IMAC) method was originally employed for the fractionation of enriched proteins with histidine and reduced cysteine residues that contributed to metal binding [181].

The IMAC method is based on the interaction that occurs between transition metal ions (e.g., Co^{2+}, Ni^{2}+, Cu^{2+}, Zn^{2+}) and precise amino acid side chains [181]. Indeed, in this interaction, the Histidine residue is a potent amino acid, which has the ability to interact strongly with immobilized metal ion matrices. Thus, in the IMAC system only peptides that are comprised of sequential histidine residues (typically 6 histidines), are efficiently retained. Typically the system uses an IMAC resin to which 6 histidine residues sufficiently bind with high affinity to the Ni^{2+}-NTA resin (Kd=10-13, pH 8.0). Therefore, in order to purify proteins with histidine tag residues, after washing of the matrix in the column, the retained proteins that contain polyhistidine strands can simply eluted by one of the following two steps:
(a) by lowering the pH of the column buffer or (b) adding 20-250mM free imidazole a competitive counter ligand into the column elution buffer[182, 193].

In this system, the nitrilotriacetic acid (NTA) adsorbent, which has four chelating sites and is particularly suitable for metal ions with six coordination sites, permits a constant interaction among Ni^{2+} and the column resin and leaving two metal coordination sites remain free to reversibly bind with functional groups of proteins (figure 2.2) [183]. Accordingly, based on this system the length of the polyhistidine strands and the solvent system are two factors that affect the efficiency of the purification. An additional advantage of this method is that the Ni^{2+}-NTA resin has also a high capacity for protein interactions, with 5 to 10 mg protein per 1ml of resin. Following binding of the fusion protein to the resin, different concentrations of imidazole, which is a precursor of histidine, can be used (0.8 to 250mM) in order to elute the bound proteins. Addition of low concentrations of imidazole to the column (0.8mM) can decrease any non-specific interactions of host proteins with histidine amino acids.

As a result, to maintain an effective elution for 6 histidine-tagged proteins, it is recommended to use an imidazole concentration within a range of 20–250mM [186, 194].

It is important however to note that, the presence of imidazole in the elution buffer can result in several disadvantages such as protein aggregation, and it can also affect competition studies, crystallographic trials as well as NMR experiments [194].

The Poly-histidine tags usually positioned on either the N- or C-terminal part of the recombinant proteins, and the best position depends on the protein specification. A number of different studies have been performed using the poly-histidine tag method for protein purification in a variety of host cell species including mammalian cells [186, 195], bacteria [196, 197], yeast [198, 199] and baculovirus-infected insect cells [200, 201]. For several expression systems, different cloning vectors have designated in order to allow fusion of the Histidine-tag to either the N- or C-terminus along with protease cleavage sites, which permit the removal of the foreign amino acid tag from the protein of interest.

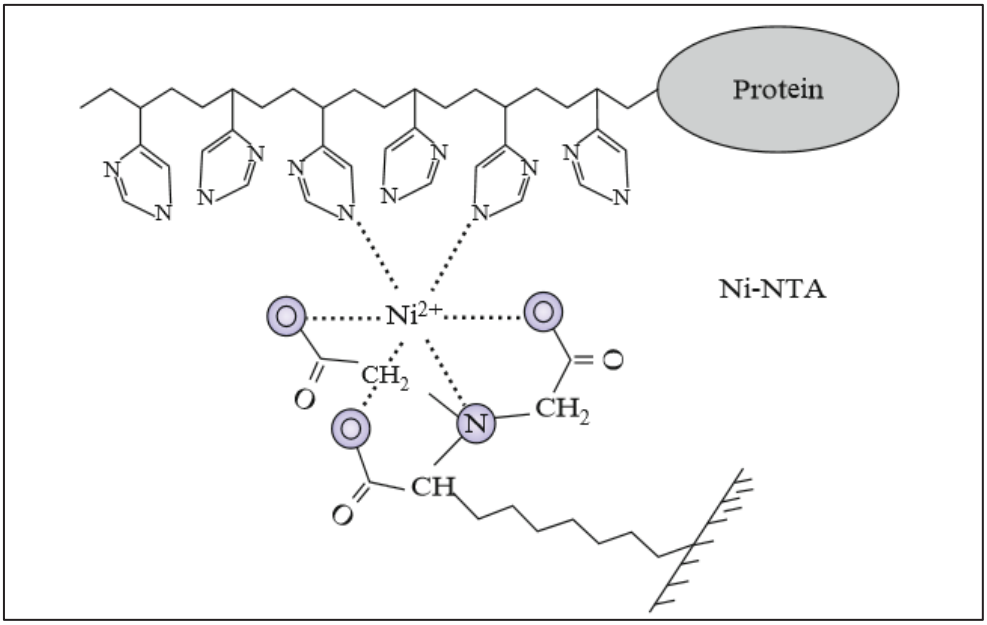


Figure 2.2: Model represents the interaction of "polyhistidine tag" residues of protein with the metal ion in tetra-(NTA). The Ni^{2+} is coordinated with four valencies which provided by NTA ligand (highlighted as spherical in this figure). Also, two free valencies remain available in Ni^{2+} in order to interact with imidazole rings of histidine residues of a tagged protein [202].

2.1.4 Size exclusion chromatography

In addition to affinity chromatography purification, alternate systems based on other physical properties of the protein sample can used to improve the purity of the final protein product. As such, partially purified proteins can be additionally 'polished' through processes such as Size Exclusion Chromatography (SEC), ion-exchange, hydrophobic interaction or hydroxyapatite chromatography [203-205].

Size Exclusion Chromatography (SEC), also known as gel filtration, gel permission or molecular sieve chromatography has developed gradually as a widespread characterisation chromatographic method for synthetic and natural polymeric materials, such as protein [206-209]. Accordingly, a seminal work has demonstrated that polymer molecule, regardless of their chemical composition and chain structure, would separate based on their hydrodynamic volume, which is defined by the Stokes radius[210] and in some cases would separate based on their molecular weight[211].

Size exclusion columns are composed of porous polymer beads, which designed with pores of different sizes. Therefore, in this regards, once a solution or a mobile phase passes through the column, the smallest particles take a longer time to travel down the column length due to their equilibration with more pores of the beads, in comparison to larger particles, which consequently results in their separation.

The first application of Size exclusion chromatography was introduced by Lathe and Ruthven, who used starch as a column matrix in order to separate substances such as sugars, amino acids and proteins and, estimation of their relative molecular sizes [212]. Later on, dextran was used as the stationary phase in order to conduct the separation [213]. Several applications of size exclusion chromatography have introduced so far. Examples of such applications include fractionation of proteins [214], determination and quantification of molecular size parameters and dimensions of membrane proteins[215, 216] as well as facilitating the identification of peptides[217]. In addition, the SEC is used in the biopharmaceutical industry for monitoring of protein or modified protein aggregation and quaternary structure [218].

When the SEC method is utilised for purification of proteins, some practical considerations need to be applied. For instance, the protein samples need to be injected in a small volume (1–2% of column volume) in order to gain sharper peaks, as migration causes peak expansion[216]. In addition, as purification of protein at low concentration is usually difficult, it is recommended that the protein sample is concentrated prior to loading into the SEC column[216].

2.1.5 Enzyme assays

Enzymes perform a vast range of chemical transformations. Hence, experimental enzyme assay procedures are used to make these enzymatic transformations visible and capable of being monitored. Such assays are essential in enzyme discovery, enzyme engineering, and the drug discovery process. In addition, these assays are important in the study of enzyme kinetics and enzyme inhibition.

Enzyme kinetics is the study of an enzyme's catalytic activity in a biochemical reaction that permits the classification and distinction of the individual enzyme. As such, kinetic studies allow us to obtain a better insight into enzyme catalytic behaviour through a systematic analysis, which involves studying several factors such as reaction rates, substrate, product and inhibitor concentrations, as well as the concentrations of any modifiers including pH and temperature [219, 220].

In this Masters study, an enzyme assay known as the HEDS assay was utilised in order to further define the oxidoreductase activity of CLIC protein members, which are newly discovered as catalytic enzymes. The bis(2-hydroxyethyl) disulfide (HEDS) assay, which was first introduced by Nagai and Black in 1968, was used for analysis of the catalytic activity of a Thiol-Disulfide Transhydrogenase from yeast[221].

The HEDS enzyme assay has since become a characteristic assay, commonly used in order to define several parameters such as presence, activity and enzyme kinetics of Glutaredoxin proteins as well as proteins with Glutaredoxin-like activity, from a variety of species [5, 70, 222-225]. The advantages of utilising the HEDS enzyme assay are twofold. First: the HEDS substrate is commercially available as an inexpensive disulfide substrate. The second it is an

easy spectrophotometric method monitoring the consumption of NADPH which reflects the glutathione disulfide (GSSG) formation, through a robust coupled assay owing to the fact that NADPH is consumed by glutathione reductase (GR)(Figure 2.3)[226].

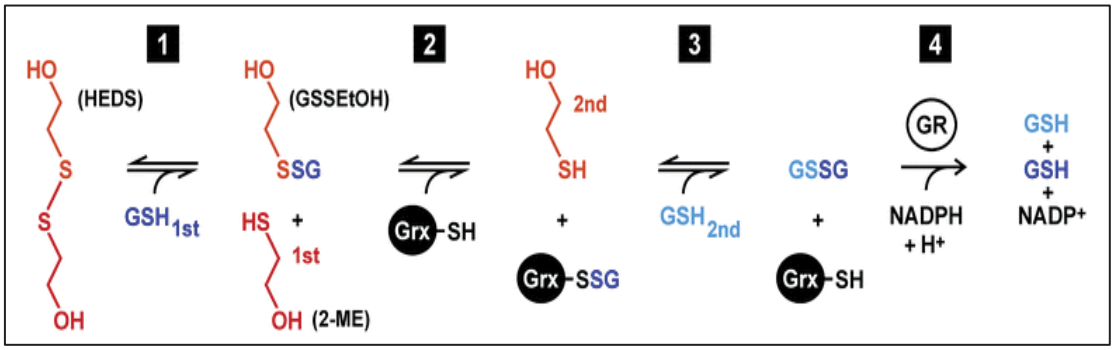


Figure2.3: The mechanistic model is representative of the HEDS enzyme assay system. In this assay system, the glutaredoxin protein acts as an enzyme by deglutathionylating the mixed disulphide between glutathione (GSH) and the beta-mercaptoethanol region of the HEDS substrate. Subsequently, the oxidised GSSG would be reduced again to GSH by the Glutathione reductase (GR), through catalysing the consumption of NADPH[226].

The HEDS substrate initially interacts with GSH in a non-enzymatic manner (reaction 1 in Fig. 2.3). After this interaction, the actual substrate of Grx enzyme would be the GSSEtOH, which is the mixed disulfide formed between GSH and 2-mercaptoethanol (2-ME). The Grx contains a conserved cysteine residue at its active site which would under reduced conditions exist in its thiolate form. During the course of the oxidative half-reaction, the thiolate part of Grx interacts with GSSEtOH and therefore becomes glutathionylated (reaction 2). Subsequently, the reduced Grx enzyme can be regenerated by a second GSH molecule through a reductive half-reaction of the ping-pong mechanism, which leads to the production of another Glutathione disulfide (GSSG) (reaction 3)[167, 227].

Several Grx proteins contain a second cysteine residue in their active site motif (CXXC) or have an additional cysteine in their GGC motif, which allows the enzyme to form a substitution intra and intermolecular disulfide bond [70, 228-231]. It has been demonstrated that mutation of this second cysteine residue in the dithiol Grx active site motif (CXXC), does not prevent the enzyme from performing its catalytic activity in the HEDS assay [167, 228, 229]. In addition, several studies have also confirmed this mutational analysis using a number of Grx isoforms and glutathionylated substrates such as L-cysteine-

glutathione disulfide (GSSCys)[13, 224, 231-235]. However, the dithiol ScGrx8 from yeast, which has only low enzymatic activity, is an exception to this theme, as the enzyme lost its activity when the second cysteine residue of active site motif (CXXC) was mutated [236]. Besides, it should be considered that several monothiol Grx-isoforms, which contain a CXXS active site motif, are typically inactive in the HEDS enzyme assay [56, 229, 230, 237, 238]. In this regards, a plausible explanation has been introduced for enzymatic inactivity of Grx isoforms, which point out that this inactivity could be due to structural peculiarities of the target protein or its geometric constraints[70].

So far, only two monothiol Grx-isoforms, the yeast ScGrx6, and ScGrx7; that localised in the endoplasmic reticulum and Golgi compartment [239, 240], have demonstrated considerable activity in the HEDS enzyme assay[54, 224, 236, 239, 240]. Both of these proteins that have the ability to form non-covalent dimers, contain a single cysteine residue per subunit and demonstrate structural similarities with dithiol Grx-isoforms[54, 224]. Thus these active and inactive isoforms of the Glutaredoxins provide useful tools to unravel the relationship between structure and function of these enzymes.

The overall aim of this Masters Project is to investigate the enzymatic profile of members of the CLIC proteins using recombinant purified His-tagged proteins. Therefore, the first part of this project was aimed at investigating whether the His-tag used in the preparation of the recombinant CLIC proteins, interferes with the functional activity of the purified fusion His-tagged protein by comparing it to the purified non-tagged protein.

As such, this chapter will describe:

- Defining the kinetic properties of the His-tagged CLIC1 and CLIC3 proteins;
- Evaluating the activity of the Histidine peptide in the HEDS Assay;
- Assessing the oxidoreductase activity of His-tagged CLIC1 in different storage buffers;
- Determining the effect of different concentrations of imidazole on the enzymatic activity in the HEDS assay of both His-tagged and non His-tagged CLIC1.
- Investigating the effect of the “Histidine tag” on the oxidoreductase activity of the CLIC1 protein in the HEDS assay

2.2 Materials and methods

2.2.1 2xYT media for bacterial growth

Standard microbial growth 2xYT medium was used for the cultivation of bacteria *E. coli*. This medium is comprised of yeast extract (10g), bacteriological tryptone (15g) and NaCl (5g) (Sigma Aldrich). These reagents were dissolved in sterile deionised water to a final volume of 1 liter and subsequently autoclaved for 40 minutes at 1.5kg f/cm^2 at 122°C.

2.2.2 Production of recombinant CLIC proteins

2.2.2.1 Small scale culture preparation

Glycerol stocks of *E.coli* BL21 (DE3) cells transformed with His-tag expressing pET28a vector (Novagen) containing the coding sequences for either the protein CLIC1, CLIC3 or mutant CLIC1-Cys24A were used to inoculate individual sterile conical flasks containing 20ml of 2xYT media and 20ul of kanamycin antibiotic with stock concentration of 30mg/ml (Sigma Aldrich). These were left to grow overnight in a shaking incubator set at 200 rpm, at 37°C.

2.2.2.2 Large scale culture preparation and protein induction

The entire volume of the small scale cultures as described above was subsequently added to individual large flasks that contained 350 ml of 2xYT medium and 30mg/mL kanamycin. After that, the flasks were left to grow in a shaking incubator at 200 rpm at 37°C for 1.5 hours. Subsequently, once the bacterial growth achieved an optical density (OD) at the 600nm value between 0.6-0.8, 1mM of IPTG (Isopropylthiogalactoside) was added to the media to induce the expression of the His-tagged protein. Finally, the bacterial cultures were incubated at 20°C for a further 16 hours with shaking at 200 rpm.

2.2.3 Harvesting the induced *E-coli* bacterial cells

The induced cells were subsequently harvested by centrifugation using a Hitachi high-speed refrigerated centrifuge fitted with a rotor R13A at 8000 rpm for 30 minutes at 4°C. The resulting bacterial pellets were collected and pooled in a 50mL falcon tube and resuspended

in about 15 ml of lysing buffer (300mM NaCl, 50mM potassium phosphate buffer pH 8.0 and 5mM Imidazole).

2.2.4 Lysing of *E-coli* bacterial cells

The resuspended E-coli cells were subsequently homogenized by an ultrasonic method using a sonicator (Sonics & Materials Company / Vibra-Cell Ultrasonic Liquid Processors) for 15-20 seconds per cycle at 700 psi; until the viscosity of solution became greatly decreased. Afterward, 20% Triton solution (8ml H_2O added to 2ml of TritonX-100) was prepared from the original stuck of TritonX-100 and then 1 ml of the 20% Triton solution was added to 25 ml of bacterial homogenites. The resulting soluble lysates cells were then collected after further centrifugation at 10000 rpm for 40 minutes at 4°C.

2.2.5 Purification of His-tagged CLIC1, CLIC3 and mutant CLIC1-Cys24A protein using Ni^{2+}-NTA resin

Adapting an affinity chromatography method described earlier in this chapter (2.1.4), purification of Histidine-tagged CLIC1, its mutant His-tagged CLIC1-Cys24A and His-tagged CLIC3 proteins was performed using a Ni^{2+}-NTA Affinity resin (Biorad). Three main steps were involved: First, crude bacterial cell lysate samples were incubated with a slurry of washed Ni^{2+}-NTA beads (Qiagen) to allow the CLIC proteins to bind to the immobilized ligand. Second, any non-bound molecules were washed from the beads. Finally, the target protein molecules were eluted from the immobilized ligand by changing the buffer conditions.

Specifically, in the case of His-tagged CLIC1 and its mutant His-tagged CLIC1-Cys24A ; 2ml of the Nickel beads were prepared by first washing with 10mL of elution buffer (250mM imidazole, 300mM KCl, 50mM potassium phosphate buffer pH8, 0.5mM TCEP), followed by a second wash with 10ml of wash buffer 2 (10mM imidazole, 300mM KCl, 50mM potassium phosphate buffer pH8, 0.5mM TCEP) and then the final wash with 15ml of wash buffer 1 (5mM imidazole, 300mM KCl, 50mM potassium phosphate buffer pH8, 0.5mM TCEP). After that, the soluble fraction of the bacterial cell lysate was added to the prepared Ni-NTA bead

and left to incubate overnight at +4°C on a rocker, which will result in the “His-tagged” part of the CLIC1 or CLIC3 or their mutants to bind with the nickel beads.

The resin slurry was then loaded into a small affinity column and allowed to settle, after which it was washed using gravity flow with 10ml of wash buffer 1 (5mM imidazole, 300mM KCl, 50mM potassium phosphate buffer pH8, 0.5mM TCEP) and then 10mL of wash buffer 2 (10mM imidazole, 300mM KCl, 50mM potassium phosphate buffer pH8, 0.5mM TCEP), in order to remove unbound proteins and impurities.

Finally, the His-tagged CLIC1 and its mutant (Cys24A) protein fraction were individually eluted from the nickel column by adding 2 ml elution buffer (250mM imidazole, 300mM KCl, 50mM potassium phosphate buffer pH8, 0.5mM TCEP) through the column. The eluted protein fraction was subsequently stored in the same elution buffer at -80 °C freezer, or run through a column size exclusion chromatography (SEC) system following the process described in section 2.2.6 from the point of injection onto the SEC column.

However, in the case of His-tagged CLIC3 purification process the same condition as described above was followed. But, different buffers have been used in order to purify this protein, which are include: lysing buffer or wash buffer1 (200mM NaCl, 5mM Imidazole, 20mM HEPES/pH7.5, 0.5mM TCEP), binding buffer or wash buffer 2 (200mM NaCl, 10mM Imidazole, 20mM HEPES/pH7.5, 0.5mM TCEP) and elution buffer (200mM NaCl, 400mM Imidazole, 20mM HEPES/pH7.5, 0.5mM TCEP).

2.2.6 Enzymatic cleavage of His-tag from CLIC protein followed by purification using size exclusion (gel filtration) chromatography

In order to remove the His-tag from the recombinant protein, thrombin enzymatic cleavage was employed prior to the elution step in the method described above. The procedure involved the addition of bovine plasma thrombin enzyme (30 NIH units per 1L of original bacterial cell culture) (Sigma Aldrich) to the resin bound His-tagged protein and this was left incubating overnight at +4°C.

The cleaved non-His tagged protein was collected as 4 x 1.5 mL fractions by washing the resin beads with PBS buffer (10mM phosphate buffer,2.7mM KCl, 140mM NaCl, pH 7.4, and

0.5mM TCEP) in order to elute the protein from the column. As the cleaved CLIC protein from the affinity chromatography column was only partially purified and contained thrombin enzyme, it needed to pass through a size exclusion column system (AKTA Explore /Amersham Pharmacia Biotech). This column has size and dimension of 60 cm × 16 mm (HiPrep™ 16/60 Sephacryl® S-100 HR/ Sigma Aldrich) and set up in cold room at +4°C.

The system was run overnight at a pressure setting of 0.15 MPa with a flow rate of 0.3ml/min using column sizing buffer (100mM KCl, 1mM NaN_3, 20mM HEPES pH 7.5 and 0.5mM TCEP) resulting in separation of the monomeric CLIC1 (WT) proteins from the thrombin and other contaminants. Purified protein samples were stored in column sizing buffer at -80°C freezer.

In addition, For purifying of His-tagged CLIC1 and His-tagged CLIC3 proteins using the Size Exclusion Chromatography method, the obtained protein sample from affinity column that was eluted by elution buffer with imidazole concentration of (250mM) and (400mM), respectively, were directly injected to the size exclusion column (SEC) in two separate experiment; without using thrombin enzymatic cleavage. (The same protocol as described above was given to the purification AKTA system, and the purified sample were stored with the similar condition). Besides, the non-Histidine tagged CLIC1-Cys24A was kindly provided by my group at UTS [5].

2.2.7 Protein quantification using UV-Vis spectrophotometer

Spectrophotometry is a quick measurement by which the quantity of a chemical substance that absorbs light is measured, so, this method is defined based on the intensity of light as a function of wavelength that passes through the sample solution. Usually, the proteins concentrations are determined by their absorbance at 280nm, mainly contributed by the aromatic amino acids residues tryptophan (Trp) and tyrosine (Tyr). Accordingly, by using a Nanodrop spectrophotometer (Thermo Fisher Scientific) the concentrations of the eluted CLIC protein fractions that pooled from the high-affinity chromatography column and size exclusion chromatography column were measured at 280nm wavelength. The blank was against the protein diluent buffer (column sizing buffer (100mM KCl, 1mM NaN3, 20mM HEPES pH 7.5 and 0.5mM TCEP) for purified protein after SEC and elution buffer (200mM

NaCl, 400mM Imidazole, 20mM HEPES/pH7.5, 0.5mM TCEP) for purified protein after affinity chromatography). The concentration was calculated based on the Beer-Lambert law:

$$c = \frac{\lambda_{280}}{\varepsilon_{280 \times l}}$$

In this equation, C represents the concentration of the protein in (mg/ml). Where, the λ280 indicates the protein absorbance at 280nm; ε is the extinction coefficient in ($cm^{-1}.mg^{-1}$. mL), which defines the power of a substance in light absorption at a given wavelength, by mass density or by molar concentration (extinction coefficient for CLIC1 is 0.6 $cm^{-1}.mg^{-1}$ and for CLIC3 is 0.4 $cm^{-1}.mg^{-1}$. ml), and finally l represents the path length in (cm).

2.2.8 Determination of molecular mass using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis is an efficient separation technique extensively used in the determination of the molecular weight (MW) of biological macromolecules, such as polypeptides and proteins.

In this procedure, the protein mixture is first denaturated, then mixed with sodium dodecyl sulfate (SDS), an anionic detergent, which can coat the protein samples with a uniform negative charge. Therefore, the proteins which are loaded onto a SDS gel matrix may migrate along an electrical field, and the molecular weights of the proteins or peptides will determine the distance they migrate, independent of the original charge of the protein[241].

In this study, the reducing SDS page gel (4–15% Mini-PROTEAN® TGX Stain-Free™ /Biorad, Australia) were used in a BIORAD Mini-protean gel electrophoresis system. Pre-stained protein ladders as reference molecular weight markers (Bio-Rad) were also used. Proteins in the gel were visualized by staining using Coomassie brilliant blue stain (Bio-Rad).

2.2.9 Enzyme assay

In this work, the condition for performing the enzyme assays was based on using a flat 96-well plate that contains a final volume of 200µL of objective reagents with an absorbance

reading at 340nm using a BioTek PowerWaveTM Microplate Spectrophotometer. All data were analysed using Microsoft Excel 2010 and GraphPad Prism 7.

2.2.9.1 HEDS (2-Hydroxyethyl disulphide) enzyme assay

Previous studies in our group have demonstrated oxidoreductase activity of the CLIC family in HEDS enzyme assay[5]. In this project, based on the protocol described in [99], HEDS enzyme assay was performed separately for both His-tagged and non His-tagged recombinant CLIC1, CLIC3 and mutant CLIC1 Cys24A (WT) proteins in the presence of glutathione reductase with concentration of the 5ug/ml. In this Assay, the reduced monomeric His-tagged CLIC1, CLIC3 (WT) and mutant CLIC1-Cys24A protein were used as test proteins while HcTrx5, which was obtained from the parasitic nematode, Haemonchus contortus and is known as a thioredoxin-like protein, was used as positive control. The same final concentration of 10uM for each protein was added to a potassium phosphate buffer (5mM/pH7) that contained EDTA (1mM), NADPH (250uM), HEDS (1mM). Then, glutathione reductase (0.5ug/ml) was added to the solution after the addition of proteins. Subsequently, a 5-minute incubation period at 37°C was applied to the mixture. Finally, after this incubation period, GSH (1mM) was added to the mixture as the final reagent to initiate the reaction by which the absorbance of NADPH decreased at $A_{340\ nm}$ as result of the decrease in its consumption.

2.2.10 The enzymatic kinetics of the His-tagged CLIC1 and CLIC3

The enzymatic kinetics of the His-tagged CLIC1 and CLIC3 were determined using different concentrations of the HEDS substrate (0, 0.25, 0.5, 1, 2, 4 and 6mM final concentration) with a fixed concentration of GSH peptide (1mM) and a fixed concentration of enzymes (10uM). The apparent Km and Vmax value were calculated from the Michaelis-Menten plot using Microsoft Excel 2010 and GraphPad Prism7.

2.2.11 “Histidine tag” purification and evaluation

The 6 amino acid polyhistidine tag was used in the HEDS assay. Two versions were used, one in elution buffer and one in column sizing buffer. The same final concentration of 10uM of this peptide was added to the mixture of 5mM potassium phosphate buffer that contained

the same conditions and reagents that are described in the HEDS enzyme assay procedure (material and method chapter 2 section 2.2.9.1). By using affinity chromatography protein purification method, we aimed to purify the target protein without its Histidine-tag, and then finally remove the "tag" from the column and keep it in elution buffer and column sizing buffer for the forthcoming experiment.

To do so, thrombin enzyme (30 NIH units per 1Liter of bacterial growth) was added to the nickel column to digest the His-tag from the target protein, which was bonded to the nickel ion via its His-tag part. As a consequence, the histidine tag was still attached to the resin, while the thrombin and the CLIC protein were in solution. Therefore, 1x PBS buffer was used to wash these two proteins from the nickel column. The poly-histidine tag was then eluted from the column with 250mM elution buffer. After this stage, some his-tag was kept in elution buffer for further evaluation. For the second condition, ultrafiltration spin column was used in order to remove the entire elution buffer. Then, the next step followed by washing the column membrane with column sizing buffer in order to remove the sticked "His tag" peptides from the membrane and finally keeping them in column sizing buffer.

2.2.12 Assaying the effect of different storage buffer on the catalytic activity of His-tagged and non His-tagged CLIC1

His-tagged and non His-tagged CLIC1 samples were prepared as previously described in this chapter sections 2.2.5 and 2.2.6, respectively. Where, the second protein sample for this experiment was prepared by using the ultrafiltration spin column; based on the same method, which conducted for "His tag" peptides preparation with column sizing buffer as described in previous section, in order to remove the elution buffer and storing the His-tagged CLIC1 protein in column sizing buffer that contains 20mM HEPES (pH7.5), 100mM KCl, 1mM sodium azide and 0.5mM TCEP. Therefore, prepared protein samples were examined in the HEDS enzyme as described in this chapter section 2.2.9.1.

2.2.13 Enzymatic activity of His-tagged and non His tagged CLIC1 proteins in the presence of different concentration of imidazole in HEDS Assay

In this experimental assay the purified CLIC1 protein from size exclusion chromatography, which was stored in column sizing buffer, and His-tagged CLIC1 protein sample that was stored in elution buffer (250mM) after affinity chromatography purification; were used and evaluated as test protein samples in standard HEDS enzyme assay. In this matter, final concentrations of 10, 20 and 50 mM of imidazole compound were added to the mixture that contained 5mM potassium phosphate buffer (pH7+ 1mM EDTA), 250uM of NADPH, 1mM HEDS, 0.5ug/ml glutathione reductase (GR) and finally 10uM of non His-tagged CLIC1 or His-tagged CLIC1 protein.

2.2.14 Evaluation of both His-tagged and Non His-tagged CLIC1 after SEC purification in the HEDS assay

In order to confirm whether the “Histidine tag” would interfere with the catalytic activity of the CLIC1 enzyme, batches of both His-tagged CLIC1 and non His-tagged CLIC1 were prepared as it is described in material and method section 2.2.2. In order to treat both proteins in the same way, both His-tagged and non-His-tagged proteins were subjected to purification through first affinity chromatography, followed by separation on a size exclusion chromatography column, as described in material and method sections 2.2.5 and 2.2.6. Both proteins that were purified using the same conditions were then, characterised using the standard HEDS assay as outlined in section 2.2.9.1 of this chapter.

2.3 Results

2.3.1 Characterisation of His-tagged CLIC1 and CLIC3 protein fractions after Ni^{2+}-NTA affinity chromatography

As described in material and method sections 2.2.5 and 2.2.6, we used a two-step procedure for the CLIC protein purification, a Ni^{2+}-NTA affinity chromatography followed by a size exclusion chromatography. We monitored the protein contents after the initial Ni2+-NTA by a spectra absorbance of eluted fractions.

The result shown in Figure 2.4, are representative traces demonstrating the absorbance profile measured over a range of wavelengths from 200 – 350 nm, for the His-tagged CLIC1 eluted fractions, collected from the Ni^{2+}-NTA Affinity column. The most concentrated protein fractions (first three samples) from this stage were pooled and injected onto the SEC column. A similar process was followed for rCLIC3 purification.

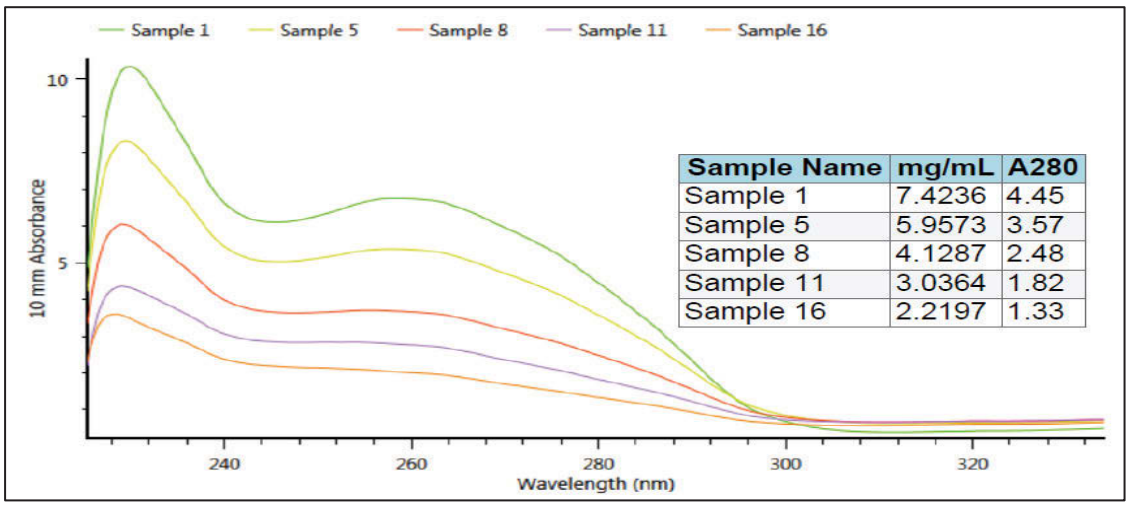


Figure2.4: A representative absorbance profile for the eluted fractions of the monomeric His-tagged CLIC1 (WT) purified by Ni^{2+}-NTA Affinity chromatography method.

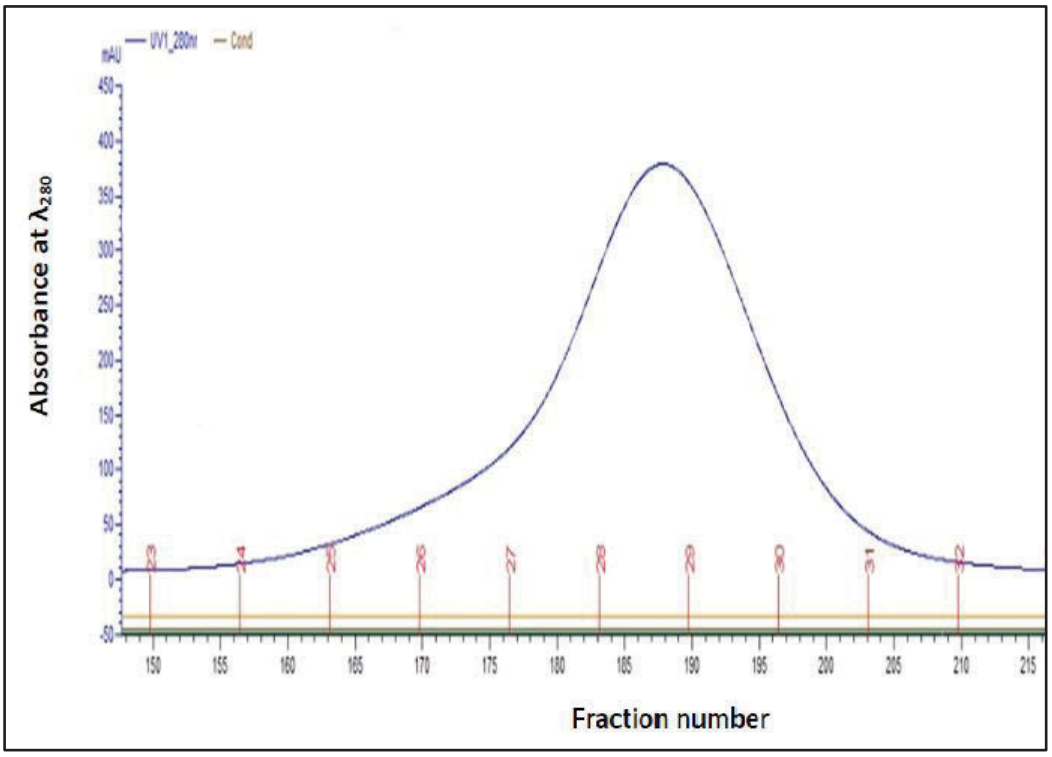
2.3.2 Size exclusion chromatography of recombinant proteins

Following initial affinity purification of the recombinant proteins (rCLIC1 and rCLIC3) using a Ni^{2+}-NTA Affinity column, as seen in Figure 2.2, rCLIC1 and rCLIC3 were then further purified following thrombin enzyme digestion, to remove the Histidine-tag by passing through a size exclusion (gel filtration) Chromatography column. In the case of CLIC1, a proportion of the protein His-tagged CLIC1 sample was kept and mock digested, no thrombin enzyme was

added. It was then run through the SEC column, in order to prepare His-tagged-CLIC1 that had been processed in an equivalent manner to rCLIC with no His tag.

Following thrombin digestion, CLIC1 (WT) monomeric protein was eluted from the SEC column as a single peak, as monitored at Absorbance of 280nm, with fractions 27 to 30 pooled together (Figure 2.5A). Where, figure 2.5B indicates the purification of monomeric CLIC3 (WT) protein, post thrombin digestion that was eluted between fractions 50 to 60, with these fractions collected and pooled. The preceding shoulder seen between fractions 28-47 likely represent aggregates of the CLIC3 protein, which may have become oxidised to cause multimerisation of the protein. The samples had been maintained in a reduced environment with all buffers containing TCEP; however, some oxidation may arise due to the lengthy SEC purification procedure.

A



B

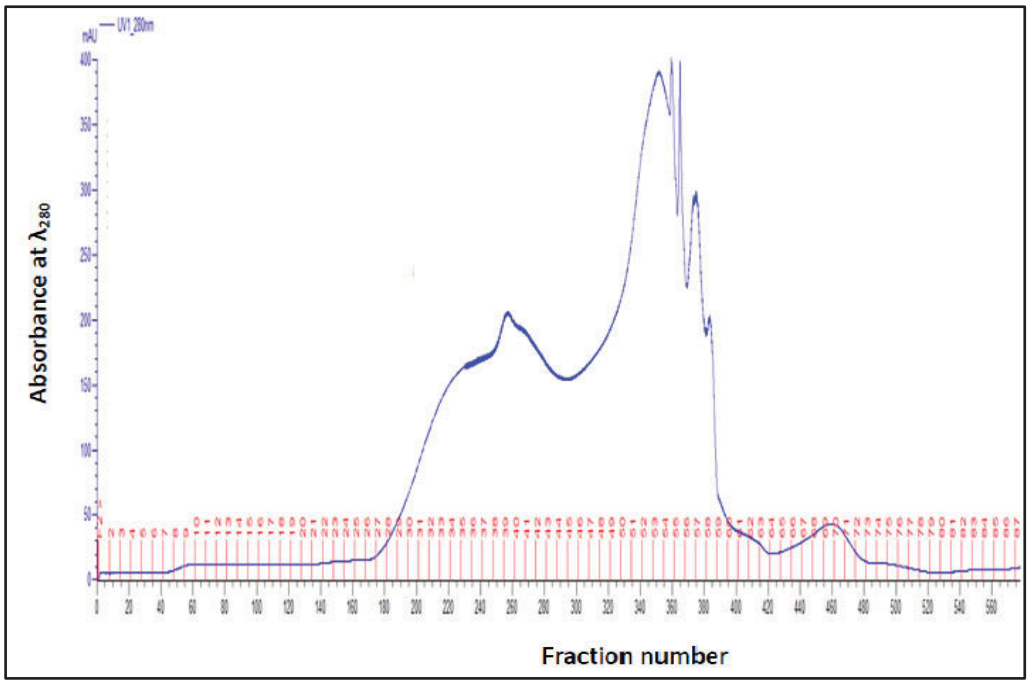


Figure 2.5: The graphs are representative profiles of the eluted monomeric CLIC1 and CLIC3 (WT) purified by size exclusion chromatography method. Figure A represents the eluted soluble monomeric CLIC1 (WT) protein at an absorbance of 280nm. Where, figure B is demonstrating the purification of monomeric CLIC3 (WT) protein following the same conditions.

2.3.3 Determining the purity of eluted His-tagged CLIC1 and CLIC3 proteins by SDS PAGE.

SDS PAGE electrophoresis was conducted in order to determine the purity and confirm the correct molecular weight, following purification of all proteins through the affinity chromatography and size exclusion chromatography columns. As it seen in Figure 2.6, both His-tagged CLIC1 and CLIC3 that were purified first through the nickel column, demonstrate molecular weight around 28kDa, as estimated by the standard protein ladder included in the SDS page gel. In figure2.7, the SEC purified His-tagged and Non His-tagged CLIC1 show an estimated molecular weight of 27.9KDa and 26.9KDa, respectively. Where the molecular weight of Non His-tagged CLIC3 is marked as 26.7KDa. We also further confirm the identity of each protein via Western blot analysis which was not included in the thesis as this is done routinely in our laboratory. These proteins are used for multiple purposes by our group.

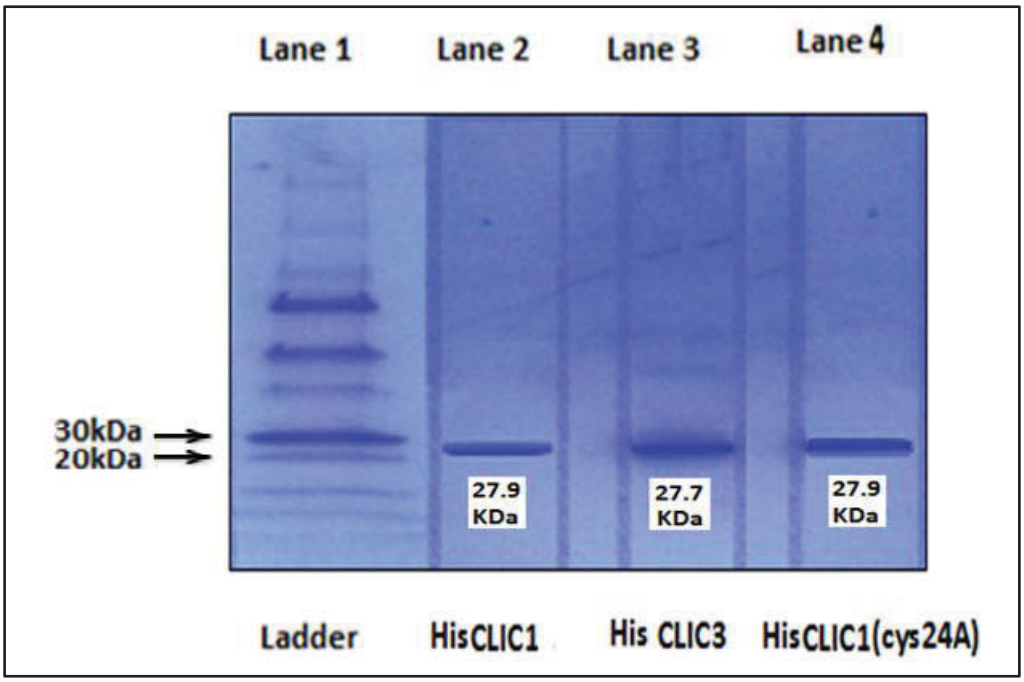


Figure 2.6: The SDS-PAGE result of purified monomeric His-tagged CLIC1 (WT), His-tagged CLIC1 (cys24A) and CLIC3 (WT) following Affinity chromatography method. In this figure, the lane1 is representing the standard Ladder with estimated molecular weights of proteins. Where, the lanes 2, 3 and 4 are representing the almost 28kDa His-tagged CLIC1, CLIC3 His-tagged and CLIC1 mutant Cys24A respectively, which purified through the affinity chromatography purification system.

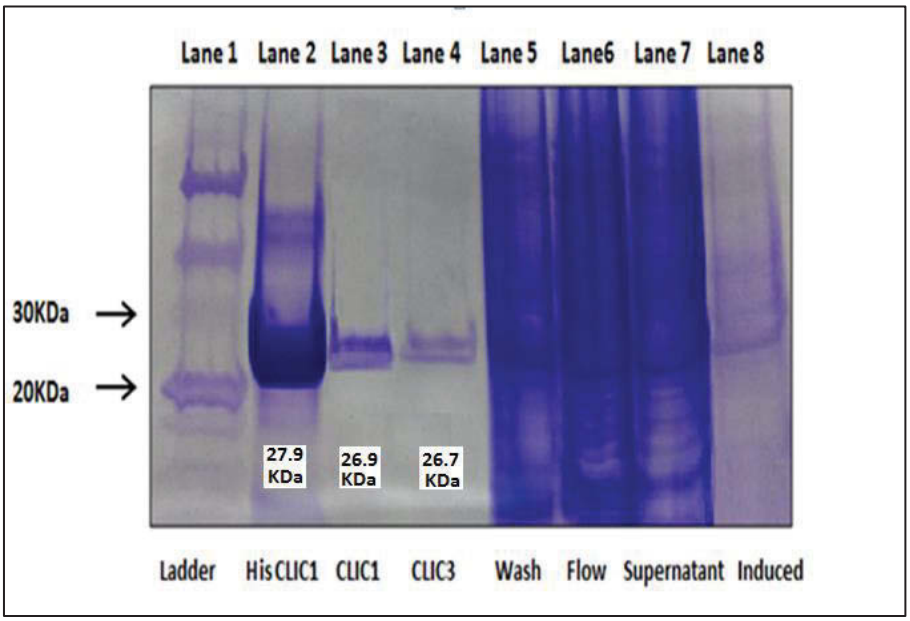


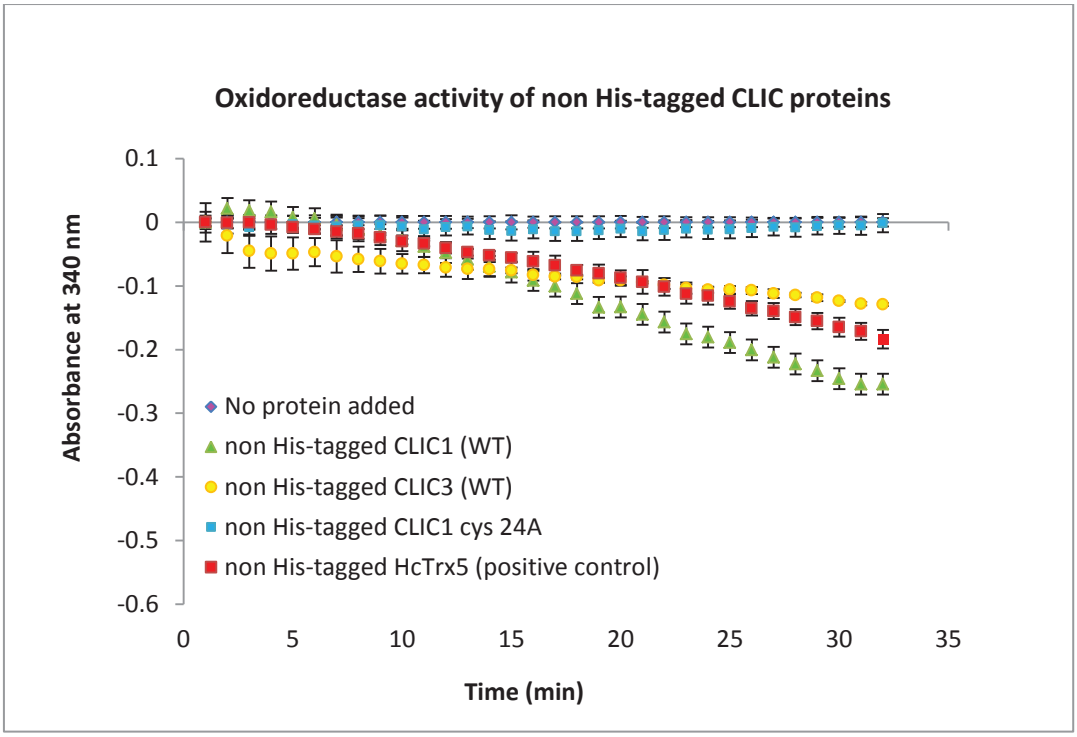
Figure 2.7: The SDS-PAGE result of purified monomeric rCLIC1 (WT), rCLIC3 (WT) using size exclusion chromatography (SEC) method. In this figure, the lane1 in the left side is representing the satandared Ladder with estimated molecular weights of proteins. The lanes 2 is representative of purified His-tagged CLIC1. Where, the lan3 and 4 are indicating the almost 27kDa non His-tagged CLIC1 and CLIC3, respectively. Besides, in turn, the lane 5, 6, 7 and 8 are representative of wash sample, flow through, supernatant and induced CLIC1 and CLIC3 proteins.

2.3.4 Observation that the purified His-tagged CLICs in elution buffer demonstrate greater oxidoreductase activity in HEDS assay

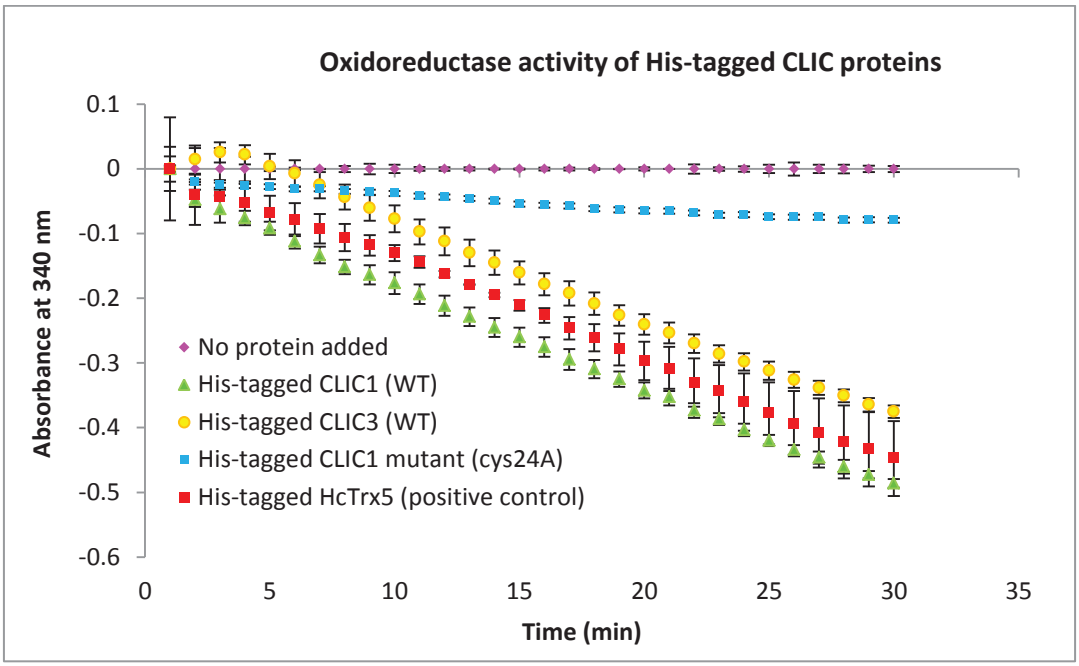
Samples from the various stages of the purification process were assessed for their enzymatic activity. This included samples that were eluted from the nickel column prior to the first round of SEC purification, which thus still remained in elution buffer containing 250mM Imidazole compound. On the other hand, the final purified samples that had been purified through the SEC column as the second step were now in column sizing buffer that contained 20mM HEPES (pH7.5), 100mM KCl, 1mM sodium azide and 0.5mM TCEP, with all imidazole now removed. As it seen in Figure 2.8A below, the purified recombinant non-His tagged CLIC1(10µM) and CLIC3(10µM) proteins demonstrated activity in the HEDS assay, while as expected, the non-His tagged mutant CLIC1-C24(10µM) A did not. These results are consistent with those previously demonstrated by our group at UTS [5, 6].

In Figure 2.8B below, the effluent protein samples of His-tagged CLIC1, His-tagged CLIC1-C24A mutant, His-tagged CLIC3 along with the positive control Histidine-tagged HcTrx5 enzyme [242] that purified after nickel column and stored in elution buffer, were also compared. Based on the results above and previously published data, it was expected that the His-tagged CLIC1-C24A mutant should demonstrate no enzymatic activity in the HEDS assay [5]. However, it was clear that in this current and subsequent repeat experiments, the His-tagged CLIC1-C24A mutant protein was enzymatically active (albeit much less active than the His-tagged CLIC1–wild type) (Figur2.8B – blue square symbols). This is demonstrated by the decrease in the absorbance measured at 340nm over 30 minutes from when the reaction commenced, which represents consumption of NADPH in the assay. Moreover, the His-tagged form of CLIC1, CLIC3, and HcTrx5 were also demonstrating higher activity in this assay when compared to their non-His tagged form seen in Fig 2.8A, which are all free of the imidazole. Thus, from these results, it was evident that the His-tagged version of each of these proteins when used at the same concentration, demonstrated higher enzymatic activity (Figure 2.8B) in the HEDS assay compared to their non His-tagged form (Figure 2.8A).

A


Oxidoreductase activity of non His-tagged CLIC proteins
Absorbance at 340 nm
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
0
5
10
15
20
25
30
35
Time (min)
No protein added
non His-tagged CLIC1 (WT)
non His-tagged CLIC3 (WT)
non His-tagged CLIC1 cys 24A
non His-tagged HcTrx5 (positive control)

B


Oxidoreductase activity of His-tagged CLIC proteins
Absorbance at 340 nm
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
0
5
10
15
20
25
30
35
Time (min)
No protein added
His-tagged CLIC1 (WT)
His-tagged CLIC3 (WT)
His-tagged CLIC1 mutant (cys24A)
His-tagged HcTrx5 (positive control)

C

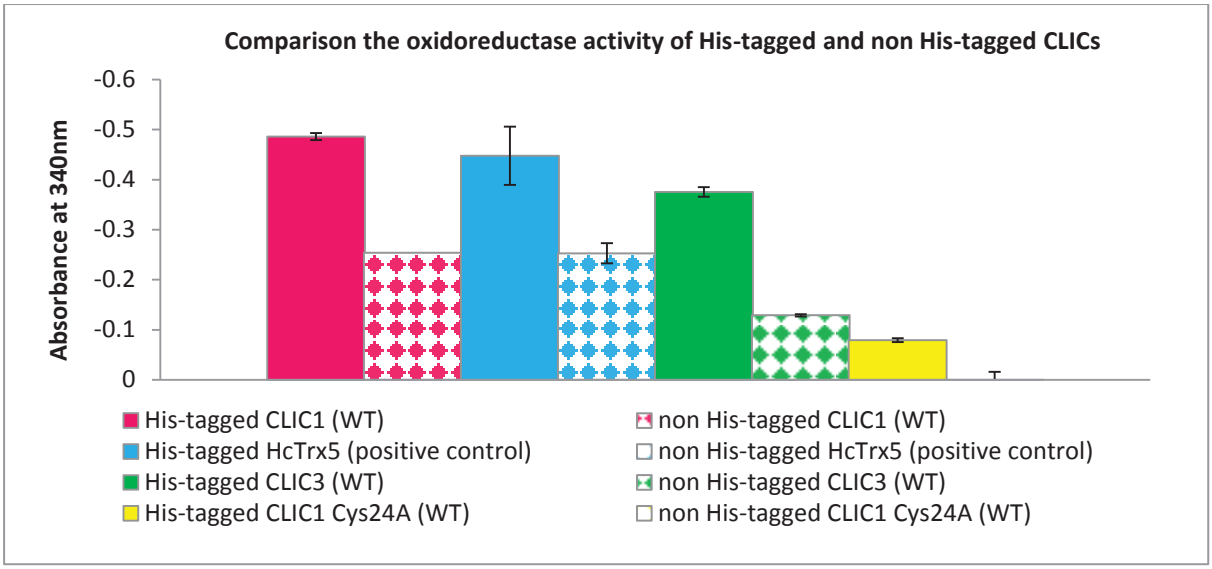


Figure2.8: His-tagged CLIC member's exhibit increased oxidoreductase activity in HEDS assay compared to their non His-tagged form. Figure A summarises the non His-tagged protein activity that kept in column sizing buffer (100mM KCl, 1mM NaN3, 20mM HEPES pH 7.5 and 0.5mM TCEP), while figure B summarises the His-tagged protein action that kept in elution buffer (250mM imidazole, 300mM KCl, 50mM potassium phosphate buffer pH8, 0.5mM TCEP). The experiments were conducted in the presence of 5mM potassium phosphate buffer with 1mM EDTA, pH 7 containing 10µM of either His-tagged or non His tagged CLIC proteins (CLIC1, CLIC1-C24A or CLIC3) or HcTrx5 protein (positive control), 250µM NADPH, 1mM HEDS and 0.5ug/ml GR. The mixture was heated for 5 minutes at 37°C. Subsequently, 1mM GSH was added to initiate the reaction, with consumption of NADPH was monitored at 340nm over 30 minutes. Error bars indicate the standard deviation from three independent measurements.

2.3.5 Observation that the His-tagged CLIC1 and CLIC3 are kinetically different to their non-His-tagged form

Following the unexpected result from the His-tagged versions of the proteins, a comparison of their enzyme kinetics was undertaken. In this regards, different concentration of HEDS substrate (0.25, 0.5, 1, 2, 4 and 6mM) was used in order to characterise and compare the kinetics behaviour of His-tagged and non His-tagged CLIC1 and CLIC3 proteins. The apparent Vmax and Km values were calculated from the Michaelis-Menten plot using Microsoft Excel 2010 and GraphPad Prism7.

The "His-tagged" form of CLIC1 and CLIC3 demonstrated distinct kinetics in comparison to their non-His-tagged form, as seen in figure 2.9. In addition, this result also shows that the His-tagged CLIC1 and CLIC3 quickly reached the maximum velocity and afterwards their

activity was started to decline. This suggests their activity was somehow inhibited by the substrate in comparison to their non His-tagged counterparts, which demonstrated a lower maximal reaction rate by about two-fold.

From this result, it was evident that the His-tagged CLIC members compared to their non His-tagged form, have a greater ability to reduce the disulfide bond of the HEDS substrate once coupled with reduced glutathione peptide (GSH) and glutathione reductase (GR), in the presence of NADPH. Their activity was also inhibited at higher levels of substrate.

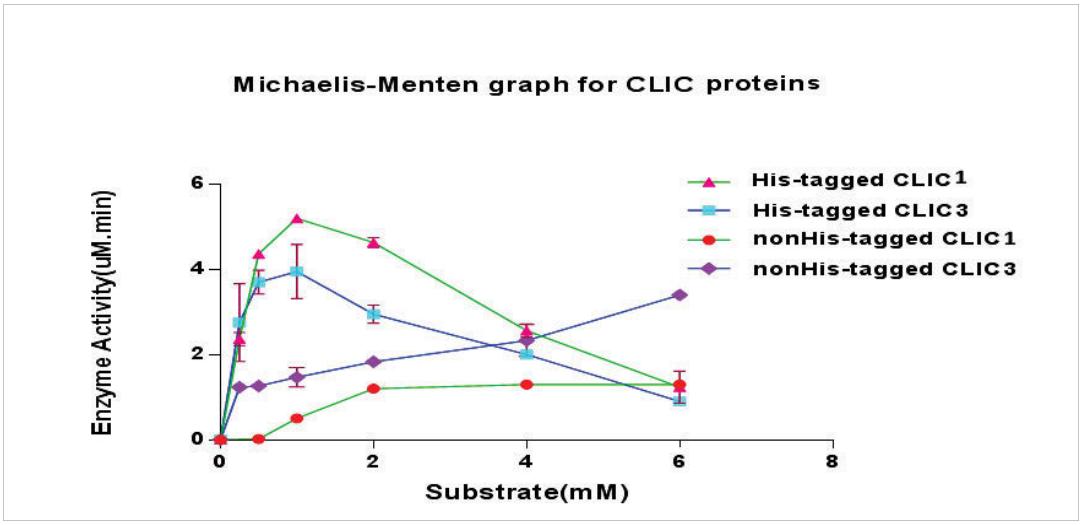


Figure2.9: His-tagged CLIC members are not following Michaelis-Menten kinetic patterns. This figure indicates that the His-tagged CLIC1 and CLIC3 proteins are more active when they stored in elution buffer with calculated Vmax of (3.4 and 2.7 µM/min), and Km value of (0.0056 and ~1459e-016mM), respectively. As the curves reached the peak very quickly and then declined, they are not characteristic of an enzyme that should reach a plateau pattern. However, the non His-tagged CLIC1 and CLIC3 counterparts that stored in column sizing buffer, demonstrate Vmax of (2.026 and 3.3µM/min), and Km value of (2.503 and 0.9941mM), respectively and fallowed a plateau pattern after reaching the maximum velocity (Error bars indicate the standard deviation of three measurements and 10uM final concentration of proteins was used in this experiment).

Given these overall unexpected findings, it was then necessary to determine whether this differential behaviour between the His-tagged versus the non-his-tagged forms of the CLICs and HcTrx5 proteins was due directly to the presence of the “6- amino acid Histidine tag”. Alternatively, there was some other factor at play, which arose due to differences in handling, processing, and storage of the different forms of each protein.

2.3.6 “Histidine tag” activity in the HEDS assay

In order to clarify the reason for this observed higher oxidoreductase activity of His tagged CLIC members, first, we investigated the effect of the “Histidine tag” itself on the HEDS assay system. In this regards, the 6 amino acid “His tag”, which was kept in two different buffers was evaluated in the HEDS assay.

We first evaluated whether 6 amino acid “His tag” contributed to the observed HEDS assay activity. For this purpose, both Histidine-tag peptides that were stored in two different solutions were used along with the His-tagged CLIC3 protein (positive control/ stored in elution buffer) in HEDS assay as shown in Figure 2.10.

The result is evident that when the same 10µM final concentration of His-tagged CLIC3 protein, and histidine tag peptide in either imidazole elution buffer or column sizing buffer, were separately added to the 5mM potassium phosphate solution, only the 6 histidine tag peptide that was stored in elution buffer along with His-tagged CLIC3 (positive control/in elution buffer) showed an increase in NADPH consumption, indicated by a reduction in absorbance measured at 340nm over the 30 minutes of the reaction.

In contrast, the 6 histidine tag peptide which was kept in column sizing buffer, showed no enzymatic activity in this assay system. This also indicated that there was no contaminating CLIC protein in the His peptide sample. Therefore, based on the obtained result it became clear that the “his tag” by itself does not have any obvious effect on the HEDS enzyme activity, however, it pointed to the imidazole buffer needing to be tested.

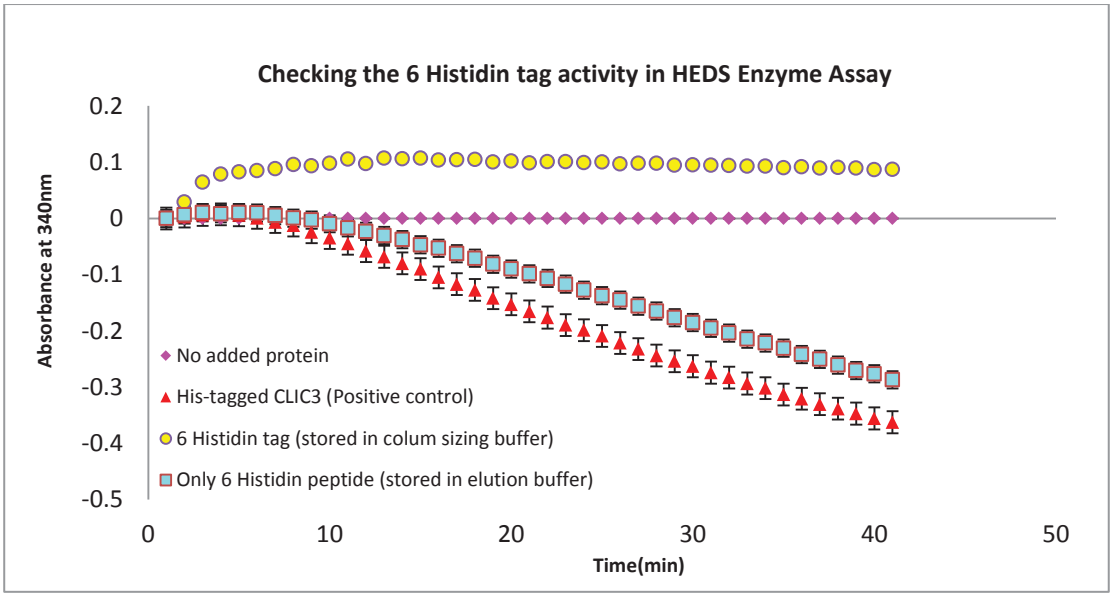


Figure2.10: Polyhistidine tag has no effect on high oxidoreductase activity of CLICs in HEDS enzyme assay. The final concentration of 10µM of His-tagged CLIC3(positive control/ in elution buffer), only 6 histidine tag peptide with elution buffer (250mM imidazole, 300mM KCl, 50mM potassium phosphate buffer pH8, 0.5mM TCEP) and only 6 histidine tag peptide with column sizing buffer (100mM KCl, 1mM NaN3, 20mM HEPES pH 7.5 and 0.5mM TCEP) were separately added to the mixture of 5mM potassium phosphate buffer that contained 1mM EDTA in pH 7, 250µM NADPH, 1mM HEDS and 0.5ug/ml GR. The mixture was incubated for 5 minutes at 37°C. afterward, to initiate the reaction 1mM GSH was added to the mixture and the absorbance of NADPH consumption was monitored at 340nm for 30 minutes. (Error bar represents S.E).

2.3.7 Analysing the effect of column sizing and elution buffers on the enzymatic activity of His-tagged CLIC1in HEDS assay

The imidazole Elution buffer, utilized in the removal or washing the unbound proteins from the nickel affinity column, is used at a higher concentration during the final purification stage, in order to releases the target protein from the ligand. It is vital that the elution buffer works rapidly to avoid any alteration on the function or activity of the target protein[243].

On the other hand, in gel filtration or SEC chromatography method the target protein is purified in the same buffer that the column has been equilibrated with. Hence, the buffer composition although not critical for the elution, it should ,however, be compatible with protein stability and activity[244].

Therefore, to analyse the effect of these two different buffers on the enzymatic activity of CLIC1, all Three-protein samples were assessed in HEDS assay. Based on the results below,

in Figure2.11 the His-tagged CLIC1 that was stored in column sizing buffer demonstrate almost identical behaviour in consumption of NADPH, compared with the positive control. While the His-tagged CLIC1 that was stored in the imidazole elution buffer revealed higher activity in contrast to the positive control.

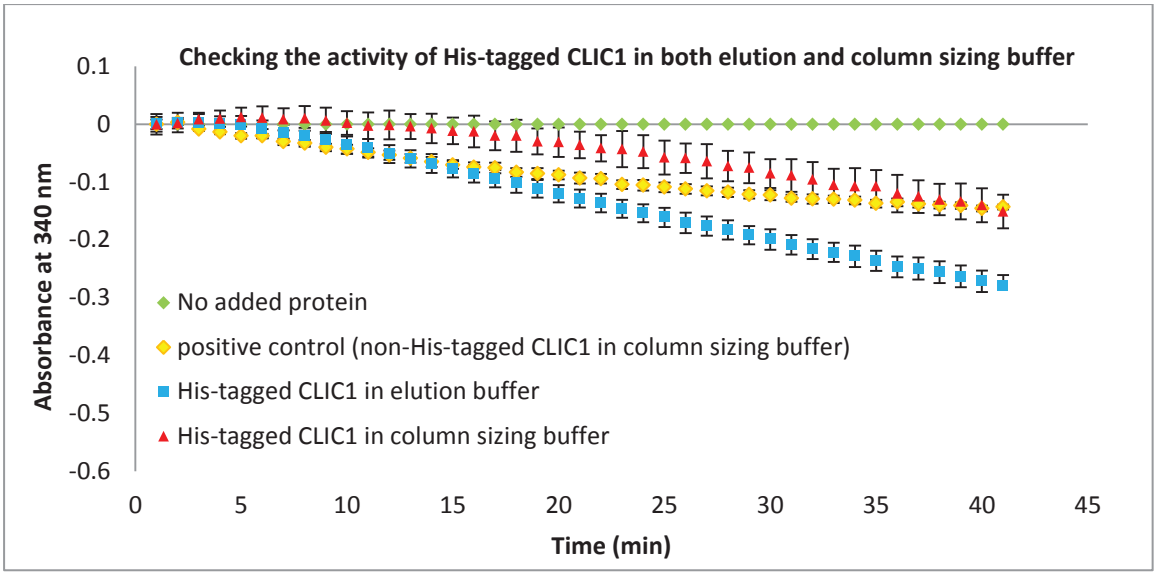


Figure2.11: Elution buffer containing imidazole, alters His-tagged CLIC1 activity in the HEDS assay. The HEDS enzyme assay was carried out by using 10uM His-tagged CLIC1 as two different tested protein samples (one sample stored in elution buffer and the other one stored in column sizing buffer) along with the 10uM of the positive control, the non His-tagged CLIC1. The proteins sample together with 250µM NADPH, 1mM HEDS and 0.5ug/ml GR were added to the 5mM potassium phosphate buffer that contained 1mM EDTA in pH 7. After 5min of incubation at 37°C, the reaction was initiated by addition of 1mM GSH. NADPH consumption was monitored at 340nm. Error bars represent the standard deviation of three independent measurements.

2.3.8 Investigation of the effect of imidazole on the enzymatic activity of His-tagged and Non His-tagged CLIC proteins in the HEDS Assay

The imidazole compound (1,3-diaza-2,4-cyclopentadiene) is incorporated with many essential biological molecules such as, the amino acid “histidine” that contain an imidazole side-chain. Several proteins and enzymes have the Histidine amino acid residue. Moreover, Imidazole-based histidine complex functions in a vital role in intracellular buffering [245]. In addition, one of the applications of imidazole is in the purification of His-tagged proteins in immobilised metal affinity chromatography (IMAC) system [246]. Furthermore, it has been shown that the Imidazole buffer has the ability to act as catalyser by catalysing the cleavage

of both RNA and dinucleotides. [247]. Hence, the initial study undertaken in order to investigate whether the imidazole compound would interfere with enzymes catalytic activity. In this experiment, the effect of different concentrations of imidazole on the oxidoreductase activity of both His-tagged and non His-tagged CLIC1 that were stored in column sizing buffer were assessed in the standard HEDS enzyme assay.

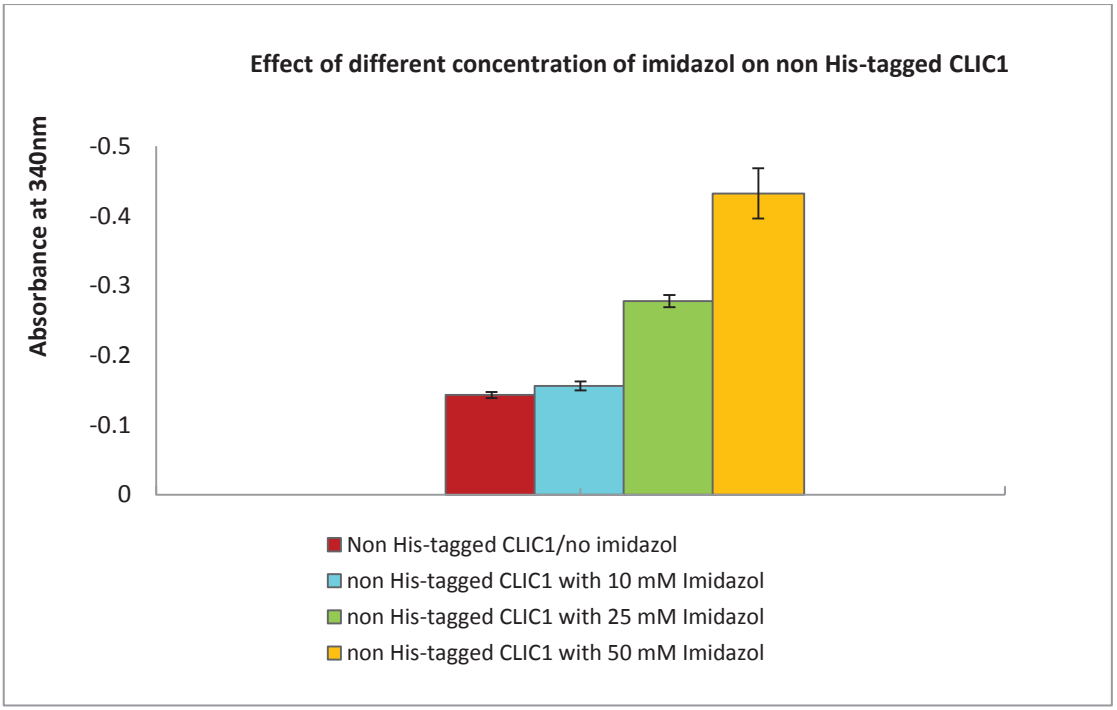
In this regards, a final concentration of 10, 25 and 50mM of imidazole was added to the mixture of 5mM potassium phosphate buffer at pH7, which contains same reagent and condition for HEDS assay protocol, as described in materials and methods section 2.2.12.

As it can be seen in figure 2.12 (A), an increase in imidazole concentration led to a significant increment in the NADPH consumption. Therefore, the non His-tagged CLIC1 oxidoreductase activity is dramatically enhanced as the imidazole concentration is increased. In this regards, the maximum point was obtained at imidazole concentration of 50mM, in contrast to the positive control of lesser activity, which does not contain imidazole compound. This behaviour is in the same line with the result that obtained from figure2.8 (B) in which the His-tagged CLIC proteins that were kept in elution buffer with the high concentration of Imidazole, were greatly active.

On the other hand, as the data in figure 2.12 (B) is shown, in the case of the His-tagged CLIC1 and CLIC3 proteins(stored in column sizing buffer), different concentration of imidazole was influenced their activity in a different manner to their non-His counterparts, by which the activity of enzymes starts to be increased at the maximum imidazole point of 25mM and then the activity decreased at imidazole concentration of 50mM.

In addition, when the activity of both non His-tagged and His-tagged CLIC1 and CLIC3 with no added imidazole (positive control), which kept in column sizing buffer was compared together, this was clear that the non His-tagged protein demonstrates almost two times higher activity in contrast to their His-tagged counterparts. So, based on this result we aimed to further investigate on the effect of “6 Histidine tag” on the activity of both non His-tagged and His-tagged CLIC1 and CLIC3 that were purified by size exclusion chromatography and kept in the same storing buffer. The next section would cover this matter.

A



B

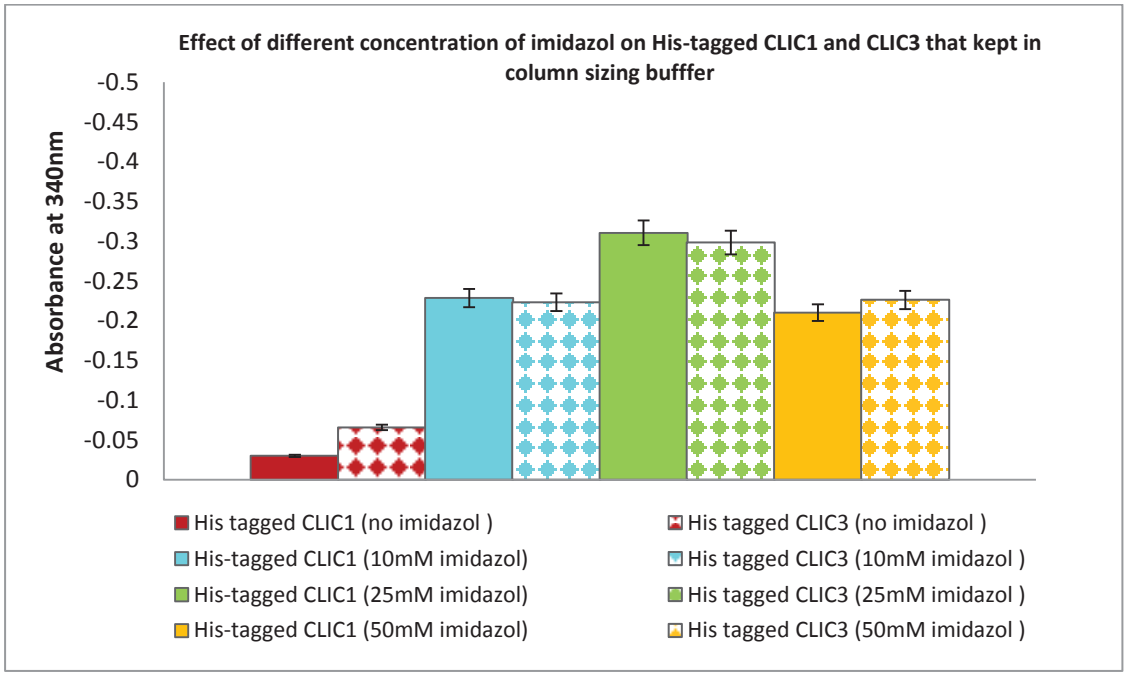


Figure2.12: Imidazole at high concentrations interferes with the non-His-tagged CLICs activity.

(A) Non His-tagged CLIC1 which was kept in column sizing buffer was evaluated in HEDS assay by addition of different concentration of imidazole. As it can be seen in this figure, by increasing the imidazole concentration the enzyme activity dramatically increased**.**

(B) His-tagged CLIC1 and CLIC3 which were kept in column sizing buffer was evaluated in HEDS assay by addition of different concentration of imidazole. As it shown in this figure, by increasing the imidazole concentration the catalytic activity raised only at imidazole point of 25mM, then the activity decreased by the addition of higher imidazole concentration**.** Error bars represent the standard deviation.

2.3.9 The "Histidine tag" interferes with oxidoreductase activity of CLIC1 in the HEDS assay

In order to definitively determine the effect of the His-tag on the protein's enzymatic activity, it was essential to compare protein samples that had been prepared and handled in an identical manner, with the only difference being the presence of the His-tag or not.

Therefore, based on the obtained result seen in figure 2.13, the His tagged CLIC1 demonstrated much lower activity compared to its Non-His-tagged counterpart in the HEDS enzyme assay. Therefore, this provides strong evidence that the six histidine peptide, attached to the N-terminus of CLIC1, is inhibiting the protein's catalytic activity.

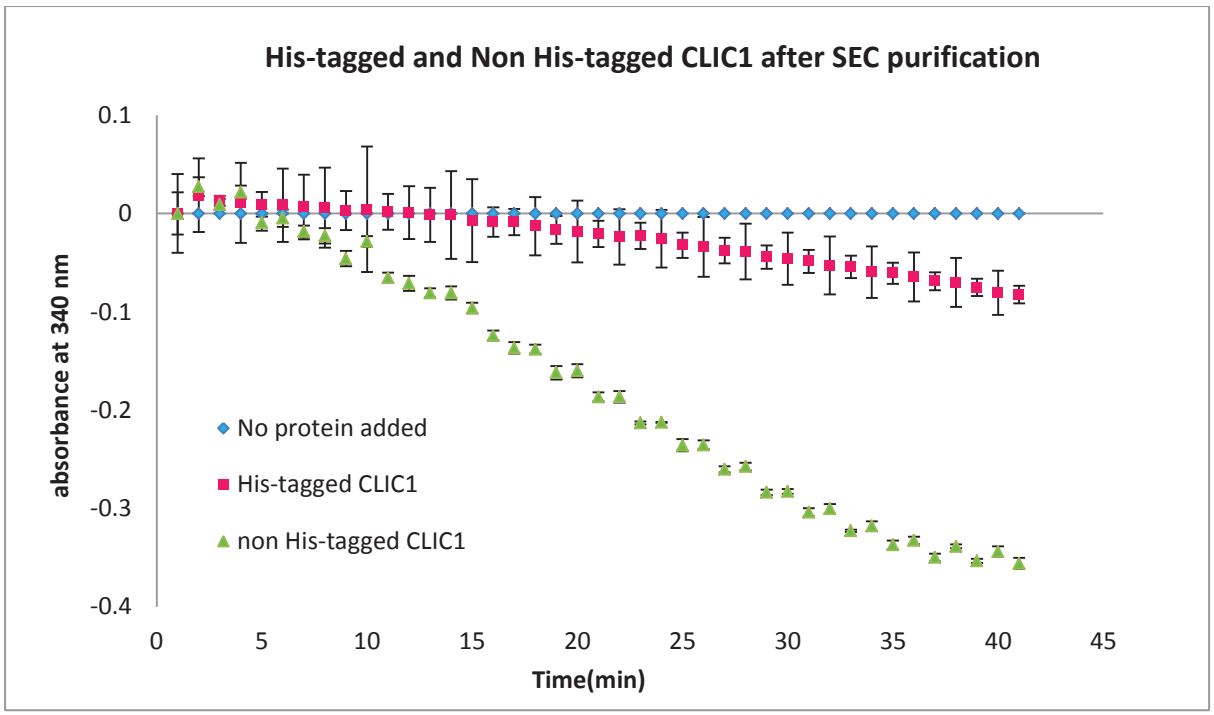


Figure2.13: Poly histidine-tag interferes with the catalytic activity of CLIC enzyme. His-tagged CLIC1 which was purified through SEC chromatography similar to its non His-tagged counterparts was evaluated in the standard HEDS assay. As it can be seen in this figure, by the existence of "His-tag" on protein the enzyme catalytic efficiency is lowered. Where the enzyme without tag has much higher activity. Error bars indicate the standard deviation from three independent measurements.

2.4 Discussion and conclusions

In recent years the application of recombinant proteins in proteomics field has been greatly increased. Recombinant hybrids comprise of a polypeptide fusion partner known as “affinity tag”, which utilized in order to assist and simplify the purification of the objective polypeptides, are extensively used. As it was described in the introduction of this chapter, different “affinity tags” exists that have diverse application.

However, several studies have been revealed that the biochemical properties of the target protein could be affected with these distinct “affinity tag” systems. In regards to these studies, it becomes evident that the “affinity tag” would be able to inhibit proteolysis [248], and therefore, can be used as an efficient method for production of the protease such as Urokinase-Type Plasminogen Activator. In addition, it would also assist in an effective protein refolding, when the on-column metal affinity chromatography method was conducted [249].

Accordingly, modifying of protein with a hexahistidine tag, which is considered as one of the affinity tagging systems, could be a beneficial method in cancer treatment. For instant, it has been shown that the antibody response in human patients was significantly reduced when the protein modified with “His-tag” peptide [250]. Besides, the “affinity tag” can also enhance the solubility of fusion protein [251-254]. Finally, it improves the sensibility of binding assays for reactivity of tagged ScFv fragments, by forming a stable dimeric fragment that led to increasing in the reactivity and yield of scFv, and as a result can be considered as a reproducible method, which would simplify the isolation and usage of scFv fragments. [255].

Despite the fact that purification of proteins using the “His-tag” is the most commonly used method, to some extent the Histidine-tagged proteins in comparison with native proteins can differ in terms of their mosaicity and diffraction [256]. In addition, another study has been revealed that the biochemical properties of different proteins were fundamentally changed due to the presence of “6 Histidine tag” on the target proteins [257], in spite of the relatively small size and charge of the “Histidine tag”.

In this regards, in contrast to the positive side effect of affinity tag usage, it has been reported that several biological processes are also negatively affected by the use of different “affinity tag” method. In this regards, the negative results of “tag” application can be exemplified as : conformational alteration in the active site of HZFB AreA protein [258], lowers the efficiency of CC49 scFv protein by negatively interfering with the antigen binding property[259], interferes with post-translational modification and crystallization of SH3 domains from the chicken src tyrosine kinase [260], drastic reduction in biological activity of recombinant tumor necrosis factor-alpha [261], unsuccessful crystallizations of maltose-binding protein (MBP), thioredoxin (TRX) and glutathione-S-transferase (GST) proteins [262], induced toxicity to normal recombinant human TRAIL cells (tumor necrosis factorrelated apoptosis-inducing ligand) [263] and prevention of exopeptidase activity in recombinant Aminopeptidase B (rAp-B) enzyme [264].

Above and beyond all advantages and disadvantages of the affinity tag, several studies were also implicated on the imidazole ring of Histidine amino acids and its effects on enzyme activity. In this regards, the recent study has been revealed that the imidazole side-chain of histidine amino aside has the ability to improve the substrate affinity of the Fe_3O_4 nanozyme, which is reported as the first nanoparticle that demonstrates intrinsic peroxidase-like activity with extensive application in biomedicine field. In this study, a single histidine residue was introduced onto the Fe_3O_4 nanozyme surface and as a result, up to twenty-fold enhancement on the catalytic efficiency was obtained [265].

In addition to this work, a number of studies have been shown that the imidazole ring of histidine amino acid has the ability by which, it can contribute to the proteins stabilities and conformational modifications, due to the alteration that occurs in the entropy of its protonation form in response to changes that happens in the pH of intracellular milieu. For instance, a study was accomplished on the responsibility of histidine amino asides on the pH-dependency of global conformational stability of human CLIC1 protein [266]. Based on the obtained result, it was indicated that the conformational stability of human CLIC1 protein, which structurally contains three histidine amino aside [138], is dependent on both His74 and His185 residues that are able to trigger the pH-induced alteration to the

conformational stability of this protein, through their protonated mechanisms by which the intermediate state is stabilized.

On the other hand, a research study was performed in order to investigate the function of the imidazole compound. In this regards, this work has been shown that the imidazole buffer compound has the ability to demonstrate a bifunctional catalytic activity and behave as a catalyser [267]. Thus, in this chapter, the effect of both “polyhistidine tag” and “imidazole” as two possible factors for high oxidoreductase activity of His-tagged CLIC protein members was evaluated that will discussed in two separate perspectives.

2.4.1 Imidazole point of view:

This study was conducted in two different conditions in HEDS enzyme assay. In this regards, based on obtained result it becomes clear that the His-tagged CLIC proteins that stored in elution buffer demonstrate higher oxidoreductase activity in HEDS enzyme assay (figure2.8 B), in comparison with their non His-tagged counterparts (figure2.8 A). This effect was due to the storage buffer with high concentration of imidazole, which the protein was kept in, and was not related to the existence of “6 polyhistidine tag” on the protein.

Accordingly, as seen in figure 2.10, when the “6 polyhistidine tag” itself with both elution buffer and column sizing buffer was separately assessed in HEDS assay the result points out that the “tag” by itself has no catalytic influence when the tag was stored in column sizing buffer. However, the stored “tag” in “imidazole elution buffer” remains highly active in a similar manner compared to the control non-His-tagged CLIC protein. Thus, from this, we can postulate that the only possible reason for this “highly activated tag” is attributable to the imidazole compound (Figure 2.14).

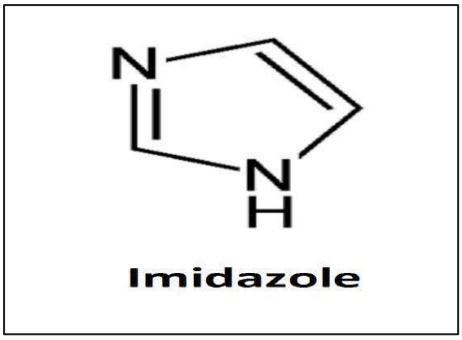


Figure 2.14: is representative of imidazole compound structure [268].

Therefore, it seems that the imidazole compound (Figure 2.14), which has been shown to have bifunctional catalytic activity [267] also interferes with enzyme activity of CLIC protein due to the presence of a large amount of this compound in the storage buffer that the protein was stored in. Consequently, in order to evaluate this activity more precisely the elution buffer should be first removed and replaced with column sizing buffer, or any storage buffer that has no effect on enzyme activity.

Indeed, after applying the buffer exchange method, the result from (figure 2.11) suggests further strong evidence that the "6 polyhistidine tag" has no impact on high catalytic activity of oxidoreductase CLIC protein, in contrast to the recent study that demonstrates the histidine residue increase the catalytic activity of Fe_3O_4 nanonzyme [265].

However, the result from figure 2.12 (A) and (B) indicates that the catalytic activity of both His and non His-tagged protein, which stored in column sizing buffer, would be influenced by the increase of imidazole concentration in the biochemical reaction. So, when the concentration of imidazole is doubled the activity is almost doubled, which suggest that the imidazole could work as a substrate for this protein (further experiment for this hypotheses is done and explained in chapter 3/ figure 3.6 of this thesis).

2.4.2 Histidine tag point of view:

In regards to the previous section, when the activity of non His-tagged CLIC1 and it's His-tagged counterparts (figure2.12 A&B / contained no imidazole compound) were compared, this become evident that even though the proteins were stored in the same storage solution

(column sizing buffer), the catalytic activity of His-tagged proteins were almost two time lesser than their non His-tagged counterparts. Accordingly, from that, we postulate that the "6 polyhistidine tag" (figure2.15) could possibly have a negative influence on the catalytic activity of oxidoreductase CLIC protein. Thus, this became our next goal practice in order to test the function of His-tagged fusion CLIC proteins to confirm its integrity, as several studies have been revealed that the histidine fusion tag has a negative side effect on the functional properties of the fusion proteins [259, 261].

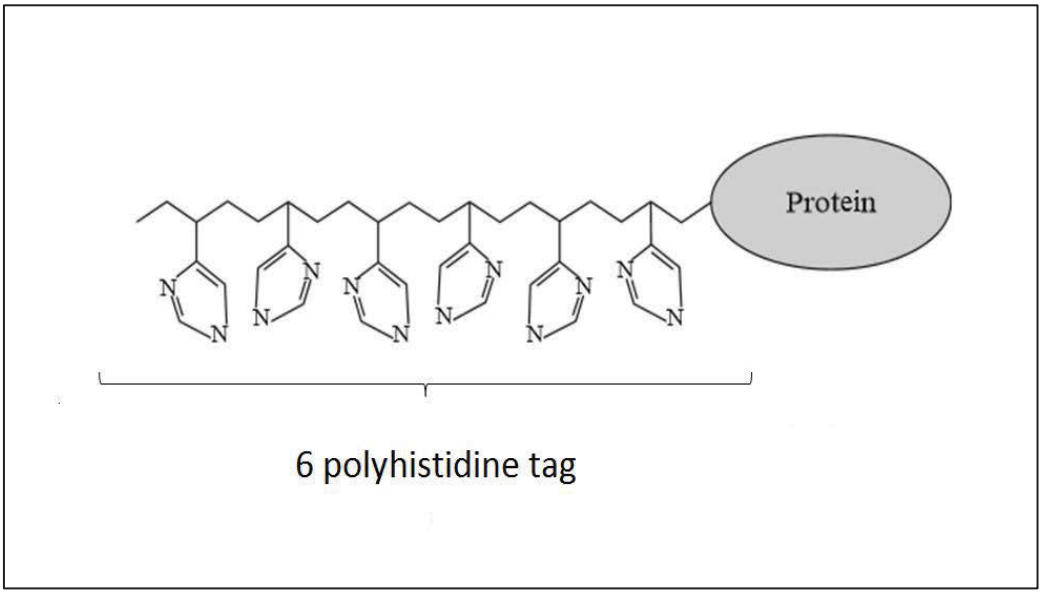


Figure 2.15: Model represents the "6 polyhistidine tag" that attached to the protein [202].

In that regards, both His and non His-tagged proteins were treated by the same purification steps for in-depth analysis, and then evaluated in the HEDS assay with the same experimental condition. In consequence, the negative influence of "6 polyhistidine tag" becomes evident, as the obtained result from (figure 2.13) is shown a significant difference between the activity of His-tag fusion protein and its non His-tagged version. a similar study has been revealed that the hexahistidine-tag interferes with tropinone reductase enzyme active site and lowers its activity [269]. Thus, based on this result, this could be postulated that the "hexahistidine-tag" somehow interferes with the local structure surrounding of the enzymes active site and as a result, can potentially affect the structure of the protein and

therefore lower the enzymes efficiency. This would indicate that structural studies would be needed to determine what the tag has done to the CLIC's structure.

To sum up, the recombinant proteins containing a polypeptide fusion sequence have been widely used in the recent year. This fusion partner, which termed as the affinity tags are essential tools in protein purification process. Nevertheless, several advantages and disadvantages of affinity tags application have been stated that have an impact on the protein activities. Hence, the study in this chapter was focused on the effect of "polyhistidine tag" and "imidazole" compound on oxidoreductase activity of CLIC protein members.

Briefly, the His-tagged CLIC proteins show high enzyme activity in HEDS enzyme assay, when they stored in elution buffer after the affinity purification. This was due to the high concentration of the imidazole complex that exists in storage buffer and has no association with the presence of "polyhistidine tag" on protein. So, it is recommended that the protein should be stored in a storage buffer that contains no imidazole in order to avoid this kind of behaviour which is not truly accurate for enzyme characterization studies.

On the other hand, the purified His-tagged fusion CLIC after SEC chromatography, which treated with same purification condition to its Non His counterparts, demonstrate very less activity that was due to the influence of the "His tag". Indeed, the results of the present study also indicate that the His-tag contributes to enzymatic activity in the HEDS assay and therefore, should be removed from recombinant protein prior to the use in such assay.

Chapter 3
Potential Physiological Substrates of the CLICs as Oxidoreductases

3.1 Introduction

3.1.1 Interaction of enzymes with a broad spectrum of substrates

Enzymes have a notable capability of selectively interacting with specific target substrates to catalyse specific reactions. This ability is crucial in order for maintaining proper functionality of most homeostatic pathways within biological organisms [270, 271]. In fact, enzymes are evolutionarily endowed with additional specific flexibility and plasticity behaviours in order to catalyse and convert an extensive range of targeted substrates [272-274]. Therefore, as there could be one or more substrates for each type of enzyme, certain amino acid side chains of an enzyme's active site interacts and binds with the substrate(s) based on the particular catalytic reaction. Indeed, it is this interaction that determines the enzymatic specificity and its ability to accelerate a catalytic reaction [275]. In accordance to that, general understanding of the structural and functional properties of specific enzymes, which undertake the same catalytic reaction by interacting with their target substrate, such as disulfide substrates has been greatly enhanced in most mammalian cells.

In this regards, structural and molecular studies of enzymes with broad substrate specificity have been undertaken in various fields, such as drug design [276, 277]. Accordingly, the multispecific or broad specific enzymes such as the glutathione S-transferases (GST's) are a good example of enzymes that perform the same type of reaction on a range of different substrates [278].

The thioredoxin (Trxs) and glutaredoxin (Grxs) enzymes are well-known master regulators of the oxidation-reduction of the functional thiol groups of a targeted substrate(s), that contribute to various essential functions of cellular processes in metabolism and cell signalling [49, 279-281]. As was discussed in the previous chapters, the thioredoxin superfamily enzymes carry out their redox catalytic activity by using their cysteine amino acid residues that are located at their catalytic active site. The members of this superfamily are classified based on two common features, first their thioredoxin fold and second their active site that consists of C-X-X-X/C sequence, which is associated with redox reactions. In many cells, in addition to the thioredoxin superfamily, there is also the glutaredoxin family, along with several other proteins that are associated with disulfide bond formation, isomerization and other reductive biological processes like peroxide inactivation and

cytochrome biogenesis [33, 282, 283]. Moreover, despite the fact that the thioredoxin and glutaredoxin enzymes generally reduce both catalytic and allosteric disulfides [284], the substrate specificity is different amongst the individual protein members of each family. However, the actual mechanisms and characteristic features that are involved in the regulation of these specificities are vague and have yet to be well explained.

Nevertheless, since the first discovery of thioredoxin (Trx) as a protein factor that demonstrated functional catalytic ability in reduction of the *Escherichia coli* ribonucleotide reductase (RNR) in the presence of thioredoxin reductase (TrxR) and NADPH [41], several studies have now revealed that this enzyme can reduce a broad range of disulfide substrates[283]. Correspondingly, the thioltransferase glutaredoxin (Grxs) enzyme have been shown to use the oxidized form of ascorbic acid, known as dehydro-L-ascorbic acid (DHA), as its substrate and catalyse the regeneration of this oxidised vitamin [285]. Also, it has been shown that the glutaredoxin enzymes have the ability to use other molecules like the selenium compounds as their substrates [286].

As mentioned in Chapter 1, the members of the chloride intracellular ion channel (CLIC) protein family demonstrate “glutaredoxin-like redox activity”, and hold significant structural similarities to the Glutathione-S-Transferase, Thioredoxin, and Glutaredoxin Enzyme Families [5, 6, 287]. Based on this, it is not surprising that CLICs may efficiently use the same or related substrate targets as the GST’s, thioredoxin, and glutaredoxin families. Some of these substrate molecules include:

Dehydroascorbic acid and Sodium selenite (symbol Se and the atomic number of 34). Reduction of these substrates by Thioredoxin and Glutaredoxin enzymes would respectively contribute to the maintenance of both ascorbic acid levels [285, 288] and selenium metabolism and therefore lead to redox control in cells and biological systems [289]. In this regards, these two substrates were chosen to further characterise whether the CLIC protein members can involve and contribute in ascorbic acid maintenance and redox control similar to Thioredoxin and Glutaredoxin enzymes.

3.1.2 Inhibition of enzymatic catalytic activity

Enzymes are important molecules by virtue that they control almost all cellular processes through their catalytic behaviour. As such, regulation of their activity is essential, including processes that can enhance or inhibit their activity. Enzyme catalytic activity can be inhibited by specific molecules such as drugs, toxic agents or ions. These inhibitor molecules bind to the enzyme, resulting in a slowing or stopping of the forward direction of the enzymatic reaction. For this reason, enzyme inhibitors are considered as one of the most important drug agents by pharmaceutical industries. For instance, aspirin (acetylsalicylate) the most widely used pharmaceutical drug hinders the catalytic activity of the enzyme cyclooxygenase 2 (COX-2) by forming a covalent bond with the enzyme. COX-2 is involved in the first step of prostaglandin synthesis, which participates in several cellular processes, including pain. Detailed investigation of enzyme inhibitor behaviour also provides valuable information with regards to enzymatic mechanisms, defining metabolic pathways and they can assist in identification of the catalytic residues of enzyme active site [11], [7].

Enzyme inhibitors are classified into two broad classes based on their mode of action: reversible or irreversible inhibition.

Reversible inhibition

Reversible inhibition is where there is rapid dissociation of the enzyme-inhibitor complex through competitive, uncompetitive or mixed inhibition types of behaviour.
In competitive inhibition, the inhibitor bears a strong resemblance to the substrate. Hence, it competes with the substrate for binding at the enzyme's active site. The enzyme would bind either the substrate (ES) or inhibitor (EI) but it cannot bind both of them (ESI) at the same time (figure 3.1.a). As a result, when the inhibitor binds to the active site, it prevents substrate interaction with the enzyme and therefore effectively reduces or stops the catalysis.

In uncompetitive inhibition, the inhibitors bind to a completely different site away from the substrate catalytic site (figure 3.1.b). Likewise, the mixed inhibitor also binds to a different catalytic site from the substrate site, but this inhibitor would bind to E or ES complex in

order to cause inhibition (figure 3.1.c). In addition, the catalytic activity of enzymes that have two or more substrates can be inhibited by either uncompetitive or mixed inhibitors.

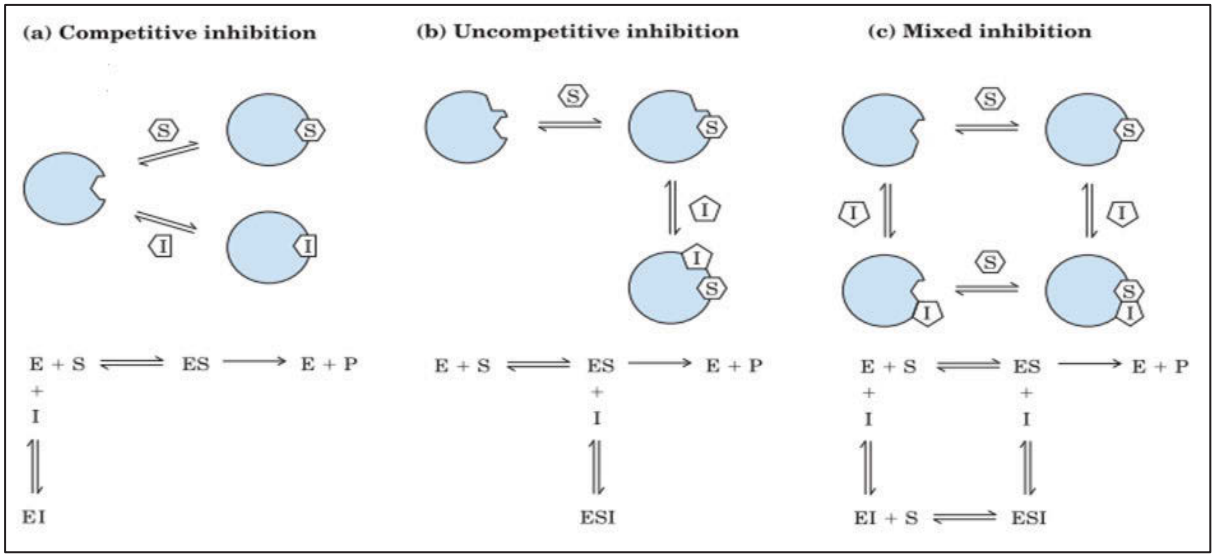


Figure 3.1: Representative of the three different reversible inhibition types. (a) Is demonstrating the competitive reversible inhibitors that bind to enzymes catalytic site. Whereas, (b) is indicating the uncompetitive reversible inhibition type through which the inhibitor binds to distinct catalytic sit of ES complex. While (c) representing the mixed reversible inhibition that also may bind to the distinct site of either E or ES complex [11].

Irreversible inhibition

On the other hand, irreversible inhibition is where dissociation of the enzyme-inhibitor complex occurs very slowly due to the fact that the inhibitor is strongly bound to the enzyme via either covalent or multiple noncovalent intramolecular interactions [11], [7]. In this regards, several studies have investigated the effect of a diverse range of drugs on enzyme catalytic activity. For instance, it has been shown that the catalytic activity of both rat and human glutathione S-transferases (GSTs) isoenzymes were reversibly inhibited by use of ethacrynic acid that is a potent inhibitory drug [290]. Moreover, it has been discovered that the specific mammalian thioredoxin reductase inhibitor, the 1-Chloro-2,4-dinitrobenzene (DNCB) compound has the ability to irreversibly inhibit and inactivate both thioredoxin and thioredoxin reductase activity preventing their reduction of disulfide bonds [291]. In a recent research study in our lab, the effects of ion channel blocker drugs A9C and IAA94 on the enzymatic activity of the CLIC1 protein was undertaken. This investigation

revealed that these drugs are capable of inhibiting the catalytic activity of chloride intracellular ion channel 1 (CLIC1) protein [5].

In this regards, this chapter will elaborate on the investigations undertaken to determine and further characterise the interaction of different substrates with CLIC proteins, along with examining the Inhibitory effect of drug IAA94 on the oxidoreductase activity of these protein members.

As such, this chapter will describe:

- Assaying the compound sodium selenite as a substrate using both non-His-tagged CLIC1 and CLIC3.
- Assaying DHA as a substrate for both non-His-tagged CLIC1 and CLIC3 in the standard HEDS assay.
- Determining the effect of ion channel blocker drug and IAA94, on the enzymatic oxidoreductase activity of both non-His-tagged CLIC1 and CLIC3 in the HEDS enzyme assay.

3.2 Materials and methods

3.2.1 Chemicals and reagents

The following reagents were purchased from *Sigma Aldrich*: Recombinant glutathione reductase (GR) enzyme from yeast, reduced glutathione peptide (GSH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), the substrate dehydroascorbic acid (DHA), and the substrate sodium selenite salt (Na2SeO3), 2-hydroxyethyl disulphide (HEDS) substrate, the drugs indanyloxyacetic acid (IAA-94) plus bovine plasma thrombin.
The following reagents were purchased from *Astral Scientific*: the reducing agent TCEP (Tris (2-carboxyethyl)phosphine hydrochloride), the inducing agent IPTG (Isopropyl-B-D-1-thiogalactopyranoside) Dioxane Free, and the antibiotic Carbenicillin Disodium Salt. The 4–15% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (10 well, 50 µl) was purchased from *Bio-rad*.

3.2.2 2xYT media for bacterial growth

Procedure for the preparation of the Standard microbial growth 2xYT medium is as described in the previous chapter (Materials and Methods sections 2.2-2.2.1).

3.2.3 Production and purification of recombinant CLIC1 and CLIC3 wild-type

The Recombinant CLIC1 and CLIC3 Wild-type, which are expressed in E. coli BL21 (DE3) using the His-tag pET28a vector, were purified based on methods described in chapter 2 material and method sections 2.2.5 and 2.2.6.

3.2.4 Production of recombinant wild-type HcTrx5 (IS5)

The cDNA clone encoding the IS5 or protein HcTrx-5, which is isolated from *Haemonchus contortus* [242] was kindly provided by Associate Professor Mary Davey (University of Technology, Sydney, Australia). The purification procedure of recombinant protein HcTrx-5 will be described in the following section.

3.2.4.1 Small scale culture preparation

Glycerol stocks of *E. coli* bacteria cells transformed with the plasmid pTrcHisB vector (*Invitrogen, Australia*), containing the open reading frame for the protein HcTrx were provided by Professor Mary Davey and her group (University of Technology, Sydney, Australia). These were used to express the HcTrx-5 (WT) protein. The glycerol stock containing the coding sequences for HcTrx-5 (WT) was used to inoculate 6 individual sterile conical flasks that contain 20ml of 2xYT media (1g Yeast extract, 1.6g Tryptone and 0.5g NaCl, pH 7.0) and 20ul of Carbenicillin antibiotic (*Sigma Aldrich*) with the stock concentration of 30mg/ml. These were left to grow overnight in a shaking incubator set at 200 rpm and at 37°C.

3.2.4.2 Large scale culture preparation and induction of expressed HcTrx-5 protein

The entire volume of the overnight small-scale cultures was used to inoculate 3 large flasks that contained 350 ml of 2xYT medium and 30mg/mL of Carbenicillin antibiotic. Subsequently, the flasks were left to grow in the incubator, shaking at 200 rpm and temperature at 37°C for about 1.5 hours. When the bacterial growth reached an optical density (OD) at the 600nm value of 0.6-0.8, 1mM of IPTG (Isopropylthiogalactoside) was added to the media culture in order to induce the expression of the HcTrx-5 protein. The bacterial cultures were left in the incubator for further growth at 20°C with shaking at 200 rpm for 16 hours.

3.2.5 Harvesting the HcTrx-5-transformed *E-coli* bacterial cells

The IPTG induced cells were subsequently harvested by centrifugation using a Hitachi high-speed refrigerated centrifuge at 10000 rpm for 30 minutes at 6°C. The resultant pellets were collected in a 50mL falcon tube and then resuspended in about 15 ml of Native IMAC lysing buffer, which was comprised of 1mg/mL lysozymes and 10% N-lauryl sarcosine from Bio-Rad Company.

3.2.6 Lysing of the HcTrx-5-transformed *E-coli* bacterial cells

The resuspended *E-coli* cells were subsequently incubated on ice for about 30 minutes before conducting the sonication process. After this incubation time, the resuspended cells were homogenized by an ultrasonic method using Sonicator machine (Sonics & Materials Company / Vibra-Cell Ultrasonic Liquid Processors) for 15-20 second per cycle at 60% output until obtaining a less viscous solution. Then, 1.2ml of 20% Triton was added to 30 ml of homogenized solution. Finally, the lysed cells were further centrifuged using a Hitachi centrifuge, at 10000 rpm speed for 40 minutes at 4°C resulting in separation of both the supernatant and the cells insoluble/membrane components.

3.2.7 Purification and quantification of His-tagged HcTrx-5 (WT) protein

The Histidine tagged HcTrx-5 (WT) protein was purified as described in chapter 2 (materials and methods section 2.2-2.2.5) by using the Native IMAC purification Kit and Ni2+-NTA affinity chromatography purification system. After purification was accomplished, the purified His-tagged HcTrx-5 protein was quantified as previously described in Chapter 2 (materials and methods section 2.2-2.2.7).

3.2.8 Purification of non-His-tagged HcTrx-5 (WT) using size exclusion chromatography

The non-Histidine tagged HcTrx-5 (WT) protein was purified as described in chapter 2 (materials and methods section 2.2-2.2.6) by using Size Exclusion Chromatography (SEC). The resultant purified proteins, which were of low concentration were then concentrated following the method that will be described in the next section.

3.2.9 Ultrafiltration spin column

Ultrafiltration is an efficient concentration method commonly used in biochemical research in order to increase the concentration of a target sample. The diluted protein solutions can

be concentrated and separated from low molecular weight compounds by filtering through a semi-permeable membrane using vacuum centrifugation or pressure by which a pressure gradient is created [292]. By applying this method, both the solvent phase and low molecular weight solutes are permeated through the membrane filter and collected on the other side of the column. Meanwhile, the target macromolecules become concentrated due to a decrease in solvent volume and subsequently remain on the top of the sample side of the membrane.

The purified non-Histidine tagged HcTrx-5 (WT) proteins that were collected from the Size Exclusion Chromatography column were concentrated using the *Vivaspin* ultrafiltration spin columns (*Sartorius Stedim Biotech GmbH, Goettingen, Germany*) that equipped with a membrane with a cut-off of 3-50,000 MWCO PES. The assembled spin column was subsequently centrifuged on a swing bucket rotor at 4500 rpm for 2 hours. The concentrated HcTrx-5 protein was again quantified as previously described in Chapter 2 (material and method section 2.2-2.2.7).

3.2.10 Determination of HcTrx-5 (WT) protein molecular mass employing SDS-PAGE

The molecular weight and purity of His- tagged and non-His-tagged HcTrx-5 (WT) protein was determined as was previously described in chapter 2 (materials and methods section 2.2-2.2.8) by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

3.2.11 Enzyme assay conditions

The condition for carrying out the enzyme assays is as previously described in chapter 2 (material and method section 2.2-2.2.9).

3.2.11.1 HEDS enzyme assay using sodium phosphate buffer

The oxidoreductase activity of both non-His-tagged CLIC1 and CLIC3 was assessed using the standard HEDS enzyme assay as previously described (chapter 2 materials and methods section 2.2.9.1). However, as the ions and ionic strength of solution would affect the

catalytic activity, therefore for optimizing the buffer solution for CLICs activity the 5mM sodium phosphate buffer, which contained 1mM EDTA at pH 7, was substituted with 5mM potassium phosphate buffer that also contained 1mM EDTA at pH7.

3.2.11.2 The enzymatic activity of CLIC1 and CLIC3 in the presence of sodium selenite

The assay was performed based on the method described in [286]. It was conducted separately for both non-His tagged recombinant CLIC1 (WT) and CLIC3 (WT) proteins. A final concentration of 10uM of either reduced monomeric CLIC1 (WT) or CLIC3 (WT) or HcTrx5 (positive control) were added to a mixture that contained 0.1mM Tris-HCl buffer (containing 1mM EDTA/ pH 7.5), 200µM NADPH, 5ug/ml GR, 0.1mg/mL bovine serum albumin and 1mM sodium selenite (Na2SeO3). After 5 minutes incubation at 37°C, the reaction was initiated by the addition of 50µM of GSH peptide. Consumption of NADPH was monitored spectrophotometrically at an absorbance reading of 340nm for 40 minutes of reaction time.

3.2.11.3 Assaying the enzymatic activity of CLIC1 and CLIC3 in the presence of dehydroascorbic Acid

The assay was conducted following the method described in [285]. A final concentration of 10uM of either non-His tagged recombinant CLIC1 (WT), CLIC3 (WT) or HcTrx5 protein (positive control) was added to 137mM sodium phosphate buffer (pH7.5), which contains 0.35mM NADPH, 5mg/ml GR and 2mM GSH. The mixture was incubated at 30°C for 1 minute and subsequently, 1mM DHA was added to the mixture in order to initiate the reaction. Consumption of NADPH was monitored at absorbance reading of 340nm for 40 minutes of reaction time.

3.2.11.4 Assaying the effect of ion channel blocker IAA-94 on CLIC1 and CLIC3 enzyme activity

IAA-94 was stored as a stock solution with a concentration of 560µM (0.2mg/mL of the drug was dissolved in deionised water and mixed until homogeneous solution attained). This stock was diluted to 10µM in 5mM potassium phosphate buffer (pH 7.5) for the assay. To demonstrate the inhibition of CLIC enzymatic activity by IAA-94, 10µM of both non-His-

tagged recombinant CLIC1 (WT) and CLIC3 (WT) as tested proteins were separately incubated with 10µM IAA-94 drug for 1 hour on ice bath prior to use in the HEDS enzyme assay. Subsequently, the HEDS enzyme assay was performed as previously described in chapter 2 materials and methods section 2.2.9.1.

3.3 Results

3.3.1 Purification of His-tagged HcTrx5 protein using Ni^{2+}-NTA affinity chromatography

The "Histidine tagged" HcTrx5 protein was purified using Ni^{2+}-NTA Affinity chromatography procedure and, the purification was followed by a size exclusion chromatography system, which will be explained in the next section. The protein contents of the eluted fractions of the initial Ni^{2+}-NTA purification were determined by absorbance spectroscopy. Figure 3.2 shows the typical recording traces of the elutant His-tagged HcTrx5 Protein, representative of changes in the absorbance over a range of wavelengths from 200 – 350 nm. The most concentrated protein fractions obtained from this stage were pooled and subsequently further purified on a SEC column.

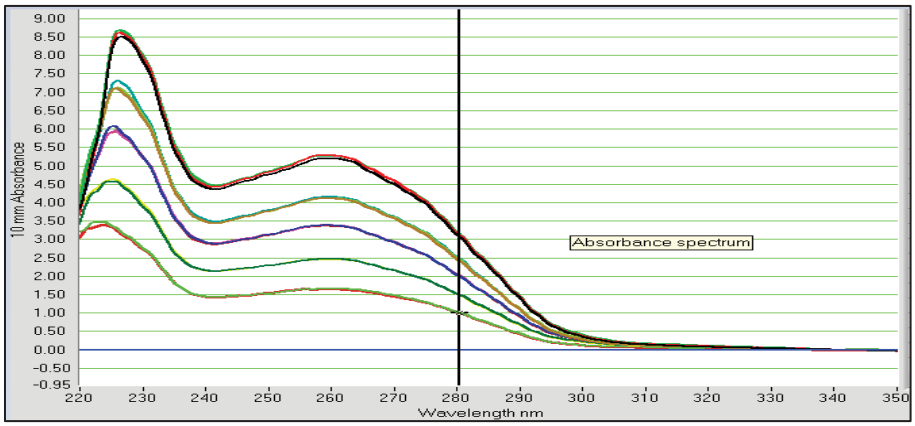


Figure3.2: The traces are representative absorbance profile at 280 nm of the eluted monomeric His-tagged HcTrx5 (WT) purified through Nickel column. Each pooled protein samples has been measured three times (as indicated in with different colors) and the average was taken in order to determine the approximate concentration.

3.3.2 Purification of non-His-tagged HcTrx5 Protein using Size Exclusion Chromatography (SEC)

The figure3.3 is a representative trace of the eluted non-His-tagged HcTrx5 protein, which was purified using the size exclusion Chromatographic (SEC) method. The monomeric non-His-tagged HcTrx5 Protein was eluted at +4°C from a Superdex75 high-performance

chromatography column (GE Healthcare, Piscataway, USA) by washing the column with column sizing buffer for 16 hours.

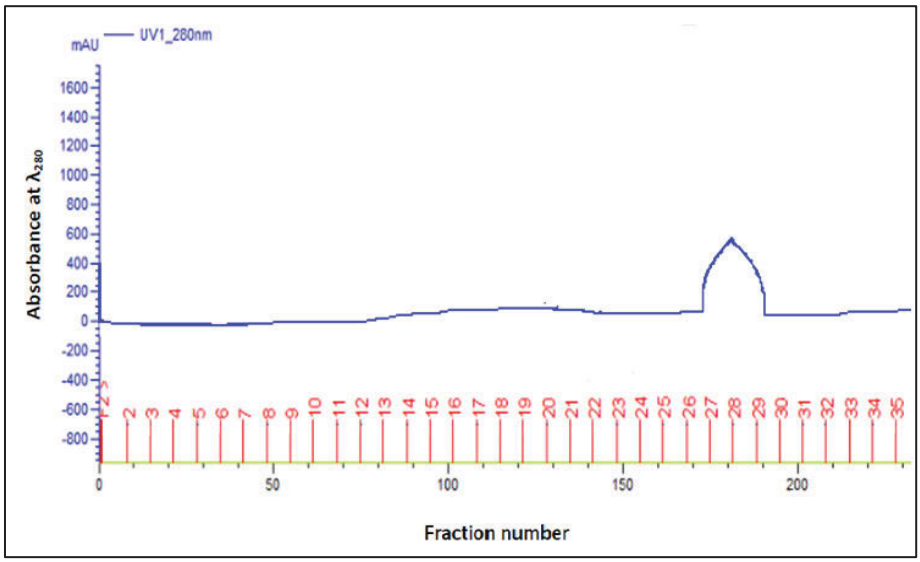


Figure 3.3: A representative trace of the eluted soluble monomeric non-His-tagged HcTrx5 Protein going through a SEC column, monitored at an absorbance of 280nm**.** As indicated by the single peak in this figure, the purified protein was collected in the fractions 27 and 29.

3.3.3 Concentration of purified non-His-tagged HcTrx5 after SEC using Ultrafiltration spin column

Since the HEDS enzyme assay requires a final concentration of 10μM of a protein sample, the relatively diluted protein fractions from the SEC column (3.3.2) were concentrated using ultrafiltration spin columns. As a result, this resulted in obtaining protein with a higher stock concentration

3.3.4 Assessing the purity of the eluted His-tagged and non-His-tagged HcTrx5 protein samples by SDS page

The purity and molecular weight of the eluted His-tagged and non His-tagged HcTrx5 Protein samples were examined by SDS PAGE. As shown in figures 3.4, both the purified HcTrx5 proteins (His tagged: A and non-His tagged: B) displayed a single prominent band which is at the correct estimated molecular weight and showed high levels of purity.

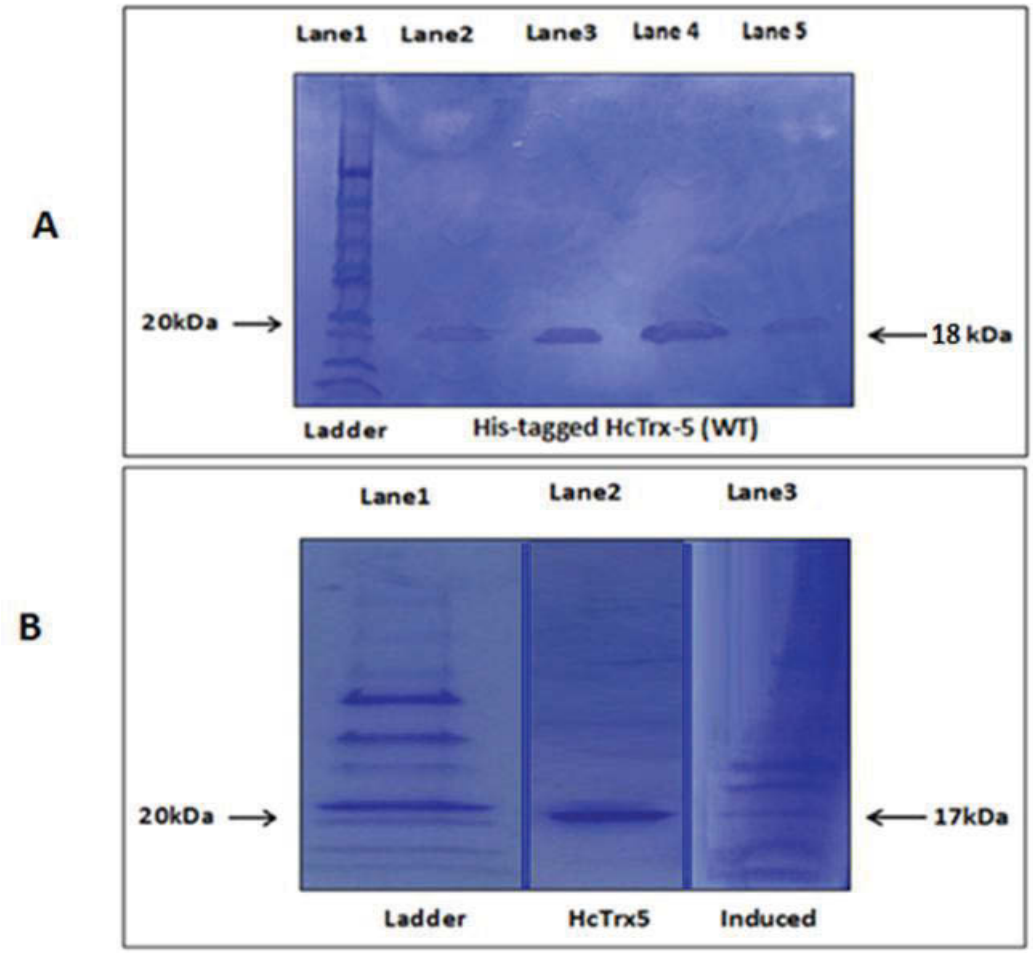


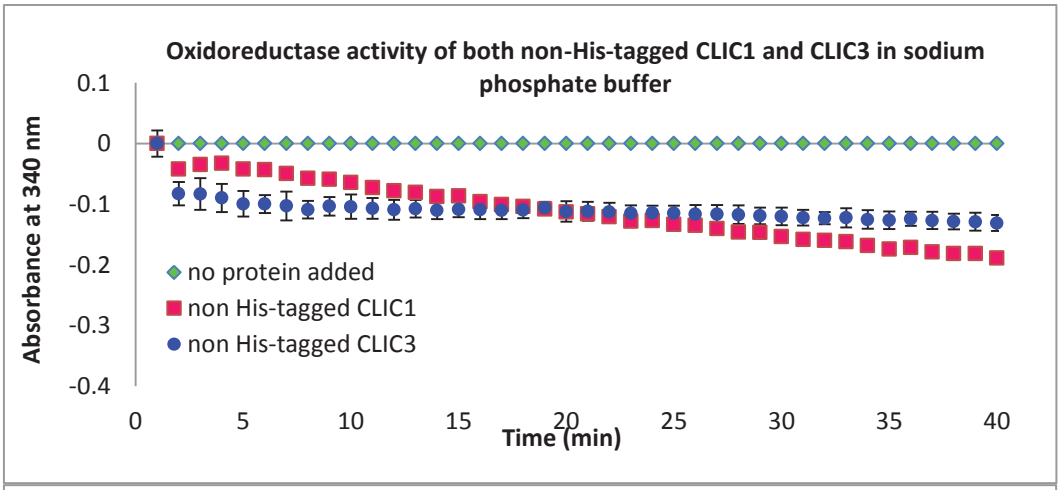
Figure 3.4: SDS-PAGE of affinity purified His-tagged HcTrx5 and subsequently SEC purified, non-His-tagged HcTrx5. (A) Lane1, protein ladder, Lanes 2-5, eluted fractions of His-tagged HcTrx5. (B) Lane1: protein Ladder standard; Lane 2: Non-His-tagged HcTrx5 (WT) eluted from the SEC chromatography column. Lane 3: Bacterial Non-His-tagged HcTrx5 that was induced with IPTG (WT).

3.3.5 Non-His-tagged CLIC1 and CLIC3 remain enzymatically active in the presence of sodium phosphate

The optimal enzyme activity is directly influenced by the identity of the ions and ionic strength in solution. Thus, as some of the other assays that published in literature used sodium based buffers rather than potassium for characterising enzyme activity[285], in this experiment the sodium phosphate was substituted with potassium phosphate, in order to compare the CLICs activity in different buffer composition and therefor optimize the best buffer solution for CLICs activity. The oxidoreductase activity of both CLIC1 and CLIC3 protein was assessed in the HEDS assay in the presence of 5mM sodium phosphate buffer

that contained 1mM EDTA at pH 7 in place of the usual buffer. This was done in order to compare and find the best buffer composition for optimal CLICs catalytic activity. As demonstrated in figure 3.5 (A), both proteins remained enzymatically active in sodium phosphate buffer. As figure 3.5 (B) indicates, no significant changes occur in non His-tagged CLIC3 when potassium phosphate buffer substituted with sodium phosphate buffer. However, the activity of non His-tagged CLIC1 slightly decreased due to this buffer substitution.

A



B

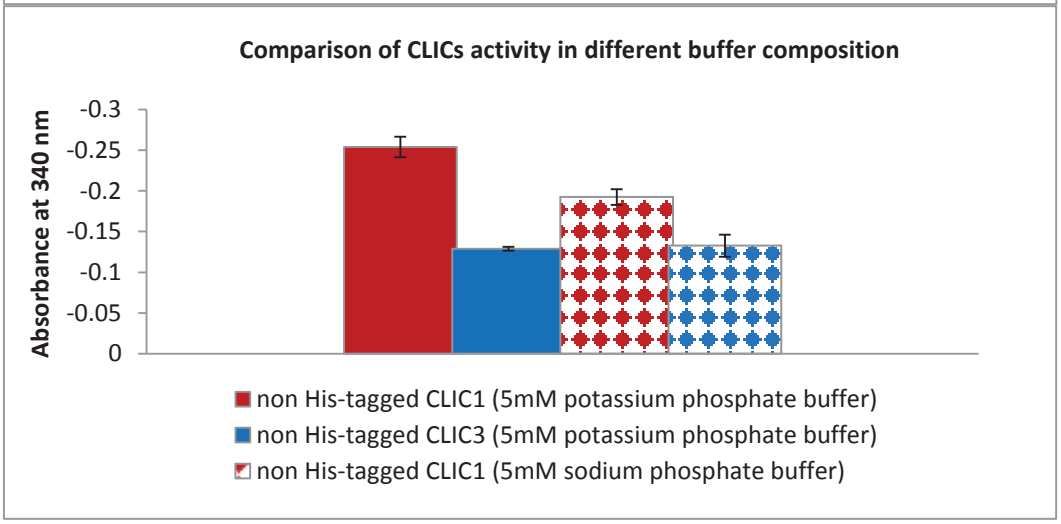


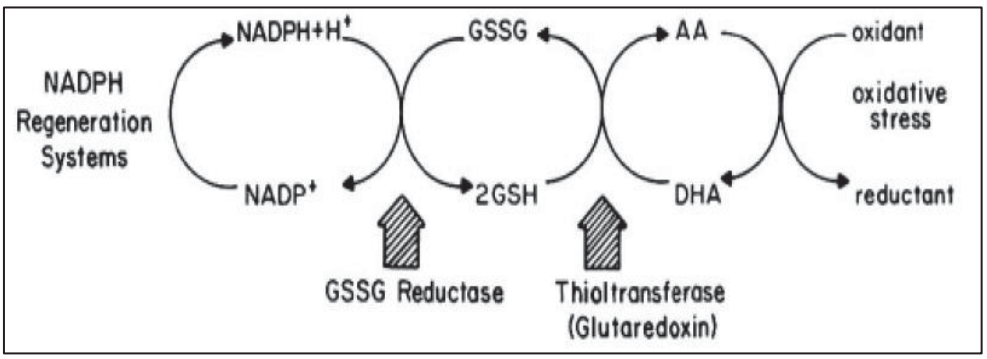
Figure 3.5: Oxidoreductase activity of non-His-tagged CLIC1 and CLIC3 in the presence of sodium phosphate buffer in vitro assay at pH7. (A): the catalytic activity of non-His-tagged CLIC protein members were measured by using a final concentration of 10µM of CLIC1 and CLIC3 that mixed by 250 µM NADPH, 1 mM HEDS and 0.5µg/ml of GR in 5 mM sodium phosphate, which contains 1 mM EDTA in pH 7, at 37˚C. The mixture was heated up for 5min at 37°C. After that, 1 mM of GSH was added to the mixture in order to initiate the reaction. Then, the consumption of NADPH was monitored for 40 minutes at absorbance of 340 nm. (B): the comparison column indicates the optimal activity of CLIC1 obtained in potassium phosphate buffer, where the CLIC3 were similarly active in both buffers. Error bars represent the standard deviation of three individual measurements / the absorbance measurement of the activity was taken for analysis at the 40th minute of the reaction time.

3.3.6 Dehydroascorbic acid and sodium selenite as substrates

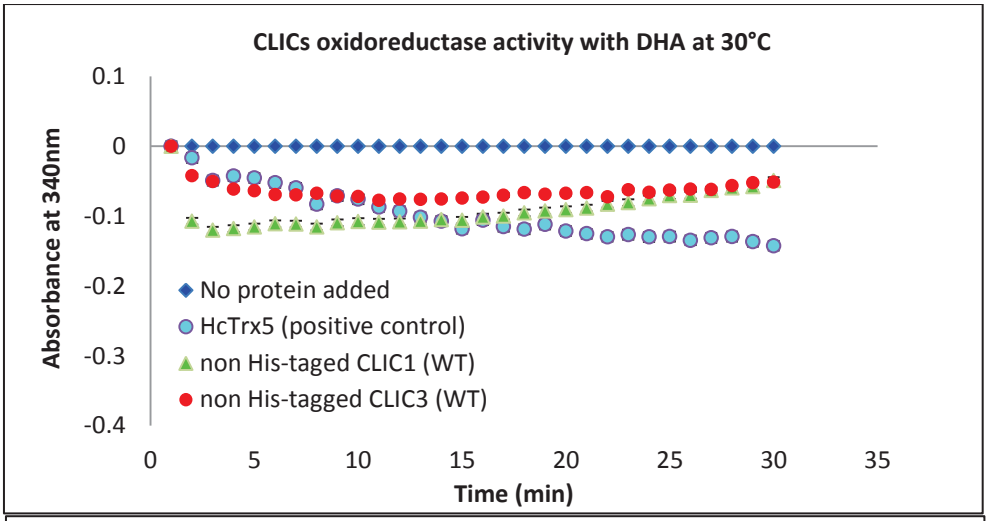
One of the well-recognized redox reactions is the reduction of the oxidized form of ascorbic acid (vitamin C), also known as dehydro-L-ascorbic acid (DHA) by a thioltransferase enzyme, including the Glutaredoxins. As illustrated in figure 3.7A below, in this reaction the glutathione peptide (GSH) is actively regenerated from GSSG that receives electrons through the reduction of NADPH by glutathione reductase (GR), which together maintain the ascorbic acid levels in cells[285, 288]. It is well known that the Glutaredoxins (Grx) also use Selenium compounds as substrates [286], and our group at UTS has previously shown that the CLIC1 is also able to reduce both DHA and sodium selenite in a manner similar to the Grxs [5]. In this study, the substrate affinity of the non-His-tagged CLIC3 protein was assayed to investigate whether it could reduce selenium anion and DHA, similar to non-His-tagged CLIC1.

In order to assay CLIC3 enzymatic activity, a glutaredoxin-like enzyme activity assay was employed using either sodium selenite (Na2SeO3) or DHA as the substrate. The reaction was initiated by addition of GSH and the consumption of NADPH was monitored at 340nm absorbance. As seen in (figure 3.7B) both non-His-tagged CLIC1 and CLIC3 proteins were able to use DHA as a substrate similar to the HcTrx5 (positive control). But, as the result in this figure indicates the catalytic reaction for both proteins start to be inhibited as the reaction reached the end. Besides, Figure3.7C demonstrates that non-His-tagged CLIC1 and CLIC3 are also able to use sodium selenite as their substrate like the HcTrx5 (positive control), however, non-His-tagged CLIC3 demonstrated lower catalytic activity with this substrate in comparison to the non-His-tagged CLIC1.

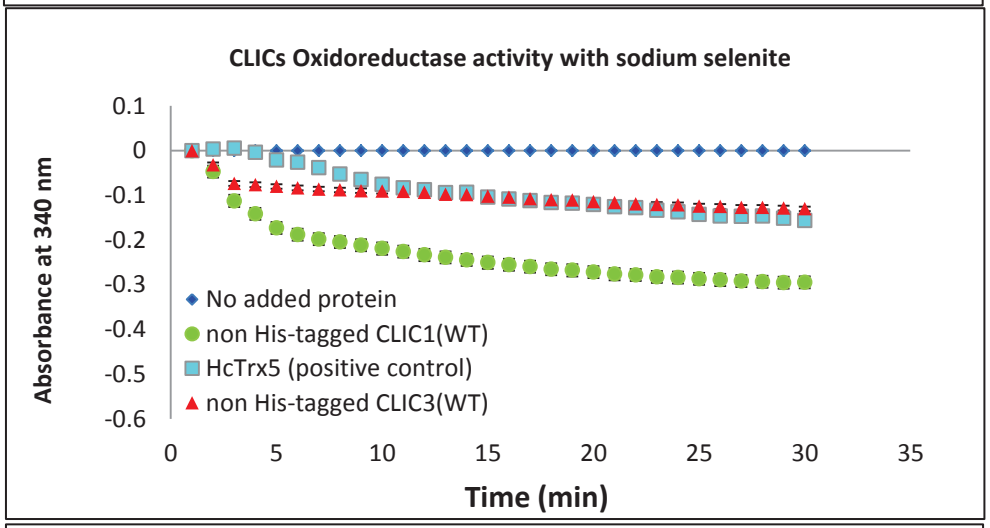
A



B



C



D

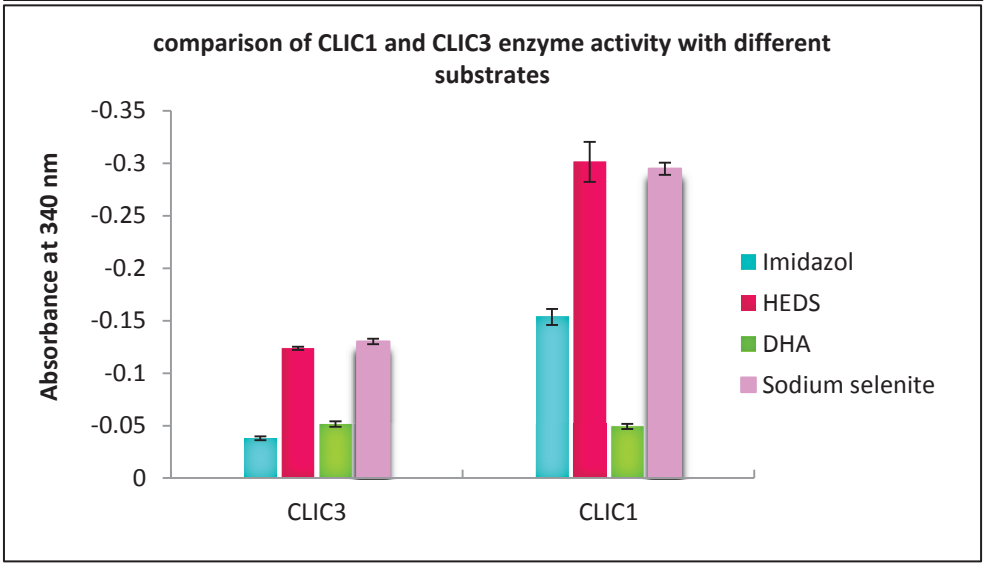


Figure3.6: Non-His-tagged CLIC1 and CLIC3 using DHA and Sodium selenite as their substrates. (A) Enzyme coupled electron transfer pathway by which the dehydroascorbic acid (DHA) is reduced through a GSH-dependent system [285]. (B) The enzymatic activity of non-His-tagged CLIC1 and CLIC3 using DHA as substrate was separately implemented for each protein in the mixture that contained 137 mM sodium phosphate buffer with 2 mM EDTA (pH 7.5), 0.35mM NADPH, 0.5µg/ml

Glutathione reductase, 1mM DHA, 2mM GSH and 10µM of either non-His-tagged CLIC1, CLIC3. HcTrx5 was included as a positive control. The mixture was incubated for 1 minute at 30°C and the reaction was initiated by addition of 1mM DHA. The NADPH consumption was measured at 340nm absorbance reading. Error bars represent the S.E. (C) The enzymatic activity of non-His-tagged CLIC1 and CLIC3 using sodium selenite as a substrate. The reaction mixture contained 0.1mM Tris-HCl with 1mM HCl (pH 7.5), 0.5µg/ml GR, 200 µM NADPH, 0.1 mg/ml BSA, 1mM Sodium selenite as well as 10uM final concentration of non-His-tagged CLIC1 or CLIC3. HcTrx5 was included as positive control. After 5 minute incubation of stated mixture at 20°C, 50µM of GSH was added to initiate the reaction by which the consumption of NADPH was monitored at 340nm absorbance. (D) Column graph comparing the enzyme activity of CLIC1 and CLIC3 with different substrates after 30 minutes of reaction time. Error bars indicate the S.E.

3.3.7 Chloride ion channel blocker drug IAA94 inhibits oxidoreductase activity of both non-His-tagged CLIC1 and CLIC3 proteins

As CLICs are known as intracellular chloride ion channel proteins, we then studied whether there is any correlation between their enzymatic function and their ion channel activity. We examined the effects of a known pharmacological Cl^- channel blocker, Indanyl oxyacetic acid 94 or IAA-94 ($C_{17}H_{18}Cl_2O_4$) on the non-His-tagged CLIC1 and CLIC3 catalytic activity. The drug IAA-94 is known to completely block both CLIC1 ion channel activity in cells and its oxidoreductase enzyme activity in the HEDS enzyme assay [118].

Likewise, *in vitro* studies have demonstrated IAA-94 blocks the Cl^- ion selective channel formed by recombinant CLIC1 in an artificial bilayer system [2, 4, 293]. As seen in figure 3.8, the drug IAA94 was found to have an inhibitory effect on the enzymatic activity of both non-His-tagged CLIC1 and CLIC3 proteins, completely inhibiting their catalytic activity in the standard HEDS enzyme assay.

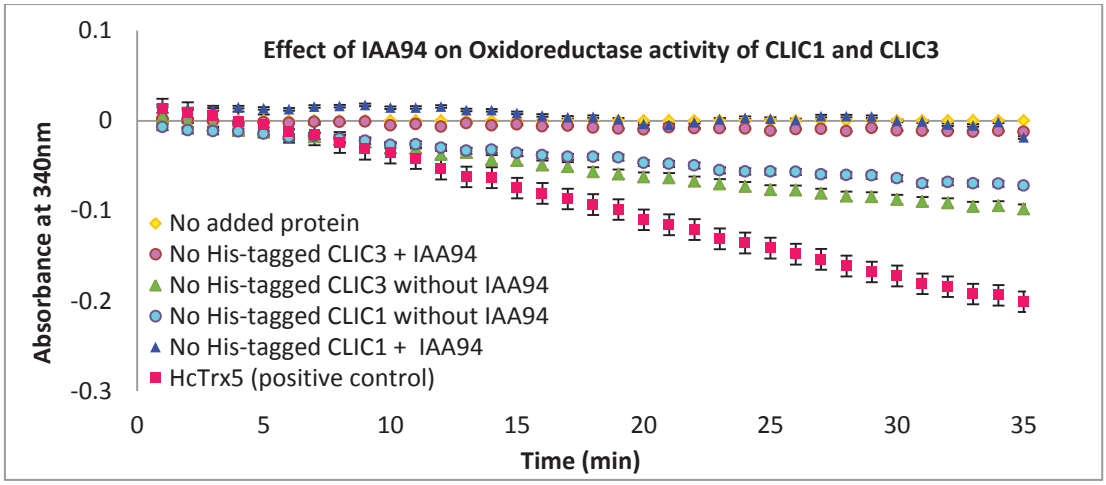


Figure 3.7: Influence of Cl^- ion channel blocker IAA94 on the oxidoreductase activity of non-His-tagged CLIC1 & 3. The same final concentration (10µM) of both non-His-tagged CLIC1 and CLIC3 protein were incubated with a 10µM final concentration of drug IAA94 for 1 hour. Then the incubated CLIC proteins were mixed with 1mM HEDS, 250 µM NADPH, 0.5µg/ml Glutathione reductase and 5mM potassium phosphate buffer that contains 1mM EDTA (pH7). The mixture was incubated for 5 minutes at 37°C and the reaction initiated by the addition of 1mM GSH. NADPH consumption was monitored at 340nm. The error bars indicate the standard deviation from three independent readings.

3.4 Discussion and Conclusion

3.4.1 CLIC3 alike its CLIC1 counterpart use sodium selenite as its substrate

The trace element Selenium is a non-metal but is occasionally also considered a metalloid. It exists in nature in both organic and inorganic forms. It exists in its inorganic forms as selenite (SeO(OH)2) and selenate (SeO2(OH)2), which are nutritionally essential for humans. In biological systems the organic forms of selenium are found as seleno-methionine (SeMet), seleno-cysteine (SeCys), which can be incorporated into seleno-proteins [289, 294, 295]. As Selenium is considered a vital element for normal functioning of the human body, several studies have investigated its functional role in the health such as its inhibitory effect on HIV progression to AIDS [296-298]. It has also been shown to have both anti-bacterial and anti-inflammatory activities [299, 300] and also has a role as an anticancer agent. It is considered as an effective chemotherapeutic agent when combined with chemotherapy medication such as cisplatin to enhance the therapeutic effect of chemotherapy [301, 302].

Selenium is a well-known compound that participates in redox reactions by sharing one or two electrons. In this regards, selenium is similar to sulfur, which has low reactivity with high oxidation states[303, 304]. Selenium is mostly found as an integral constituent of seleno-proteins in biological systems, where these proteins are involved in oxidoreductase reactions via their seleno-cysteine residue (SecU) as catalytically active sites [305]. Although a different type of seleno-proteins such as glutathione peroxidase (GPx), thioredoxin reductases, deiodinases (DIO) and one of the methionine sulfoxide reductases (Msr) exist in mammalian cells, many other proteins that contain a CXXU motif and demonstrate putative oxidoreductases activity are also considered as seleno-proteins. Thus, it is not surprising that selenium compound is increasingly involved in redox signalling [306, 307].

Furthermore, in a cellular environment in the presence of Glutathione (GSH) and thioredoxin (Trx), the selenium compounds such as selenium dioxide can be reduced and converted to the elemental form of selenide (HSe-, RSe-) [308]. In this regards, selenium reduction occurs via the thioredoxin system, through which the covalent connection between GSH and selenium results in a seleno-di-glutathione (GS-Se-SG) species, that subsequently is transformed to selenide [309, 310]. Furthermore, in the presence of oxygen,

the Selenide thiol group undergoes redox reaction resulting in the generation of reactive oxygen species (ROS)[311, 312]. As a consequence, the increase of ROS and superoxide production can significantly destroy healthy cells and result in DNA mutations, physiological malfunctions or cell apoptosis [313, 314]. As such, despite the previously mentioned positive effects of selenium, it can also be considered as a toxic element when present at high concentrations. Accordingly, in experimental animals the normal selenium concentration with narrow sufficient range of between deficient and excessive does has been shown to be in the range of 0.1–1.0μg/g in diet or ml drinking water.[289].

Similar to work by Wallenberg et al (2010) that demonstrated the oxidoreductase activity of human Glutaredoxin (Grx) enzymes with Selenium compounds as substrates [286], our group has previously demonstrated the monothiol glutaredoxin-like activity of CLIC1 using either HEDS or sodium selenite as a substrate [5]. In this Masters project, it was of interest to determine if the dithiol CLIC3 protein, could also reduce selenite, in a manner similar to CLIC1. Therefore the compound sodium selenite (Na2SeO3) was used as a substrate, which is the most common water-soluble selenium compound and is readily reduced to selenide by GSH peptide in a Glutaredoxin catalytic system [289]. As it was seen from the results (figure3.6C), both non-His-tagged CLIC1 and CLIC3 were able to catalyse the reduction of sodium selenite. This for the first time demonstrates the catalytic activity of the dithiol CLIC3 using this selenite substrate. However, as determined via direct comparison of the reaction profile (figure3.6D), the non-His-tagged CLIC3 was less effective in the reduction of sodium selenite compared to CLIC1.

Therefore, these findings presented here further strengthen the proposal that the soluble CLIC protein members; like CLIC3 and CLIC1, could use biologically available selenium compounds by catalysing and reducing these substrates in the presence of GSH and as such contribute to the maintenance of selenium metabolism and redox control within cells and biological systems.

3.4.2 Dehydroascorbic acid considered as a substrate for the CLIC3 protein similar to its CLIC1 counterpart

Vitamin C, also known as ascorbic acid is a water-soluble vitamin that is essential for several biological activities. In general, this vitamin is considered to have several roles and is able to act as a free radical scavenger, an electron donor and acceptor in electron transfer reactions and as a cofactor for several enzymes, which participates in crucial functions such as synthesis and metabolism of microsomes, collagen, carnitine, neurotransmitters and tyrosine [315-317]. In addition, it has been revealed that the ascorbic acid has other potential functions such as involvement in endothelial cell proliferation and apoptosis, endothelial permeability barrier function as well as smooth muscle-mediated vasodilation [318].

Several studies have shown that vitamin C deficiency can have a significant effect on different conditions like cancer, diabetes, cataract, HIV infection, systemic lupus erythematosus (SLE) disease and reduction of oxidative stress in the body [319-321]. In addition, ascorbic acid acts as a reducing agent and scavenger in the removal of free radicals from biological systems [322-324].

In view of that, the mono anion form of ascorbic acid (ascorbate) at physiological pH is considered as one of the predominant chemical species. This anion undergoes two sequential reversible reactions, one oxidation reaction by which dehydroascorbate (DHA) is generated and the other reaction leads to the production of the intermediate ascorbate free radical (AFR). Indeed, the AFR that is considered as an unreactive free radical has a low reduction potential in comparison with the other potent radicals such alpha-tocopherol radical, the glutathione radical and almost all known reactive oxygen and nitrogen species that are produced in several human diseases [325]. This activity of ascorbate as an efficient electron donor is vital in many biological redox reactions. Thus, the ascorbate has the ability to substitute the highly harmful and potent radicals with the poor reactive ascorbate radical[326].

In addition, ascorbic acid is capable of recycling other crucial antioxidant molecules like glutathione and a-tocopherol from their particular radical species. Besides, the oxidized

form of ascorbic acid (DHA) can be reduced back by glutathione and other thiols using NADPH dependent reaction. Thus, the reduction and regeneration of the oxidized form of ascorbic acid is an important process with the intention of maintaining the normal level of vitamin C within the cell environment [327].

On the other hand, at physiological pH, the DHA is an unstable molecule, so it needs to be reduced back into ascorbate or it could be also irreversibly hydrolysed into 2,3-diketogulonic acid. The reduction of DHA to ascorbate would occur through one of these two steps, first a direct non-enzymatically reduction by glutathione (GSH) [328, 329], second by a glutathione-dependent DHA reductase, glutaredoxin enzyme [330], or by the NADPH dependent thioredoxin reductase enzyme [331].

Moreover, it has been shown that a plant dehydroascorbic acid reductase (DHAR) enzymes also has the ability to undergo the reduction of DHA to ascorbic acid [332]. Accordingly, phylogenetic studies of CLIC proteins have demonstrated that plant DHARs share a high degree of similarities with this family [134]. Therefore, based on these structural similarities it has been postulated that the plant DHAR proteins could adopt a three-dimensional structure alike to the soluble form of the CLIC1 protein [137, 332]. In this regards, DHAR from Populus tomentosa was recently demonstrated to have an oxidoreductase catalytic activity [332]. Indeed, this was further strongly supported by the mutagenesis studies through which the catalytic activity of protein PtoDHAR2 was totally abolished as the Cys20 residue (predicted GSH binding site in the PtoDHAR2) was substituted with alanine amino acid [332]. These results are in correlation with the recent mutagenesis study with CLIC1 mutants C24A and C24S in the standard HEDS enzyme assay in which both C24A and C24S demonstrated no enzymatic activity [5].

Our data from the previous study [5] and from the current Master's project (Figure3.6B), have shown that the non-His-tagged CLIC1 and CLIC3 proteins can catalyse the reduction of DHA. Thus, based on these studies and results, we speculate that protein members of the CLIC family would likely function in cellular protective roles due to their ability to metabolize substrates like DHA and as a consequence maintain the normal level of ascorbate within the cell. However, it should be noted that as the figure 3.6(B) indicates, the reaction rate of DHA reduction by both CLIC1 and CLIC3 enzymes starts to undergo a level off by the end of

reaction time. This phenomenon is a common issue for many enzymes and could occur due to product inhibition. Where the product of catalytic reaction binds with the catalyser and as a result inhibits the rat of the forward enzymatic reaction. This would be considered as a crucial regulatory action that controls the metabolic pathways through its negative feedback [333].

In conclusion, as the CLIC members share structural similarities along with their DHA reductive ability, similar to that of the plant DHAR proteins [134, 137, 332], as well as their high binding affinity with the DHA substrate [5], this points to DHA being a likely physiological substrate for these proteins, where this ability to reduce DHA reduction can assist in the prevention of several diseases, like cataract formation [334].

3.4.3 IAA94 an inhibitor of the oxidoreductase catalytic activity of CLIC3 and CLIC1

Ethacrynic Acid ((2,3-dichloro-4-(2-methylenebuty-ryl) phenoxy) acetic acid), is known as a potent sulfhydryl-reactive loop diuretic drug [335, 336]. A number of studies have been extensively undertaken in order to understand the effect and mechanisms of action of this inhibitor drug. For instance, it has been found that Ethacrynic Acid (EA) can effectively inhibit the catalytic activity of GST isozymes, such as rat GST isoenzymes [337-339]. The nature of this inhibition occurs due to the interaction of ethacrynic acid (EA) with the GSTs by representing itself as a substrate or non-substrate ligand to the enzyme. Following binding, the drug can be conjugated with the GSH peptide and forms an ethacrynic acid-GSH (EA-SG) conjugate; spontaneously or by GST-catalysed reactions [290, 340-342]. In this regards, a comparative Inhibition study has indicated that the EA-SG adducts in comparison with the EA have a more potent inhibitory effect on GST enzyme with distinct mechanisms [341].

In view of these studies, it was defined that the GST-π class of enzyme has a ligand binding site (H-site) that is adjacent to the GSH binding site (G site) in the enzyme and it occupies part of substrate binding site [342, 343]. In this regards, crystallographic studies of GST proteins have shown that in these enzymes, the EA component binds to the H-site where the EA-SG conjugate binds to the specific GSH binding site [343-345]. In addition, based on

studies that applied a structural superposition between GST-π enzyme that contained EA complex and the CLIC1 structure; a structural homology between these two proteins was revealed in which the CLIC1 protein shares a similar empty ligand binding site (H-site) that was thought to be a site for a blocker or an inhibitor agent [108, 343].

Indanyl oxyacetic acid 94 or IAA-94 ($C_{17}H_{18}Cl_2O_4$), which is also known as a diuretic drug, shares a resemblance to ethacrynic acid and has the ability to inhibit or block chloride selective ion channel [346]. It has also been shown to block the activity of the intracellular Chloride Ion Channels (CLIC proteins) [5]. A cell study using IAA94 demonstrated that in the presence of IAA-94 the cell growth of Chinese hamster ovary cells (CHO-K1) resulted in the arrest of the cell cycle in the G2/M phase and was likely due to the blocking of the CLIC proteins and the CLIC1 in particular [118]. More recently, a study of the oxidoreductase activity of the CLIC1 and CLIC3 proteins [5, 6] along with the obtained result from this Masters study (as seen in figure 3.7) demonstrate that this ion channel blocker drug IAA-94 can also inhibit the catalytic activity of members of the CLIC family.

The mechanism of function on the IAA-94 inhibition of the CLIC enzymatic activity is currently yet to be fully understood. It may be caused by the following factors:
first, the CLIC proteins are structurally closely related to the GSTs [108, 343] with ethacrynic acid (EA) having an inhibitory effect on the catalytic activity of the GSTs by binding to electrophilic substrate site (H-site), which is in the vicinity of the GSH binding site [338, 347]. Second, owing to structural homology between both ethacrynic acid and IAA-94, this drug (IAA-94) would possibly bind to the CLICs binding slot, and therefore likely involved in the GSH mechanism [4]. Third, the study on bovine retinal cortex vesicles has revealed displacement of ethacrynic acid with IAA-94, which resulted in a good inhibitory potency by this drug [348].

Overall, this evidence suggests a possible mechanism for this inhibitory effect on CLICs catalytic activity. Due to the binding of IAA-94 a non-substrate ligand into the H-site, which is in the neighbouring of the catalytic active site of soluble CLIC1 and CLIC3 proteins, and binding site of GSH, this drug may possibly be conjugated (spontaneously or via CLICs-catalysed reactions) with the GSH to form IAA-94-GSH conjugate. Therefore, reversibly inhibit the enzymatic activity and similarly the channel activity of these two proteins [5].

Thus, based on the fact that the cell membrane is permeable to these drugs, their effect on cellular inhibition and arrestment of the cell cycle progression could be owing to the inhibition of the catalytic activity of both CLIC1 and CLIC3 proteins instead of direct blocking their ion channel activity.

In conclusion, as the proteins of the CLIC family are well known for their functional ability as ion channels, they are also recently identified to be capable of acting as oxidoreductase enzyme in their monomeric soluble form. This finding presents an additional intracellular activity to these protein members that could be involved in processes such as cell detoxification and oxidoreduction. In the last part, as the same CLIC1 ion channel blocker (drug IAA94) that inhibits the enzymatic activity of both non-His-tagged CLIC1 and CLIC3 proteins, this could be postulated that the activity of the ion channel form of these proteins could be under control of their oxidoreductase catalytic activity.

Chapter 4

Effects of Temperature and pH on the Enzymatic Activity of the CLIC Proteins

4.1 Introduction

4.1.1 Influence of temperature on enzymatic activity

Study of an enzyme's behaviour and its activity under varied environmental stresses such as changes in temperature, pH or other, is fundamental in understanding the physiological role or other activities performed by enzymes in many areas of biology. In recent times, the relationship between temperature and enzymatic activity has been studied in different aspects. For instance, it has been shown that for some enzymes, their catalytic activity increases to a certain point as the temperature is raised. But, at the same time, this increment subsequently leads to irreversible inactivity of the enzyme due to denaturation [349-352]. In that regards, the effect of temperature on enzyme activity has been described by an equilibrium model. This model introduces a mechanism by which, the active enzyme (E_{act}) would be converted to an inactive enzyme (E_{inact}) due to thermal stress. Therefore, it is the inactive enzyme that undergoes to the thermally denatured state. This view has led to a better understanding of the experimental data and the catalytic process for all enzymes that follow Michaelis–Menten kinetics[353, 354].

This novel equilibrium model, explains an additional mechanism through which the temperature would have an impact on the enzymes catalytic activity, with implication for several factors such as ecological condition, metabolic system, enzyme structural architecture and applied studies of enzymes [355].

Additional evidence is also revealed that the above-mentioned mechanism takes place at the enzyme active site motif [356]. Indeed, the active-site localisation of the transition is an expectable fact, given that the conformational flexibility is an intrinsic ability, which occurs mostly in an enzymes catalytic mechanism. Consequently, it seems that the active site motif could be one of the most sensitive parts of an enzyme in regards to temperature-induced conformational changes[355].

Besides, notwithstanding the fact that several possible molecular bases exist for the E_{inact}/E_{act} interconversion, the temperature changes would effects the charge of amino acid residues as a possible factor in the observed side effect [357, 358]. In fact, the pKa values of

amino acid side chains are sensitive to changes in ambient temperature. Particularly, the basic residues like histidine and lysine that are one of the main prevalent components of active sites and their surroundings as well as N-terminal amino group, demonstrates relatively major changes in their charge by changes in temperature. Therefore, with a 30°C temperature change the pH would shifted up to a pH unit and significantly effect on catalytic activity of many enzymes [355].

Accordingly, the temperature changes have also the potential to alter the charge and charge distribution of the active site, due to the interaction fact that occurs between the ionisable residues of amino acid side chains with the charges on proximate ionised residues, neighbouring peptide, polar residues and or bound water molecules. Several enzyme catalytic activities are closely dependent on the charge of particular catalytic residues[355].

Therefore, in this regard, a temperature-driven pKa shift would directly have a significant effect on many catalysts. It is notable that alteration in pKa value would lead to conformational changes. For instance, if an ionic bond preserve the conformational structure of active site and its surrounding (like Asp-Lys), subsequently, if the pKa of Lysine (Lys) dropped down as a result of increment in temperature, the positive charge of this α-amino acid would be decreased along with weakened in the ionic bond, which consequently resulting in a conformational change[355].

On the whole, it is evident that the effect of charge changes on enzyme activity is pH dependent, so based on that significant changes would happen in enzyme activity by alteration of pH solvent with even less than a single pH unit. For the majority of enzymes, the interconversion of E_{act}/E_{inact}, which is defined via the Equilibrium Model, is demonstrated an ΔHeq (enthalpy for the E_{act}/E_{inact} transition) between the range of 100–300 kJ/mol [356, 359]. Therefore, by a 10–20 °C reduction in temperature beneath that at which almost 50% of the enzyme is in its inactive form, this resulting in a shift in the Eact/Einact phase transition at a point in time where 90% of the enzyme is inactivated[355]. Indeed, this is not in contradictory with the concept that the alteration at an active site that may arise out of a change in pKa value upon this temperature change[350, 358].

4.1.2 What is the significance of understanding the influence of temperature on enzymes?

As is well known, almost all chemical transformations are catalysed by enzymes within cells. Thus, it is vital and fundamental fact to comprehend the enzyme catalytic behaviour and their chemical reactivity under different conditions. This understanding not only provides a better insight into the study of normal cellular activities but also assists in the ability to manipulate these activities, via enzyme and metabolic engineering.

In addition, this comprehension also leads to obtaining beneficial information on enzyme concentrations, functions and regulatory reactions within the cell, living organism or systems, which would be essential in order to control or remedy in any serious incursion that occurs in biological systems. One of the key factors in these different fundamental studies is to characterize the effect of changes in temperature on enzyme activity.

In this regards, the alterations that occur in enzymatic activity due to the changes in temperature have been studied widely. Based on these studies, the enzyme reaction rate is generally increased exponentially due to this thermal alteration. But, after a certain point, the concentration of active enzyme is simultaneously decreased, which led to the formation of inactive enzyme and therefore, led to irreversible thermal inactivation and denaturation [349, 351, 353, 360].

The first study on this effect was done by Thomas and Scopes, who investigated the influence of temperature on the kinetic parameters and stability of both mesophilic and thermophilic 3-phosphoglycerate kinases[361]. In principle, based on their work it was demonstrated that when the assay temperature was increased above the temperature optimum of the enzyme, the kcat was decreased.

4.1.3 Influence of pH on biological activities and study of pH variety within the cell

Evidently, in addition to a defined temperature adaptation, biological macromolecules like enzymes need to be adapted to other biophysical factors such as the characteristic pH, in

order to perform their function within the specific subcellular compartments or tissues. Most of the biological activities are pH dependent that indicates the significance of the local pH on all cellular processes. Therefore, the pH of the surrounding fluids certainly influences these biological processes. Examples of such effect include: subcellular displacement of α-tocopherol transfer protein (α-TTP) between hepatic cytosol and late endosomes [362], functional pH-dependence of talin–actin binding [363] as well as structural conformation changes accompany with the function in transition of *Bacillus anthracis* protective antigen with differential oxidative protein[364, 365].

In addition, it is demonstrated that the concentration of hydrogen ions play an important role in protein stability through acidic/basic unfolding [366-369] as well as pH-dependent protein stability [370-375]. Moreover, it has been shown that alteration in pH causes precipitation and the formation of amyloid fibers and aggregation phenomenon [376-380]. Furthermore, several biological processes like protein–protein interactions [381-383], protein–ligand binding[382, 384, 385], protein interactions with membrane [385-388] as well as peptide-membrane interactions[389] are also significantly influenced by changes in pH.

Furthermore, these cited examples are indicative that the pH of the cellular aqueous milieu plays a crucial role on most biological processes, which involve macromolecules and their assemblages. On the other hand, as it shown in figure 4.1 the physiological pH differs within individual intracellular compartments[390]. For instance, the pH of the cytoplasm, nucleus and endoplasmic reticulum is nearly neutral[390, 391]. Whereas, the pH of lysosomes and the Golgi compartment is more acidic[390], while the mitochondria and peroxisomes demonstrate a basic pH [392-394].

These variations in the pH of intracellular compartments arise from regulation in the balance between proton pumping, counterion conductance as well as proton permeation across cellular membranes. Accordingly, the function and localization of proteins and enzymes would be affected by these differences in pHs of cellular and sub-cellular organelles[395]. Indeed, if the charge distribution of proteins have been optimised for particular functional purposes, the protein may possibly also exhibit an adaptation to precise subcellular conditions or pH, as was seen in the case study of monomeric proteins

which demonstrated that the pH of maximal stability, correlates with subcellular characteristic pH, where histidine ionisation is the key factor that underlies this pH-dependence correlation[396, 397].

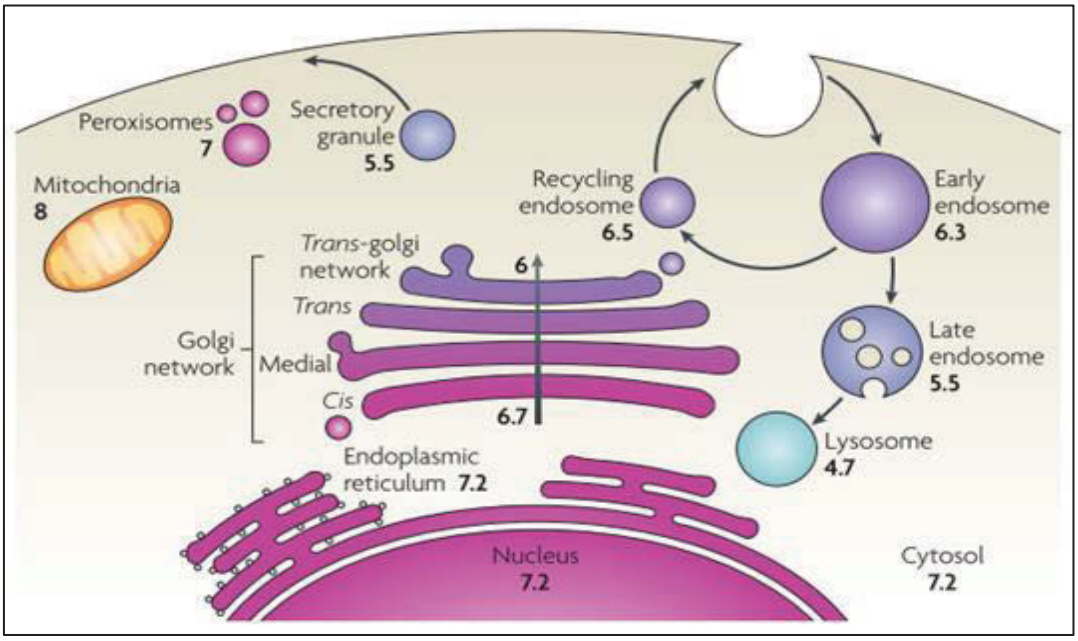


Figure 4.1: Intracellular compartments have different physiological pH. This figure is representative of the prototypical mammalian cell and indicates a differentiation of pH within individual subcellular compartments [390].

4.1.4 The relationship between enzyme activity and pH

As described in the previous section, pH is another factor that influences an enzyme's catalytic activities. On the one hand, alterations in pH can affect the shape of an enzyme; on the other hand, this alteration can also affect the enzyme substrate, by altering the shape or the charge properties of the substrate. Therefore, either the enzyme loses its ability to perform catalysis or the substrate becomes unable to bind to the active site.

In general, different enzymes demonstrate their optimal catalytic activity at different pH conditions - known as the optimum pH - which is dependent on where the enzyme normally functions. Thus, if the pH rises or falls from this optimum point, the bonds that hold onto each other in order to form the tertiary structure of the enzyme would consequently break. Therefore, the enzyme loses its structure and shape as a result of this alteration. Besides, under conditions of extremely high or low pH most enzyme catalytic activity would

dramatically reduce or be completely abolished. For instance, it was shown that an extreme reduction in pH resulted in the decline of enzymatic activity caused by the irreversible inactivation of the soil enzymes like urease, acid phosphatase, and phosphodiesterase [398].

In fact, many of common fundamental energy transduction processes such as enzymatic catalysis [399] and redox reactions [400, 401] are under the control of ionisable groups, which have different pKa values. Under the appropriate pH conditions, these ionisable groups would control the enzymatic activity as they titrate with highly perturbed pKa.
In contrast, as the pH is also a crucial factor in the enzymes stabilities, the pH-dependence of protein stability could be dramatically affected by only one of these perturbed pKa ionizable groups [402]. Hence, this could be the reason that the pH optimum of catalytic reactions is mainly not associated with the pH optimum of stability. In this regards, the protein adaptation to subcellular pH may lead to involvement of the tuned pKa values of active-site residues[395].

In this regards, it has been shown that the activity of phosphofructokinase enzyme is highly sensitive to minor alteration in pH in the physiological range, where a low pH reducing the affinity of enzyme to its substrate [403]. Furthermore, it has been revealed that the recombinant CLIC1 (NCC27), which demonstrate oxidoreductase enzyme activity[5], assembles in lipid bilayers via a pH-dependent behaviour[139]. In this regards, this chapter will define the investigations undertaken to determine the optimum pH, temperature, and stability of both purified CLIC1 and CLIC3 proteins at which their oxidoreductase activity is maximal.

As such, this chapter will present studies that seek to determine:

- The optimum pH for both purified CLIC1 and CLIC3, using different pH range of potassium phosphate buffers;
- Evaluate the thermal stability of both purified CLIC1 and CLIC3;
- Assess the residual activity of purified CLIC1 and CLIC3 following heat treatment;
- Define the optimum temperature for enzymatic activity of both purified CLIC1 and CLIC3.

4.2 Materials and methods

4.2.1 Chemicals and reagents

The following reagents were purchased from *Sigma Aldrich* Company: Reduced glutathione peptide (GSH), recombinant glutathione reductase (GR) enzyme from yeast, reduced nicotinamide adenine dinucleotide phosphate (NADPH), 2-hydroxyethyl disulphide (HEDS) substrate. Mono-potassium phosphate (KH_2PO_4), dipotassium phosphate ($K2HPO_4$) and Hydrochloric acid (HCl).

4.2.2 2xYT media for bacterial growth

Method for the preparation of the Standard microbial growth 2xYT media is as outlined in chapter 2 (Materials and Methods Sections 2.2-2.2.1).

4.2.3 Production, purification, and quantification of monomeric CLIC1 and CLIC3 (WT)

The recombinant monomeric non His-tagged CLIC1 and CLIC3 Wild-type proteins, which were previously expressed in E. coli BL21 (DE3) using the His-tag pET28a vector, were purified through size exclusion chromatography, as well as their purity and quantification were based on the same procedures as those outlined previously in chapter 2, materials and methods, sections 2.2.2 to 2.2.7.

4.2.4 Enzyme assays

The conditions employed in order to conduct the biochemical characteristic enzyme assays are based on the same conditions that were previously defined in chapter 2 (materials and methods section 2.2.9).

4.2.4.1 Biochemical properties of CLIC proteins (single factor tests)

4.2.4.1.1 Residual activity of purified CLIC1 and CLIC3 (WT) in HEDS enzyme assay

In order to characterise and define the optimal catalytic activity of the purified CLIC1 and CLIC3 (WT) proteins, different biochemical conditions were applied using the standard HEDS

enzyme assay procedure. By doing so, the optimal temperature for both purified recombinant CLIC1 and CLIC3 (WT) proteins were determined. This included, pre-heating the protein samples across a range of temperatures, specifically: 0°C, 30°C, 37°C, 42°C, 50°C and 60°C for 10 minutes. After that time, the residual activity of the CLIC proteins was assessed using the standard HEDS enzyme assay as previously described in chapter 2 (materials and methods section 2.2.-2.2.9.1).

4.2.4.1.2 Thermal stability of catalytic activity of purified CLICs

The thermal stability of purified recombinant CLIC1 and CLIC3 (WT) were measured by incubating the proteins at 37°C for 10, 20 or 30 minutes in 5mM potassium phosphate buffer (pH 7.0). Following this incubation time, the protein samples were then kept in an ice water bath prior to their use in the HEDS assay. Subsequently, the residual stability of CLIC1 protein was measured by carrying out the standard HEDS enzyme assay as formerly described in chapter 2 (materials and methods section 2.2.-2.2.9.1).

4.2.4.1.3 Assaying the thermal activity of CLIC1 and CLIC3 (WT)

In order to characterise the thermal activity of CLIC1 and CLIC3 (WT), the proteins were used in the HEDS assay performed at three different temperatures, 30°C, 37°C and 42°C in three individual experiments, following essentially the same procedure as previously described in chapter 2 (material and method section 2.2.-2.2.9.1).

4.2.4.1.4 Assaying the catalytic activity of CLIC1 and CLIC3 across a range of pHs

In order to find the optimal activity of purified CLIC1 and CLIC3 (WT) proteins, the proteins were assessed in different basic and acidic conditions using HEDS enzyme assay as previously defined in chapter 2 (materials and methods section 2.2.-2.2.9.1). In this matter, the optimal pH condition for both purified recombinant CLIC1 and CLIC3 (WT) was determined separately at 37°C by using (5mM) potassium phosphate buffer, which contains (1mM) EDTA and has a pH range of 5.0, 6.0, 6.5, 7 and 8.0 respectively.

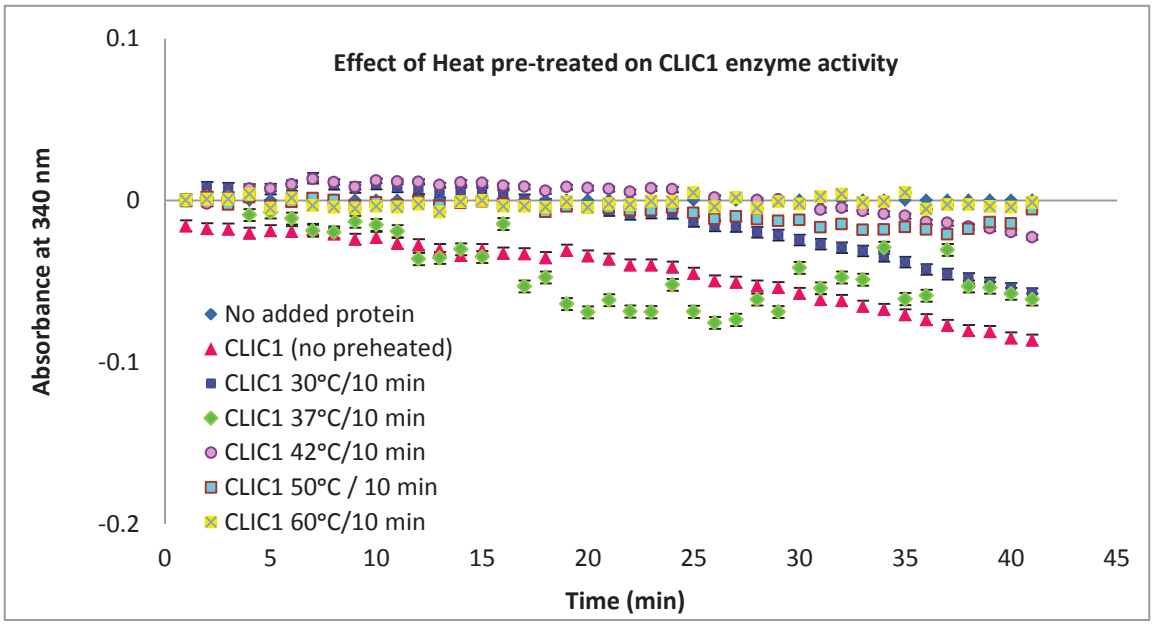
4.3 Results

4.3 CLIC3 demonstrates higher heat tolerance compared to CLIC1

We studied the effects of a range of temperatures on the oxidoreductase activity of both purified CLIC1 and CLIC3 in the standard HEDS enzyme assay. As seen in figure 4.2 (A) pre-heating CLIC1 at different temperatures ranging from 30°C to 60°C prior to its addition in the HEDS assay, caused its enzymatic activity to be dramatically reduced or completely abolished when the temperature was above 37°C. However, in the case of the CLIC3, as seen in figure 4.2 (B), there was little to no observable change in its catalytic activity as result of pre-heating the protein at different temperatures, ranging from 30°C to 60°C.

Based on these results CLIC1 does not appear to tolerate heat above 37°C. On the other hand, CLIC3 appears to tolerate heating up to 60°C with no discernible changes to its enzymatic activity. In this regards, as seen in figure 4.2 (C), comparing the catalytic activities of both CLIC1 and CLIC3 across different pre-heating temperatures, it is evident that CLIC3 shows more heat resistance in comparison to CLIC1.

A



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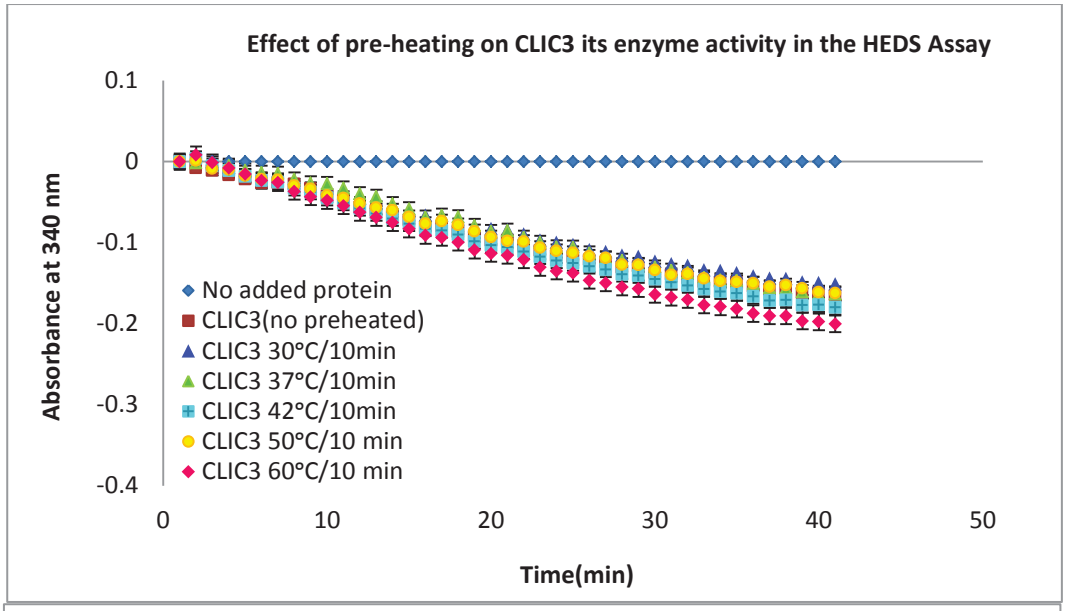

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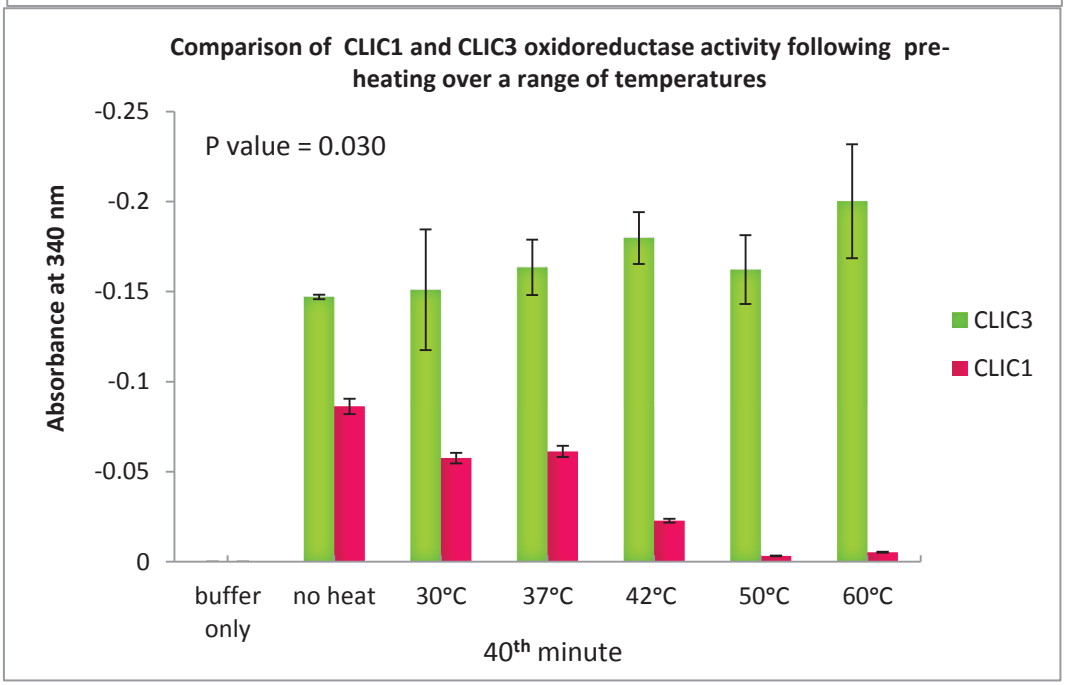
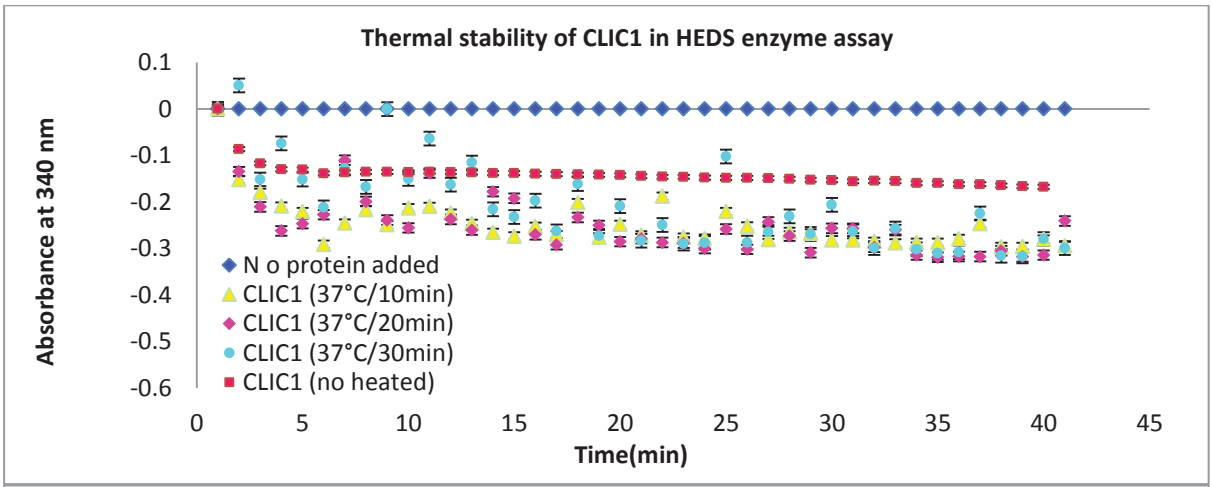


Figure 4.2: Summary graph of the effects of pre-heating on the catalytic activity of CLIC proteins. (A) is demonstrating the catalytic profile of pre-heating CLIC1 across a range of 0°C, 30°C, 37°C, 42°C, 50°C, and 60°C for 10 min. Whereas, (B) is indicating the profile of the catalytic activity of CLIC3 following incubation at temperatures (0°C, 30°C, 37°C, 42°C, 50°C and 60°C) for 10 min. (C) is a comparison of oxidoreductase activity of both CLIC1 and CLIC3 following pre-heating.

4.4 Stability testing of CLIC1 and CLIC3

In this experiment, the thermal stability of CLIC1 and CLIC3 was assessed over a variety of time points. As seen in figure 4.3 (A) pre-heating CLIC1 at a constant temperature for varying time the protein becomes more reactive compared to the non-heated sample, however, at increasing temperatures there is greater fluctuation in the assay profile. This may be due to the formation of intermediate states of the protein or aggregates or precipitation that arise due to the heating process. On the other hand, CLIC3 appears to be unaffected by heating and has a much higher thermal tolerance compared to CLIC1.

A



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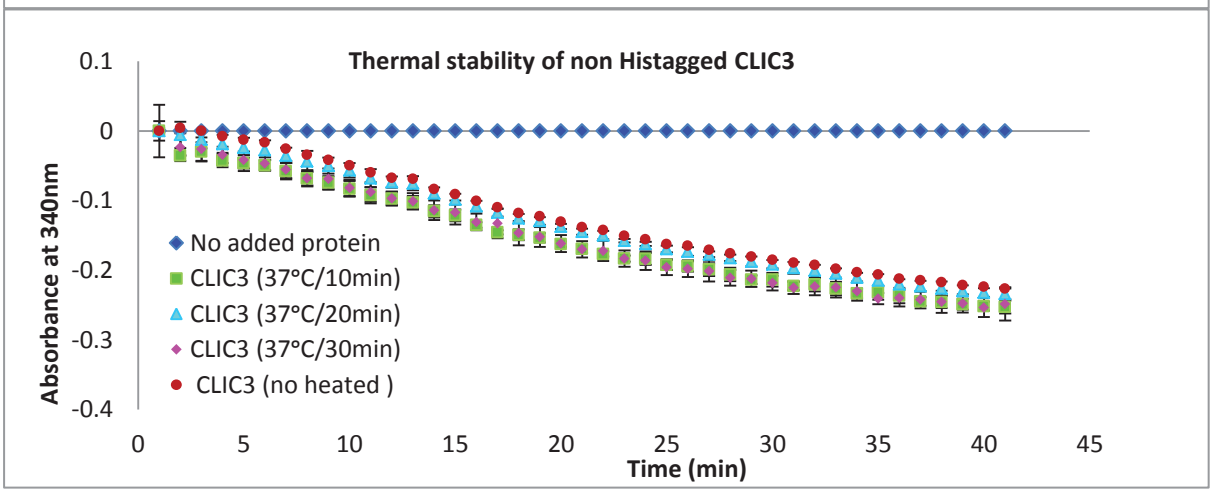
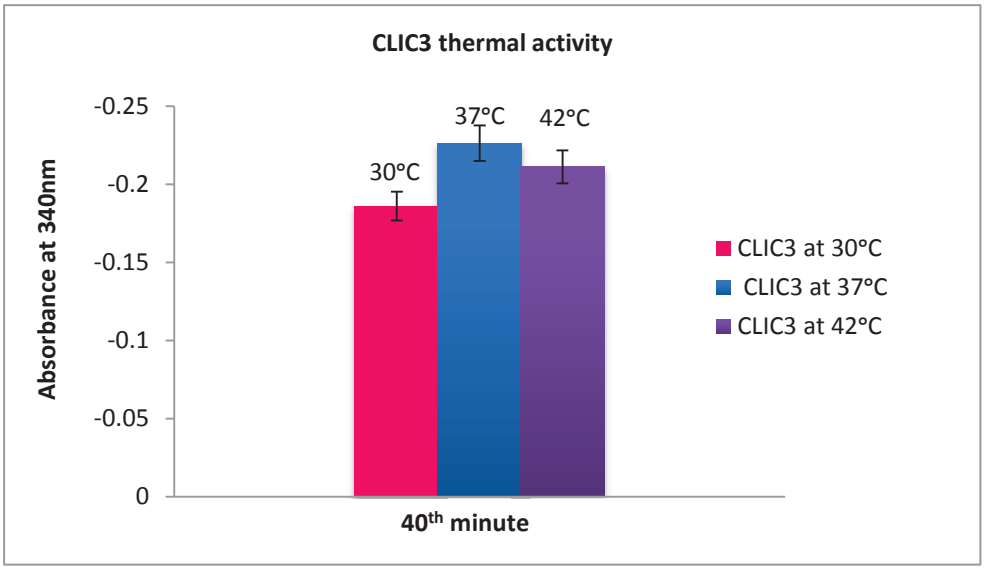


Figure 4.3: Thermal stability of CLIC1 (A) and CLIC3 (B). (The error bars are representative of standard deviation of triplicate samples run at the same time).

4.5 Optimal reaction temperature for CLIC1 and CLIC3 in the HEDS assay

In this experiment, the effect of two different thermal conditions on the catalytic activity of CLIC1 and CLIC3 (WT) proteins were characterised. This was performed by running the HEDS assay at 30°C, 37°C and 42°C degrees in three separate experiments. As seen in figure 4.4 (A and B) both CLIC1 and CLIC3 demonstrated increased catalytic activity at 42°C compared to 30°C. However, it seems 37°C may be the best optimum temperature for both proteins.

A



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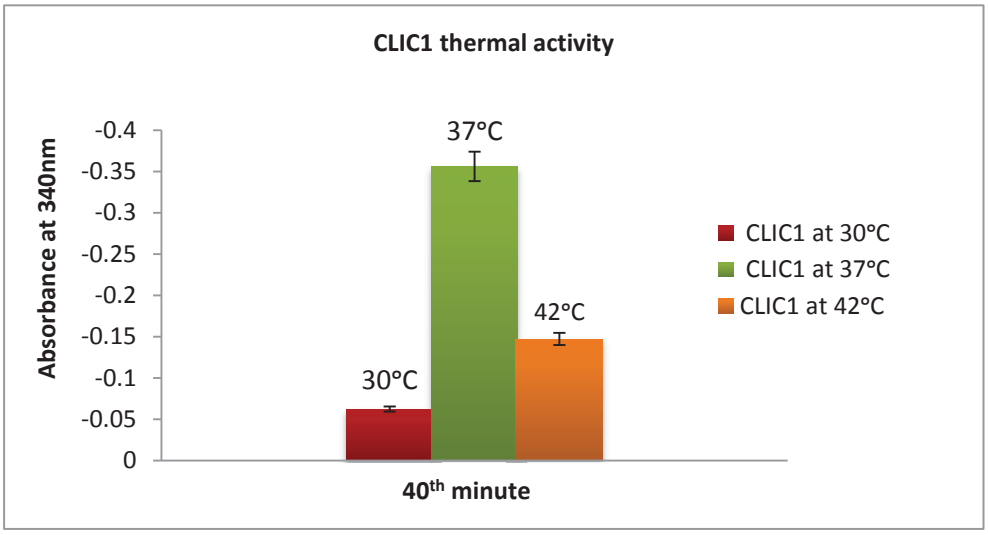


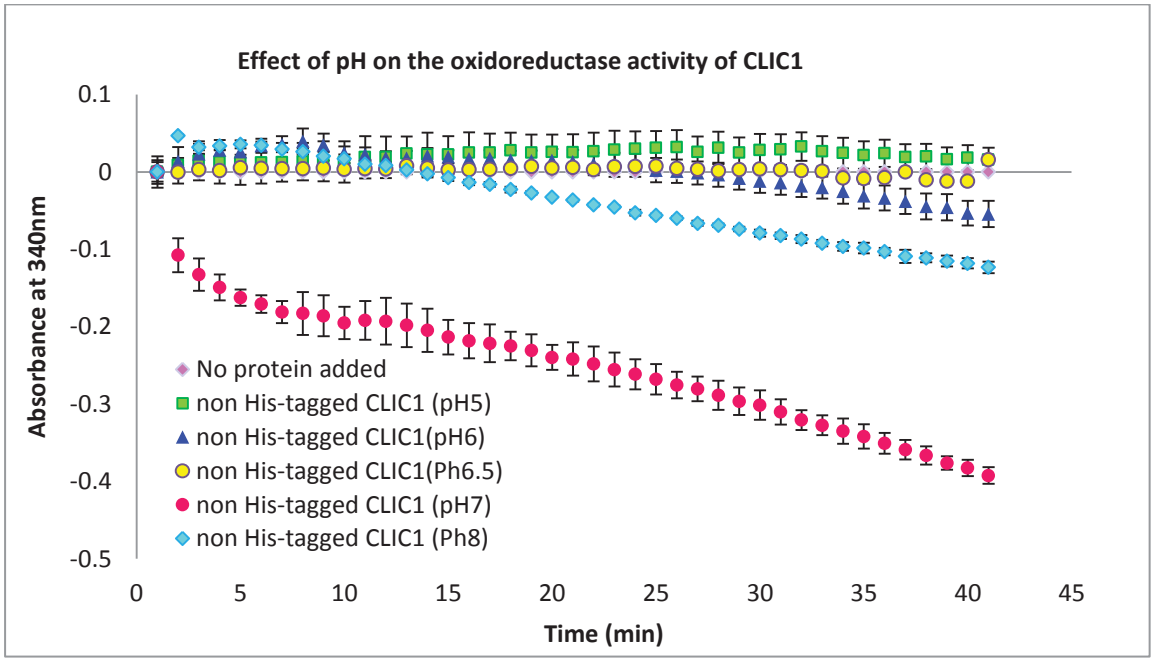
Figure 4.4: Graph of thermal activity of CLIC1 and CLIC3 proteins at 30°C, 37°C, and 42°C. The blue column in figure A and the green column in figure B are indicating the high oxidoreductase activity of CLIC1 and CLIC3 (WT) respectively at 37°C during 40 minutes of reaction. But, as the pink and red column are showing these two proteins are less active at 30°C (the error bar are representative of the standard deviation of triplicate samples run at the same time).

4.6 CLIC1 and CLIC3 demonstrate distinct enzyme activity at different pH

In order to examine the effects of pH on the oxidoreductase activity of both CLIC1 and CLIC3, the proteins were assayed at a constant temperature of 37^{o}C in the standard HEDS assay using a potassium phosphate buffer prepared across a range of pHs (pH 5, 6, 6.5, 7 and 8). From figure 4.5 (A), it is clear that CLIC1 is sensitive to both alkaline and acidic pHs, and prefers more neutral pH. CLIC1 catalytic activity is greatly reduced or completely abolished under more acidic conditions (pH 5 & 6) and at the more basic pH8 it is also less active, with the greatest activity noted at pH7.

Interestingly, CLIC3 seen in figure 4.5 (B) demonstrates optimal activity in more acidic conditions of pH5-6, while losing its oxidoreductase activity when the pH becomes more alkaline. From the velocity comparison column graph in figure 4.5 (C), the optimal oxidoreductase activity of CLIC1 is in potassium phosphate buffer at pH 7. In the case of CLIC3, the optimal oxidoreductase activity is pH 5-6.

A



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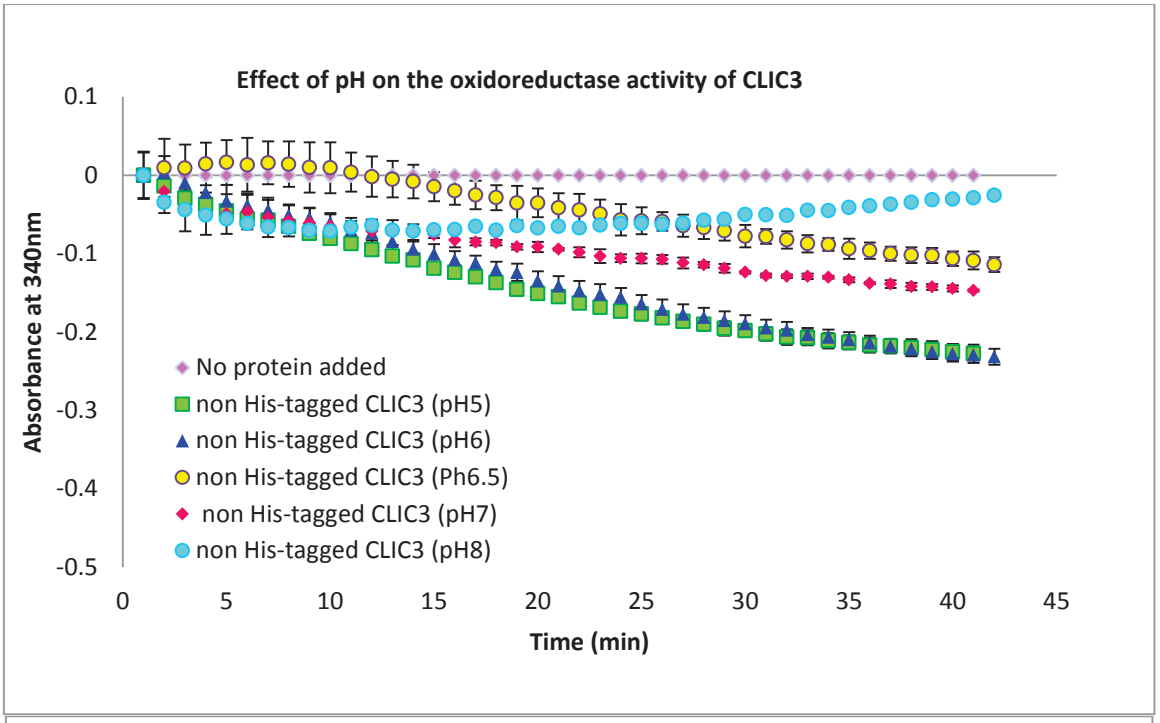

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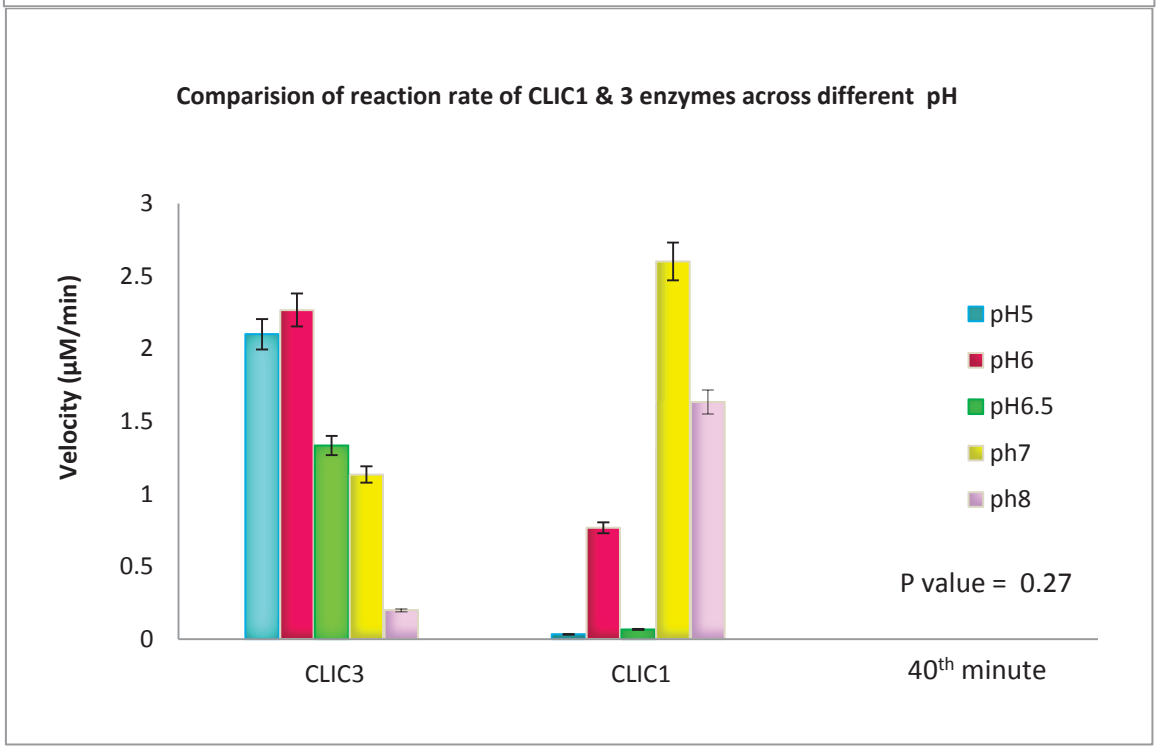


Figure 4.5: CLIC1 & 3 catalytic activities across different pH conditions. (A) pH profile analysis for CLIC1, (B) pH profile analysis for CLIC3, (C) column graph comparing reaction rate of CLIC1 with CLIC3 at different pH conditions.

4.4 Discussion and conclusions

4.4.1 CLIC3 demonstrates superior heat-stability in contrast to CLIC1

All living organisms have adapted a variety of responses to counteract sudden external and internal stresses including changes in temperature and pH, which impact on the function of biomolecules such as enzymes. Indeed, as was mentioned previously, these biomolecules have an optimal point at which they perform to their maximal activity, hence the importance of homeostasis, which is critical to ensure the correct temperature and pH are maintained.

Understanding enzymatic function involves defining the relationship between these factors and enzyme functional properties. For instance, different experimental observations indicate that the enzymes catalytic site is tuned to the effect of temperature on enzyme activity, and as a result, this would point out that any transformational changes, which occur at the active site are likely constrained through its dependency on temperature. This opinion in every respect is consistent with, and may perhaps offer part of the reasoning for views, which state that the catalytic active sites have a tendency to have more conformational flexibility due to their structural properties that are made of relatively weak molecular interactions in comparison with the whole enzyme itself [404-406].

In brief, a precise study of the effect of temperature on enzyme activity has given rise to some unpredicted novel insights such as the recognition of a new specific mechanism by which the loss or alteration in enzyme activity, due to influence of temperature alteration could be obtained, which is distinct from denaturation factor, and also the catalytic active site that is considered as a vital point of action for these special effects could be determined as well. So, based on the proposed Equilibrium Model (EM)[407]; which was elaborated upon in the introduction of this chapter, the association between the enzyme's thermal properties and the effect of temperature on physiology and evolution of the host living organism can be quantitatively studied.

In this regards, in various organisms, it has been shown that enzymes have distinct responses to environmental stresses and represent themselves as heat resistant or heat sensitive enzymes under specific conditions. For instance, the rPsGrx protein that was found to be considered as a "cold" active enzyme demonstrates a low thermo-stability [17].

Whereas, the first discovered glutaredoxin enzyme from *Escherichia coli* bacteria was identified as a small heat-stable oxidoreductase[53]. On the other hand, enzyme members from within the same family as the thioredoxins, can differ in several features like their heat stability [408]. In this regards, based on the obtained results from the current work (Figure 4.3) both purified CLIC1 and CLIC3 proteins demonstrate a different level of heat stability based upon their characterisation in the HEDS enzyme assay. As the results indicate the protein CLIC3 (Figure 4.3 (B)) in comparison to CLIC1 (Figure 4.3 (A)) is thermally a more stable enzyme, following pre-heating at 37°C for 10, 20 or 30 minutes prior to use in the HEDS assay.

Moreover, as the biochemical analyses of the recombinant Glutaredoxin (CsT-89Grx) protein in the HEDS assay revealed, the optimal temperature for catalytic activity of this enzyme was 50°C, and it retained high thermal stability at 80°C for 30 min [409]. Besides, the study on Chlorella virus Glutaredoxin (Grx) has been revealed that the optimum temperature for this protein is obtained at 37^{o}C degree [223]. Accordingly, our studies indicate an optimum thermal activity of the CLIC1 and the CLIC3 as 37°C (Figure 4.4 A and B). Hence, the experimental result in figure 4.4 indicates that the thermal activity of both purified CLIC1 and CLIC3 increases as the assay temperature rises from 30°C to 37°C within 40-minutes. However, this increment was observed to a lower extent for CLIC1 compared to CLIC3. Moreover, the thermal activity of both enzymes starts to decrease as the temperature rises at 42°C and beyond.

Several studies have previously shown that the Grxs mostly considered as heat-stable proteins. For instance, in the case of dithiol glutaredoxin from *Trypanosoma cruzi*, approximately 50% of its activity was retained at 100^{o}C for 8.5 minutes [410]. Likewise, it has been demonstrated that the Thioltransferase Grx (from *Brassica campestris*) remained catalytically active after applying heat treatment of 95^{o}C for 30 minutes [411]. Whereas, it was shown that the activity of *Cryptococcus neoformans* Grx at 60^{o}C or higher temperatures was to some extent deactivated [412]. In addition, a biochemical study by Ken et al (2009) on the residual activity of TcGrx revealed that at approximately 50% of the catalytic activity of this enzyme was retained following heating at 100 °C for 8.5 minutes [413]. Likewise,

similar work was revealed that the 60% activity of the Grx protein from *Ipomoea batatas* was maintained after heating it at 80°C for 16 minutes [414].

Similarly, our results demonstrate that by increasing the pre-incubation temperature, the residual catalytic activity of the CLIC3 protein enhanced and it remained highly active after pre-heating the protein across a broad temperature range from 30°C, 37°C, 42°C, 50°C to 60°C degree for 10 minutes. Whereas, unlike to CLIC3 the residual catalytic activity of the CLIC1 protein was gradually decreased when the temperature of the pre-heating process was increased, as seen in Figure 4.2C.

Thus, the obtained result from CLIC3 is in correlation with high thermal activity of CsT-89Grx enzyme, which was proposed as the most heat-stable Grx protein [409]. As a consequence, the evidence from this work suggests that the CLIC3 could be also considered as a heat-stable protein. Beside more recently, CLIC3 has been demonstrated to function as glutathione-dependent oxidoreductase in the extracellular space in order to activate extracellular transglutaminase-2 (TGM2), and as a result of that drives cancer progression[6]. So, based on this behaviour this could be speculated that CLIC3 heat stability is a factor through which it can respond to such sudden environmental changes.

However, the evidence for CLIC1 suggests that this protein is a heat sensitive protein in comparison to CLIC3, although it did remain catalytically active at 42°C for 40 minutes of reaction time. As a consequence, this activity of CLIC1 could be in correlation with its antioxidant oxidoreductase behaviour [415] by which it can respond to oxidative stress that initiates via an increase of oxygen consumption level due to an increment of environmental temperature [416].

4.4.2 CLIC1 is maximally active at physiological pH in contrast to the optimum activity of its CLIC3 counterparts at acidic phase

Virtually all biological processes are pH dependent; therefore, pH significantly affects the subcellular translocation, functional pH-dependence and structural conformations associated with the function of proteins [362]. In addition, intracellular pH is an essential factor in the catalytic activity of several enzymes, for example, in the productivity of contractile elements as well as the conductivity of cellular ion channels. Furthermore, it

seems that pH alteration is a vital regulatory factor in cell cycle and cellular proliferative capacity [417]. In connection with the enzymatic activity, the pH sensitivity of phosphofructokinase is well known as one of the most crucial enzymes can be exemplified as an elevation in its activity due to increment in the physiological pH [418]. Moreover, synthesis of DNA, RNA, and proteins also influenced by internal pH oscillations. For instance, it has been reported that the activity of DNA polymerase which, generally has a high pH optimum, would rise due to an increase in pH from 7 to 8 [417].

In this regard, several studies have investigated the effect of pH as a biochemical factor on the catalytic activity of the oxidoreductase class of enzymes. Indeed, there are numerous examples in the literature that state the glutaredoxin enzymes have alkaline pH optima. For instance, the Glutaredoxin (CsT-89Grx) enzyme was shown to be optimally active at alkaline phase (pH 8.5) in the HEDS assay [409]. Similarly, a study of human Grx2 was determined that the maximal catalytic property of this protein was obtained at pH of 8.0 [419] in parallel to its Grx1 counterparts [420]. Whereases, in the case of the wild-type mammalian Thioredoxin Reductase (TrxR) it has been revealed that this enzyme has a pH optimum of 7 [421]. Thioredoxin from *Escherichia coli* that catalyses the reduction of insulin disulfides, demonstrates its maximal activity at pH 7.5, which is near physiological pH phase [422].

The results of the present study indicate that the maximum relative enzyme activity of CLIC1 is pH 7, as determined in the HEDS enzyme assay shown in figure 4.5A. Also, this result demonstrates that under acidic conditions (pH 5-6.5), the oxidoreductase activity of the CLIC1 enzyme is totally abolished within 40 minutes. Similarly, at pH 6 it only showed minor activity after 30 minutes. However, at the alkali pH8, CLIC1 remained active albeit, to a lesser extent than its optimum.

Through the course of this investigation of the biochemical characterization of the catalytic activity of the CLIC3, the optimum pH for this enzyme was determined to be pH 5-6 in 5mM potassium phosphate buffer (figure 4.5B). In addition, the obtained data also demonstrates that the CLIC3 is also highly active in acidic phases like pH 5 and pH 6.5. Likewise, this protein also demonstrates activity at neutral pH7. But, the CLIC3 enzyme starts to lose its oxidoreductase activity when the pH of the solution was increased to an alkaline phase like pH 8 as seen in figure 4.5B. This is clearly illustrated by the comparison column graph in

figure 4.5C, which demonstrates the reaction velocity of CLIC3 is higher in acidic pH, while, it appears to be inhibited when in a basic pH8 phase. Where, in contrast to CLIC3, the CLIC1 enzyme has a lower reaction rate in acidic pH while it has a maximum velocity at pH 7 and pH 8 respectively (Figure 4.5/C).

Indeed, previous studies have shown that the members of Chloride intracellular channel proteins (CLICs) that reside in endosomal membranes are considered as a functional group of acidifiers. For instance, CLIC3 as one of the protein members that is proposed as an acidifier in the late endosomal pathway plays a vital role in preventing the tumour metastasis in breast cancer by its ability to controls recycling of late endosomal MT1-MMP [423]. Likewise, CLIC3 can drive cancer progression thtough its collaboration and co-localization with LAMP1 in late endosomes and lysosomes (Figure 4.6)[424]. A similar study has shown that CLIC3 has the ability to recycle the endosomes which to some extent have acidic luminal pH, however, the pivotal role of acidic pH phase of recycling endosomes in tumor metastasis remains obscure [425, 426]. The recent work on CLIC3 was revealed that this protein can drive cancer progression through its glutathione-dependent oxidoreductase activity [6]. In this regards, as our results demonstrate that the CLIC3 enzyme has a preference to function in an acidic environment (figure 4.5 B/C) one could speculate that this behaviour is in correlation with its functional role in above-mentioned cancer progression.

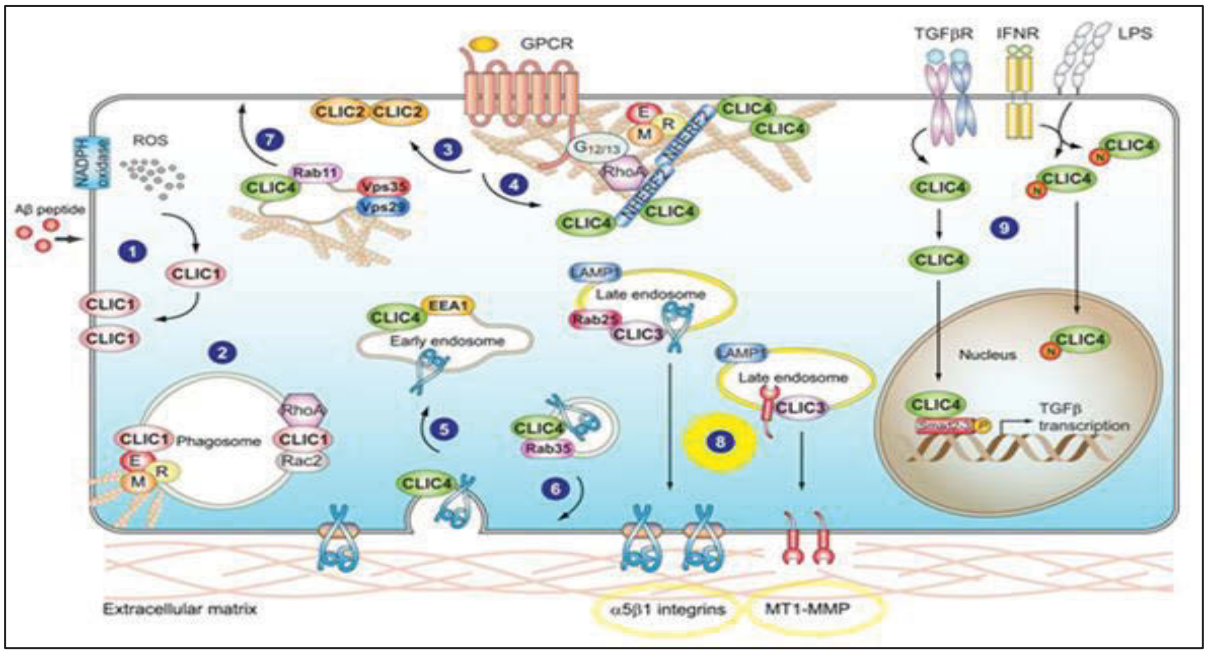


Figure 4.6: Representetive of Intracellular activities of 4 members of CLIC proteins[427]. number (8) in yellow Indicates the colocalization of CLIC3 protein in late endosomes and its association in recycling of α5β1 integrins (highlighted in yellow) in a Rab25-dependent fashion [424], as well as its regulatory role in recycling of matrix metallopeptidase 14 (MT1-MMP(highlighted in yellow)) in a Rab25-independent manner[423].

Above all, in the case of CLIC1 as it was previously mentioned (table 2 of chapter 1) the cellular localization of this protein is dependent on different factors such as the cell type or the different phases of the cell cycle. So, as this protein localizes to the nuclear envelope, endoplasmic reticulum and cytoplasmic environment, which share the same physiological pH of 7.2 (Figure 4.1) [101, 140, 390], it can be speculated that the CLIC1 optimal oxidoreductase activity that was obtained at pH 7 (Figure 4.5A) is in correlation with its subcellular localization.

Besides, it was recently shown that CLIC1 possesses intracellular antioxidant oxidoreductase activity [415], and this protein was described as a protein that belongs to the antioxidant balance elements within the peroxiredoxin category [428]. Also, as the full complement of antioxidant systems occurs in peroxisomes organelles that have physiological pH of 7 (Figure 4.1) due to the presence of GSH and ascorbate [169], this could be another speculation that the maximal catalytic activity of CLIC1 that was obtained at pH 7 could describe the functional role of this enzyme within this organelle.

In addition, as the figure 4.5 (C) indicates, the CLIC1 remain highly active at pH 8 in HEDS enzyme assay, which is in correlation with the optimum activity of Glutaredoxin enzymes and GSTs with glutaredoxin- like activity in alkaline pH of around 8 value [429, 430].

In summary, the effect of pH on the oxidoreductase catalytic activity of both CLIC1 and CLIC3 proteins was investigated via standard HEDS enzyme assay. The results of this work indicate that the optimal activity for CLIC1 and CLIC3 (in 5mM potassium phosphate buffer containing 1mM EDTA at 37°C) is pH7 and pH 6, respectively. Thus, based on the obtained results the evidence suggests that these optimum points may well reflect the respective location and the function of each of these proteins within the cell environment.

Chapter 5

Enzyme Kinetic Study of the CLICs

5.1 Introduction

5.1.1 Enzyme kinetics definition and its history

Enzymes are soluble protein molecules, which are known as biological catalysts. Catalysts are substances that have the ability to enhance the rates of chemical reaction by lowering the activation energy. The enzyme molecules are not consumed during the catalytic reaction, therefore are recovered at the end of the reaction. Through kinetic studies, the rate of the enzymatic reaction can be measured and the influence of different conditions of the reaction can be determined. Moreover, knowing the enzyme's kinetic profile would also be useful for understanding the enzyme catalytic mechanism, which can in turn assist in determining its role within cellular metabolism and how this action could be regulated or inhibited. In view of that, as the needs for this precise understanding was raised, a quantitative theory about enzyme kinetics was proposed by Victor Henri in 1902 [431]. But, despite all the remarkable experimental observations that were undertaken by Henri's investigations, many questions still remained unanswered [432].

Therefore, in 1913 Leonor Michaelis and Maud Menten proposed a mathematical description that was in line with Henri's work. However, the challenge they faced for their model was that they needed to indicate the full-time course of the reaction progress and of product formation based on their postulation, which refers to this view that the rate of the catalysed reaction and the concentration of the enzyme–substrate complex was in proportion to each other. Later on, they did overcome this challenge despite not knowing the enzyme's concentration or even its chemical nature. Thus, the consequence of their work pointed out that "the final aim of the kinetic research, namely to obtain knowledge of the nature of the reaction from a study of its progress"[433].

In that regards, the study of enzyme kinetics becomes an essential factor in the characterization of enzyme activities, since the accomplishments of Michaelis and Menten [434]. According to the reaction below that is explained comprehensively in chapter 1:

(1)

The enzyme (E) attracts and combines reversibly with the substrate (S), resulting in the formation of an enzyme substrate or (ES) complex. The ES complex is activated via catalytic activity (ES→ (ES–EP)) and subsequently proceeds in the formation of product (P) and free enzyme by the rate constant k_{cat} [12]. So, the enzymatic kinetics can be expressed based on the Michaelis–Menten equation:

$$(V_0 = V_{max} (S/ K_M +S)) \quad (2)$$

In this model, two constants that are known as the Vmax and Km value are respectively representing the maximal catalytic velocity and the concentration of substrate at the half of this maximum velocity. Indeed, the maximal point or V_{max} is the point at which the enzyme catalytic active site is saturated with substrate molecules. Hence, the V_{max} value represents how fast the enzyme would be able to catalyse the chemical reaction. On the other hand, the K_m value could be defined as an inverse measurement of the enzyme's affinity or power of binding with its substrate[12].

In fact, the K_m describes the substrate concentration at the point when half of the active sites are occupied. As a result, this can be interpreted that the lower the K_m the larger the affinity. So, a low value of K_m means that small quantity of substrate is needed in order for the active site be saturated which, indicates a high affinity of enzyme binding to its substrate. Therefore, the higher the K_m, means the lower the affinity[12].

In addition, for most of the enzymes, the reaction rate or V_0, which indicates the number of moles of product that is made per second, differs as the substrate (S) concentration changes. So, as the concentration of substrate increased the reaction rate would also be raised in a linear manner until it has reached the maximum point of its activity and then starts to level off as indicated in figure 5.1. Therefore, the study of these values is crucial in understanding both enzyme activities on the macro scale level and the influence of different types of inhibitors on an enzyme's function [7, 432].

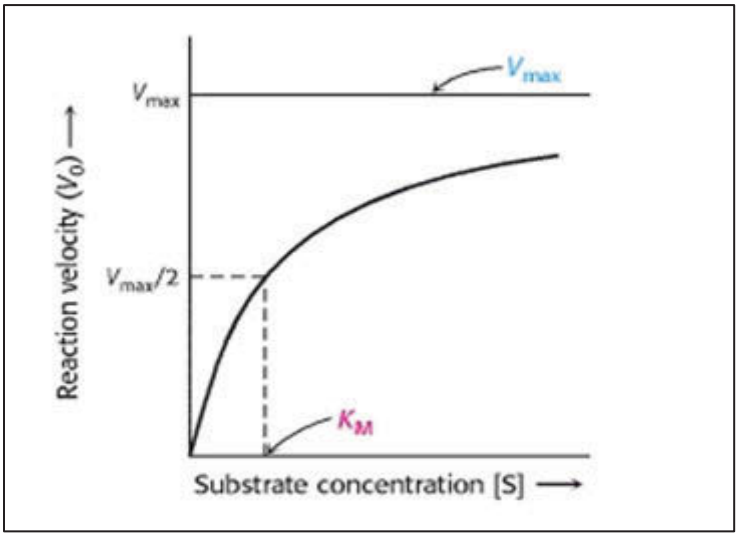


Figure5.1: Graph is representative of Michaelis-Menten kinetics. The reaction rate (V_0) of an enzyme that obeys this model is proportional with the substrate concentration, and maximum velocity (V_{max}) is reached when all substrates saturate the enzymes active sites. The K_m value indicates the substrate concentration at the point of $V_{max}/2$ [7].

Since that discovery, several enzymes have been kinetically characterised based on the proposed Michaelis-Menten Model. For instance, an *in vitro* study has been shown that the thioredoxin enzymes have a functional catalytic behaviour by which they are able to saturate their target substrate. Accordingly, it was proposed that the kinetic factors of Michaelis-Menten model would be considered as fundamental descriptors for analysing the thioredoxin activity. As a result, these factors have been used in order to define the specific roles that are performed by individual redoxins within the cellular process [435, 436].

In addition, the *in vitro* and *in vivo* descriptions were reconciled in order to indicate that the purported catalytic behaviour of thioredoxins originated from the saturation of its redox cycle, and also the ratio aspect of reduced thioredoxin to its oxidized form, which would reveal the steady-state rate of its catalysed redox reaction [437]. Moreover, based on the *in situ* Kinetic characterization study, it has been predicted that the variation in local GSH concentration would affect the deglutathionylation activity of human Grx enzyme. Besides, the kinetic analysis would also contribute to understanding the effect of environmental elements on Glutaredoxin activities in a living organism [64, 438].

Consequently, this can be expressed that Michaelis and Menten's mathematical description would be considered as a powerful tool in analysing the catalytic behaviour of enzyme

activity. So, by using these analytical kinetics we can indirectly obtain information about the saturation of enzymes active site, calculate the maximal rate of a given catalyser which subsequently can lead to the observation of alternative pathways, and as a final point we can measure and compare the substrates affinity of different enzymes [439, 440].

5.1.2 Reaction order and its relationship with enzyme concentration

In studying a chemical reaction, knowing the chemical properties of the reactants is not the only important aspect to consider, but also considering other factors such as the conditions or mechanism under which the chemical reaction takes place, and the reaction rate at which it occurs are an important aspect to review. According to that, changing the concentration of substances that participate in chemical reactions usually led to changes that influence the rate of the reaction. This effect could be explained by analysing the order of reaction, which is part of the rate equation. In this regards, in chemical kinetics, the reaction order in respect of a given substance could be defined as the index, or exponent, in which its concentration is increased in the rate equation.

In view of that, the chemical reactions are classified based on their reaction order, which is known as zero-order, first-order, second-order, mixed-order and higher-order reaction. In the first place, these different classifications of reaction orders are defined based on the initial rate of reaction effect. For example, in zero-order type of reaction, no effect occurs on the initial rate of reaction by changing the concentration of the substance. But, in the first-order reaction type the rate of reaction by a linear manner is directly proportionate with only one concentration of given substances like the reactant, catalyst or product. Indeed, as the concentration of the substance doubled the reaction rate would also be doubled. Whereas, in second-order reaction type the reaction rate is proportionate with two concentrations of substance. So, as substance concentration is doubled the initial rate of the reaction quadruples. On the other hand, it has been noted that the reactions of order that are proportionate with more than 2 concentration of substance are very rare.

In addition, the reaction order can be determined by defining the rate or velocity at different concentrations of the given substance. So, the kinetic description of enzymes

catalysed reactions would also be characterised based on this determination method [432]. In view of that, several studies have been investigated on the first-order reaction. For instance, DNA transcription and RNA translation process have been found to operate as first-order reactions [441]. It has been shown that the RNA and proteins, which have different conformational states, are following the first-order type of reactions when they are transformed among their various folding states [442, 443]. In this regards, this chapter will state the investigations undertaken to define the kinetic description of Oxidoreductase catalytic activity of non His-tagged CLIC proteins based on the Michaelis-Menten kinetics module.

As such, this chapter will outline:

- The study of the effect of different concentrations of the HEDS substrate and the CLIC proteins in the HEDS assay;

- Determining the kinetic properties (V_{max} and K_m) for the proteins CLIC1 and CLIC3;
- Determining the type of reaction orders for both the CLIC1 and the CLIC3.

5.2 Materials and methods

5.2.1 Growth of bacterial cultures

The general procedure for the preparation of the 2xYT medium microbial growth is based on the protocols that outlined in chapter 2 (Materials and Methods Sections 2.2-2.2.1).

5.2.2 Production and purification process of recombinant CLIC1 and CLIC3 (WT)

The recombinant CLIC1 and CLIC3 (WT) proteins that were previously expressed in E. coli BL21 (DE3) using the His-tag pET28a vector, were purified using size exclusion chromatography method as it is described in chapter 2 materials and methods sections 2.2.5 and 2.2.6 protocols.

5.2.3 Determination and quantification of monomeric CLIC1 and CLIC3 (WT)

After the protein purification process performed, the obtained monomeric CLIC1 and CLIC3 (WT) proteins were subsequently quantified based on the method that described in Chapter 2 (materials and methods section 2.2-2.2.7). Besides, the protein purity and molecular mass of these proteins were indicated following the protocols and method that previously defined in chapter 2 (material and method sections 2.2-2.2.8) using precast SDS-PAGE gel (Bio-Rad).

5.2.4 Enzyme assays conditions

The required conditions for the kinetics characterisation enzyme assays were followed as previously defined in chapter 2 (materials and methods sections 2.2-2.2.9).

5.2.4.1 Kinetics characterization of CLICs using a different concentration of substrate and enzymes in HEDS assay

In this assay, the catalytic activity of both the CLIC1 and the CLIC3 enzyme was assessed in order to define and characterize their kinetic behaviour. In this regards, the test protocol

and condition was followed as previously described in chapter 2 material and method section 2.2.9.1. But, the only difference was using either different concentrations of HEDS substrate (0, 0.25, 0.5, 1, 2, 4 and 6mM final concentration), or different concentrations of CLIC1 and CLIC3 (0, 5, 10, 15, 20 and 25mM final concentration), along with a fixed concentration of GSH peptide (1mM), NADPH (250uM) and fixed concentration of GR (0.5ug/ml).

5.3 Results and discussion

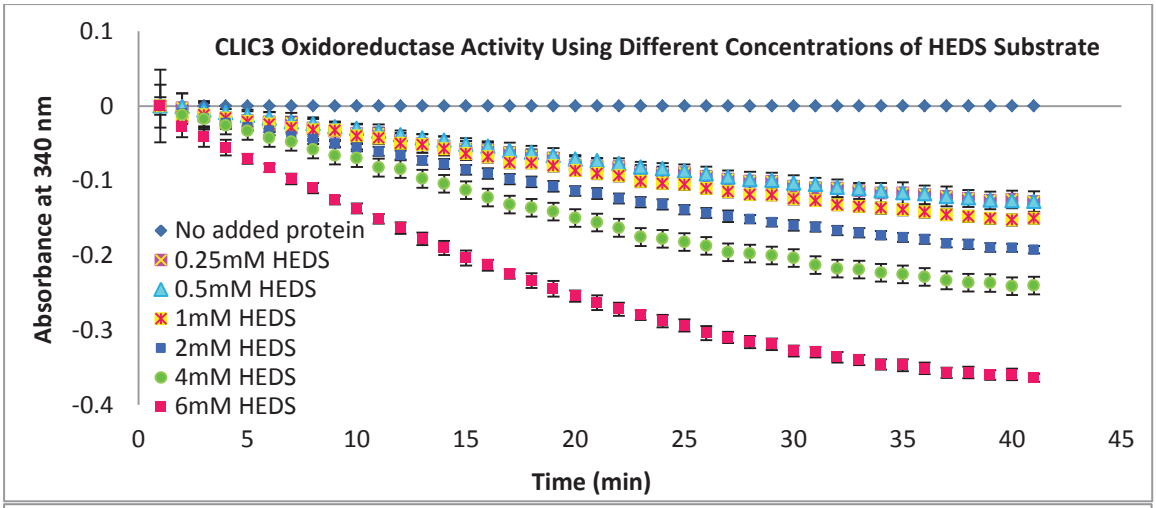
5.3.1 CLIC1 and CLIC3 obey Michaelis-Menten kinetics

Several studies have been carried out to study the kinetic behaviour of several redox proteins, characterized using different substrates. For instance, the study of insulin reduction by thioredoxin has been reported to have a K_m value of (11µM) and a V_{max} of (4 µM·min^{-1})[435]. Similarly, the reduction of HED substrate by yeast dithiol Grx1 and Grx2 has been characterized based on a Michaelis–Menten kinetic study, which indicates that these enzymes are able to reduce the mixed disulfide formed between GSH (1mM) and the HED substrate, with an apparent k_m of 0.12mM and 0.7mM, respectively [444].

In this regards the kinetic properties for both recombinant CLIC1 (WT) and CLIC3 (WT) were determined at different concentrations of HEDS substrate (0, 0.25, 0.5, 1, 2, 4 and 6mM final concentration) Figure5.2(A-C). Based on our characterization study, kinetic constants (V_{max}, K_m) for both CLIC1 and CLIC3 were determined. By comparing these catalytic efficiencies, it becomes evident that both CLIC1 and CLIC3, obey Michaelis-Menten kinetics module with V_{max} value of (2.026, 3.33 µM/min) and K_m value of (2.503, 0.9941 mM) respectively. Therefore, based on these obtained results, this can be interpreted that, as CLIC1 demonstrate a higher K_m value (2.503mM) in comparison to its CLIC3 counterpart (0.9941 mM). This means that CLIC1 has a lower affinity for the HEDS substrate, and thus a higher quantity of substrate is required in order to make its active site to be saturated. Whereas, in the case of CLIC3, this protein indicates lower K_m value, which means that this enzyme has a higher affinity for this substrate and as a result, less substrate is required for active site saturation.

Moreover, CLIC3 was demonstrated to have a higher V_{max} (3.33 µM/min) in comparison with CLIC1 (2.026 µM/min). This pointed out that the CLIC3 enzyme catalytic active site is saturated with substrate molecules more quickly and as a result, the enzyme would be able to catalyse the chemical reaction faster.

A



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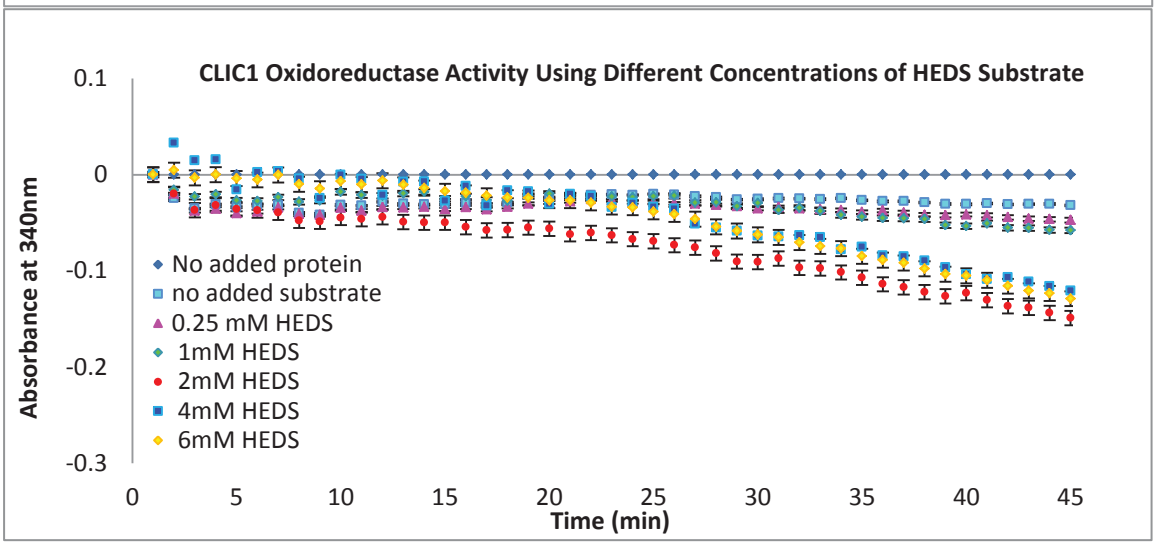

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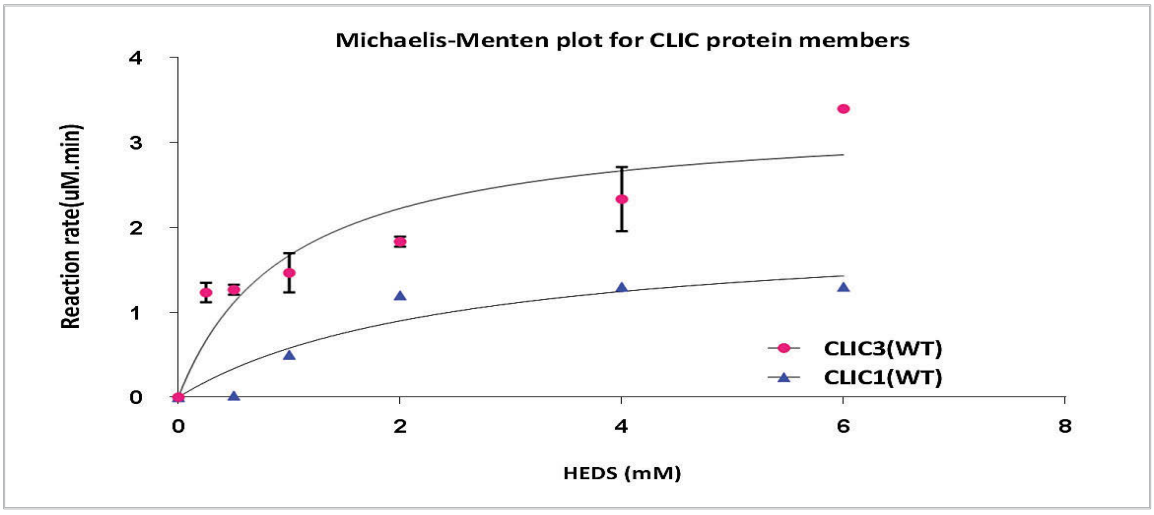


Figure5.2: Representative graphs of CLIC1 and CLIC3 reduction of HEDS substrate and their different kinetic behaviours. In both (A) and (B) a final concentration of 10uM of each CLIC protein was added to the mixture of potassium phosphate buffer that contains 1 mM EDTA in pH 7, 0.5ug/ml of GR, HEDS (0, 0.25, 0.5, 1, 2, 4 or 6 mM) and 250 uM of NADPH. After 5 minutes incubation at 37˚C, the reaction was initiated by 1 mM GSH addition. Subsequently, the absorbance of NADPH was monitored at A340 nm. The error bars are representing of the standard deviation of three samples replicate. (C) CLIC1 and CLIC3 obey Michaelis-Menten kinetics.

5.3.2 Other kinetic behaviours of the CLIC protein redox system and their reaction order

In biological systems, a systematic construction of kinetic models of catalytic reactions is becoming progressively an essential aspect through which the regulatory process of complex systems like cellular pathways can be quantified. In view of that, aside from substrate saturation effects that were described in the previous section, additional characteristic factors such as the effect of different concentration of enzyme on redox kinetics cycle have been studied extensively.

In that regards for instance, from an *in vitro* study on the thioredoxin system, it has been revealed that the kinetics of the Michaelis-Menten parameters would be altered when the concentrations and activities of the components within this redox system are changed [435]. Where, in the *in vivo* thioredoxin system these changes on the component concentration are in correlation with diverse physiological modification [435, 445, 446]. Moreover, it has been stated that in Glutaredoxin redox system by an increment in the enzyme concentration the reaction rate would also be elevated in a linear manner[235]. Likewise, the study on redoxin systems (Thioredoxins, glutaredoxins, and peroxiredoxins) has been revealed that despite of using relatively low concentration of enzyme in the experiment, as the enzyme concentration was increased the rate of catalytic reaction was also linearly raised [437].

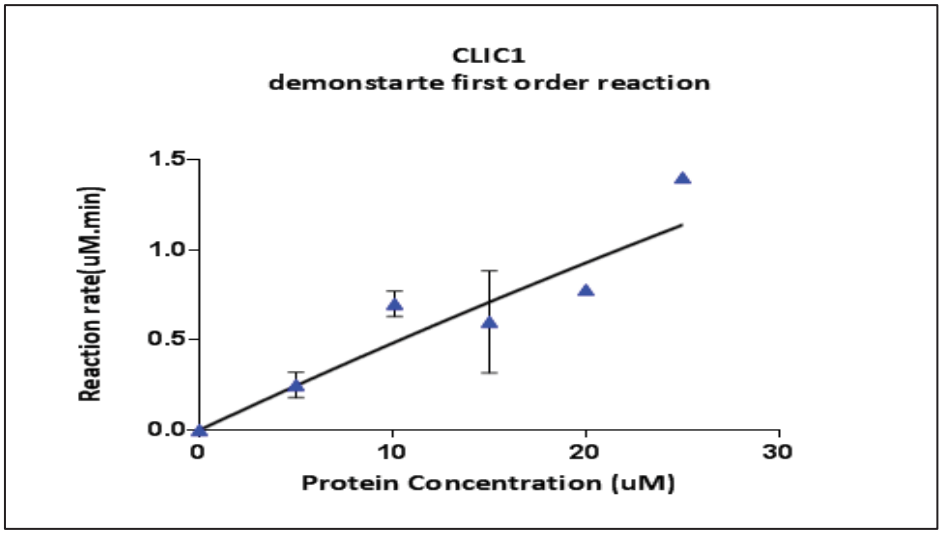
In fact, the Kinetics behaviour of reaction order type for different regulatory oxidoreductase enzymes has been studied based on the variation of enzyme concentration in catalytic reaction as well as the effect of other variant factors. For instance, the study on the kinetics reaction order of thioredoxin (Trx) was revealed that this enzyme follows an irreversible second-order reaction [447]. Besides, the reduction of DHA with oxidoreductase activity of protein disulfide isomerase (PDI), was described to be a second-order type of reactions [448]. A similar study was mentioned that the oxidation of reduced Trx1 and Grx1 by DHA have been shown to fallow rates of a similar reaction order type to PDI enzyme [448].

On the other hand, further studies on the oxidoreductase thioltransferase enzyme have been shown to have different reaction order, which is based on the different variant condition. For example, the thioltransferase Grx that catalyse the glutathione/glutathione

disulfide interchange reaction demonstrates pseudo first order kinetic reaction [449]. Similarly, it was shown that the Glutathionylation Activity of Glutaredoxin enzyme fallows pseudo first-order kinetic reaction [235].

Accordingly, in this work, we aimed to find the reaction order of CLIC enzymes by using a different concentration of the CLIC1 and CLIC3 (0, 5, 10, 15, 20 and 25mM final concentration) in HEDS enzyme assay. Based on the obtained results as the figure 5.3 (A) and (B) are showing, when the concentration of both the CLIC1 and CLIC3 enzyme was increased in the assay the rate of reaction was also increased in a linear manner. Therefore, as the first order chemical reaction is defined based on the direct proportional relationship between the reaction rate and the concentration of the reacting substance; here the CLIC1 and CLIC3 enzymes are considered as reacting substances, the reaction order for these enzymes is determined to be first order kinetics based on the shape of the obtained graphs figure 5.3 (A) and (B). The comparison column in Figure 5.3 (C) is showing CLIC1 demonstrates higher reaction rate in comparison to its CLIC3 counterparts, when the concentration of enzymes was increased. In this regards, the kinetics of this reaction were analysed and the V_{max} value of (1.4mM/min) and (0.7mM/min) was obtained from the figure (C) for CLIC1 and CLIC3, respectively.

A



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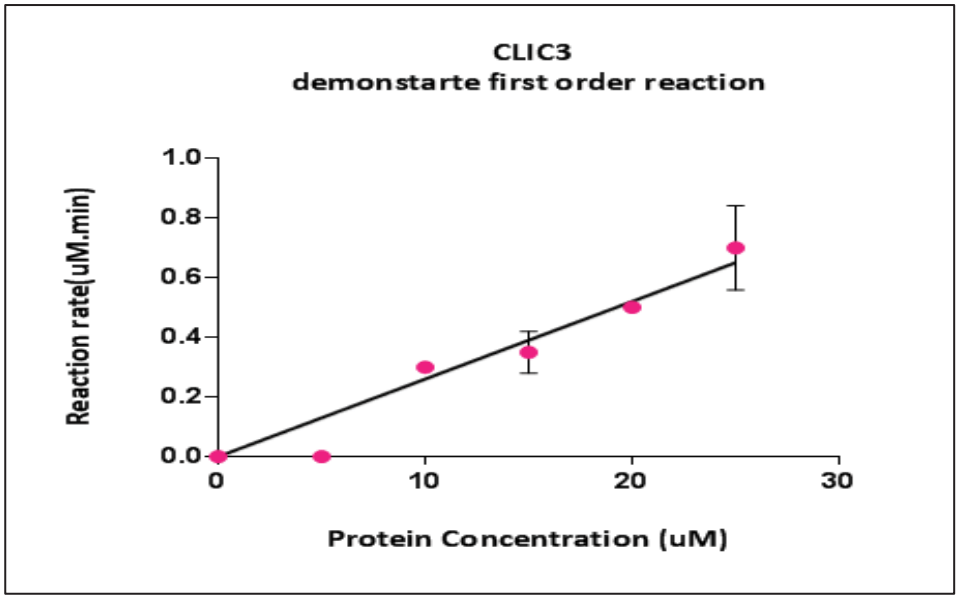

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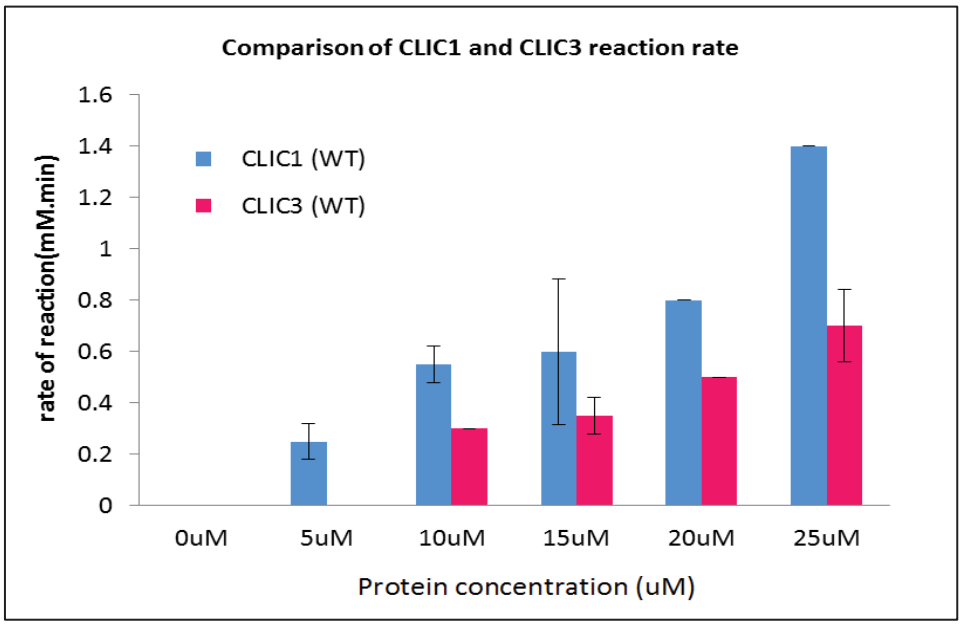

Figure5.3: Representative graph of CLIC1 and CLIC3 first order kinetics reaction. In both (A) and (B) different concentrations of CLIC1 and CLIC3 (0, 5, 10, 15, 20 and 25mM final concentration) enzyme was plotted versus their reaction rate in the HEDS assay. (C) Comparison of CLIC1 and CLIC3 reaction rates. Error bars are representing the standard deviation of three sample replicates.

Based on our characterization studies, kinetic constants (Vmax) of CLIC1 and CLIC3 reaction order were determined by using different concentrations of proteins with a constant concentration of substrate. This experiment was conducted in order to determine the effect of enzyme concentration on the reaction rate. It is evident that by increasing the concentration of both CLIC1 (Vmax of 1.4 mM/min) and CLIC3 (Vmax value of 0.7 mM/min) the reaction rate is increased. This demonstrates that the enzyme concentration and the rate of reaction are proportionately related. Therefore both protein reactions follow first

order kinetics. The enzyme concentration plotted against the rate of reaction (Figure 5.3) also supports this proposed relationship.

5.4 Conclusion

The kinetic considerations would provide important and fundamental information for the specific control of the redox state of regulatory CLIC proteins. Therefore, the significance of knowing the K_m and V_{max} values of CLIC1 and CLIC3 can be described based on different aspect. For example, by knowing the K_m value of these proteins it can be predicted whether the cell needs more of these enzymes, or more substrate needed to be presented in order to speed up the catalytic reaction. In addition, if the CLIC enzyme would be able to catalyse a chemical reaction with two similar substrates within the cell, it will prefer that substrate for which the enzyme has lower K_m value. In addition, the K_m value would give an approximate measure of the concentration of substrate of the enzyme in that part of the cell where the reaction is occurring. Moreover, by knowing the CLICs kinetics, the index of their catalytic efficiency from different tissue or organisms can also be predicted. Therefore, this information might be very useful in the discovery and designing an inhibitor drug.

Chapter 6

Conclusions and Future Direction

6.1. Key findings and conclusions

Enzymes as biological macromolecules have been extensively studied and are considered vital for the survival of all living organisms. These catalyst proteins have a critical region known as the active site, which is often comprised of highly conserved and critical residues that perform various actions, of which, the catalytic activity is the most important. We describe here the characterization of a novel family of enzymes called the chloride intracellular ion channel protein family, also known as the CLIC proteins. These proteins are unusual existing in both monomeric soluble and integral membrane bound states. These proteins can spontaneously insert into phospholipid membranes from their aqueous soluble state, where they are capable of transporting both anionic and even cationic species by acting as ion channel proteins [2].

In addition, the CLIC proteins have structural similarities to the Glutathione S-Transferase (GST) superfamily, especially with the GST-Omega class [3, 4]. The CLICs also demonstrate structural homology to the Glutaredoxin (Grx) enzyme family by sharing a similar active site motif (Cys-X-X Cys/X) that is located in their N-terminal CLIC domain. This active site couples with the Glutathione peptide (GSH) that is used as a cofactor in the redox reactions catalyzed by members of the Grx family. As a result of this activity, the disulfide bonds in protein targets and other component substrates are reversibly reduced, assisting to maintain a "healthy" reduced state within cells' intracellular environment[21].

Recent studies have demonstrated by *in vitro* assay systems that members of the CLIC protein family (CLIC1, CLIC2, CLIC3, and CLIC4), exhibit similar "oxidoreductase" activity to the Glutaredoxin family [5, 6]. This enzymatic function was demonstrated for the monomeric soluble state of the proteins and appears to be independent of their integral membrane, ion channel activity. The active site of the CLIC enzymes was confirmed, along with the identification of certain critical residues [5]. In addition, the enzymatic activity of these CLIC members was inhibited by the same drugs that block their ion channel activity, drug IAA94 [5]. Thus, following on from these new discoveries, further detailed characterization of their enzymatic activity was required. So, based on these aspects, the objective of the present work was to characterize the biochemical properties and kinetic profile of the CLIC protein members.

In that regards, the *in vitro* HEDS enzyme assay that is considered as a characteristic assay for the glutaredoxin proteins was employed in this work in order to study the enzymatic behaviour of two members of the CLIC family, CLIC1, and CLIC3. Indeed, by using this assay the parameters such as optimal pH and temperature for these two proteins was determined. In addition, their substrate affinity and inhibition of their activity was shown, using different substrates and inhibitor drug. Moreover, their structural sensitivity to the "6 polyhistidine-tag" and "imidazole" compound was also described in this thesis. Finally, the kinetic description of their oxidoreductase activity was defined based on the Michaelis-Menten kinetics module.

The conclusions from the research and this thesis are:

- **Presence of the His-fusion tag on the CLIC protein and/or imidazole in storage buffer interferes with CLIC's catalytic activity**

In the course of this investigation our results demonstrate that the partially purified His-tagged CLIC1 and its mutant CLIC1 (Cys24A) and CLIC3 proteins, which were stored in imidazole elution buffer with "6 polyhistidin tag" attached to them, showed distinct activity in enzyme assay by comparison to their non-His-tagged counterparts (chapter 2, figure2.5 C). Hence, the effect of both "6 Histidine-tag" and "Imidazole" compound on CLIC enzyme activity was examined in chapter 2 of this thesis. The results of the present study indicate that the reason for observed highly activated His-tagged CLICs is due to the presence of a high concentration of imidazole in the storage buffers. Also, as imidazole can catalyse the reaction of NADPH reduction of the enzymatic activity therefore, CLICs cannot be reliably measured in its presence. In addition, based on the comparative study of this work, it was observed that the presence of the His-fusion tag on the recombinant protein contributes to changes in enzymatic efficiency, by specifically lowering the enzyme catalytic activity.

This evidence therefore, suggests that the "6 polyhistidine-tag" somehow interferes with the enzymes active site and its local structure surrounding and consequently, influences the protein structure in a manner that lowers the enzyme's efficiency. This view would draw attention to further comparative structural studies needed comparing both tagged and non-tagged CLIC proteins in order to determine the location of the His-tag relative to the enzyme

active site and /or other critical regions within the protein. Regardless of this, it is clear that the presence of the "His-tag" negatively contributes to the proteins' enzymatic activity. Similarly, the presence of "imidazole" contributes to inaccurate catalytic measurements. Therefore, these two factors should be removed from the recombinant protein prior to the use in such assay systems.

- **Determination of dehydroascorbic acid and sodium selenite as potential physiological substrates for CLIC1 and CLIC3 and the inhibitory effect of the drug IAA94 on their activity**

Given that recent studies indicate soluble CLIC proteins have the functional ability to act as general antioxidants and oxidoreductases enzymes [5, 415], further investigation of their catalytic activity in the presence of potential physiological substrates was required, which was the second aim of this study. Therefore, the capabilities of CLIC1 and CLIC3 in the presence of alternate substrates was investigated in the standard enzyme assay. Based on the obtained results, it becomes evident that the soluble CLIC3 and CLIC1, were able to reduce Imidazole, DHA, sodium selenite as well as HEDS as their target substrate.

Indeed, the information drawn from such studies offers important knowledge about the physiological roles of CLIC proteins and could lead to an investigation of their potential involvement in disease processes. Accordingly, from the obtained results, one can postulate that CLIC1 and CLIC3 are likely involved in cellular protective roles owing to their ability to metabolize substrates like DHA. Their ability to reduce sodium selenite also suggests a role in contributing to the maintenance of selenium metabolism and redox control in cells and biological systems.

Thus, the significance of understanding such functional ability of CLIC1 and CLIC3 would have potential public health impact especially in the treatment of selenite-induced cataract in the lens. For example, selenite cataract occurs as a result of an oxidative stress in the lens of the eye. In these circumstances, sodium selenite that is the most common water-soluble selenium compound, would lead to the formation of cataracts due to the reduction of this substrate to selenide [289], which at high concentration causes selenite cataract in the lens. On the other hand, this phenomenon could be prevented by the presence of Vitamin C

(ascorbic acid) [450]. But then again, over time, this vitamin would be oxidised in order to form the dehydroascorbic acid (DHA), which is considered a toxic agent at high concentrations in cells. So, by pointing out the ability of CLIC1 and CLIC3 to reduce both sodium selenite and DHA, one could postulate that these proteins defend against selenite cataract formation, as well as keeping the normal level of ascorbic acid balance in the ocular lens when this vitamin is oxidized to DHA.

In addition, the drug IAA94 was found to demonstrate an inhibitory effect on CLICs enzymatic activity in the HEDS assay, and as a result, prevented the oxidoreductase activity of soluble CLIC3 and CLIC1 due to the binding of this drug as a non-substrate ligand with the enzyme. Indeed, the CLIC family has great potential to be used in therapeutic treatment as drug targets, due to their involvement in vital physiological functions. Consequently, as demonstrated by the recent study that the CLIC proteins can drive cancer progression through their oxidoreductase catalytic behaviour[6], as such, inhibition of their catalytic activity especially in cancerous cells would be a significant approach in cancer treatment. Also, based on the fact that the catalytic activity of CLIC1 and CLIC3 proteins are inhibited by the same drug that also blocks their ion channel activity, future studies must take this into account in order to distinguish between CLICs catalytic activity versus their ion channel activity.

- **Purified recombinant CLIC1 and CLIC3 revealed distinct biochemical properties**

One of the primary aims of this project was to investigate the biochemical properties of two members of the CLIC family, CLIC1 and, CLIC3 and to determine their optimal catalytic activity under different environmental conditions such as pH and temperature. The characterization of wild-type CLIC1 and CLIC3 reported here reveals that the biochemical properties of two enzymes can distinguish one from the other. The CLIC3 was found to be a heat stable protein, while CLIC1 demonstrated heat sensitivity as the experimental temperature was increased. However, both proteins demonstrated similar optimal thermal activity at 37°C in the HEDS enzyme assay. Indeed, the heat-resistant behaviour of the CLIC3

enzyme is comparable to that previously stated for other Glutaredoxins such as CsT-89Grx enzyme [409].

In a recent study, the glutathione-dependent oxidoreductase behaviour of the CLIC3 was shown to be involved extracellularly in order to trigger extracellular transglutaminase-2 (TGM2) activity, and therefore initiate cancer progression[6]. In that regards, all together with the heat resistance and stability of the CLIC3, this could suggest that this protein may use these feature in order to be able to respond to such sudden environmental changes. Although the CLIC1 in this study demonstrated heat sensitivity in comparison to CLIC3 protein, its catalytic oxidoreductase action remained active at 42°C for 40 minutes of reaction duration. As a result of that, the obtained evidence is in support of the antioxidant oxidoreductase behaviour of this protein [415] through which, it can enzymatically react to oxidative stress induced by an increase of oxygen consumption due to an elevation in environmental temperature [416].

In this research project, the pH property of CLIC1 and CLIC3 was also optimized. As the obtained result in chapter 4 indicates, these two proteins have distinct pH optimal point. CLIC3 enzyme prefers a more acidic environment and demonstrates its maximal catalytic activity around pH6. Whereas, CLIC1 enzyme favours more neutral conditions and was optimally active at pH7.

Indeed, the study of the basic biological effect of temperature and pH on CLICs catalytic function would directly or indirectly lead to understanding the influence of CLICs on tumour microenvironments such as stress response, gene-expression or metabolic function. Such information would be highly important in the thermal medicinal field like cancer treatment using heating or freezing applications. In addition, the significance of thermal study on CLICs catalytic activity would give rise to some unpredicted novel insights about the functional and structural properties of this family. For instance, a new specific mechanism related to the function of different members of this family could be recognized through which, the loss or change in their enzymatic activity occurs as a result of thermal alteration. Also, such study could lead to the recognition of critical residues that may have a vital role in their catalytic function.

Moreover, as CLICs are shown to have different cellular localization [139], future studies could investigate the involvement of these proteins in, for example, skin protection against oxidative damage considering their glutathione-dependent oxidoreductase ability. In that regards, as a different layer of skins, and skins in a different part of the body have distinct pH profiles [451, 452], also different cellular compartments have distinct pH, knowing the optimal pH for catalytic activity of CLIC proteins would provide basic information that may provide insight required for such studies.

- **Enzyme kinetic studies revealed the CLIC1 and CLIC3 enzymes obey Michaelis-Menten kinetics module and following first order kinetic reaction rates**

As the final aim of this project, the investigation was undertaken to define the kinetic description of the oxidoreductase catalytic activity of CLIC proteins by characterizing their activity based on the Michaelis-Menten kinetics module. As such, different concentrations of the HEDS substrate, CLIC1, and CLIC3 were examined in the standard HEDS enzyme assay in order to determine the kinetic properties (V_{max} and K_m) and reaction order of these two proteins.

Based on the obtained result from chapter 5, it becomes clear that both CLIC1 and CLIC3 catalytic reaction rate is increased when the concentration of enzymes was increased in the chemical reaction. As there was a linear proportional increase in the rate over the increased enzyme concentration of each protein, the reaction order for both CLIC1 and CLIC3 enzymes is best described as being a first order kinetic reaction type. In addition, the evidence indicates that both CLIC1 and CLIC3 enzymes obey Michaelis-Menten kinetics module with calculated V_{max} values of (2.026 µM/min, 3.33 µM/min) and K_m values of (2.503 mM, 0.9941 mM), respectively, with the HEDS substrate. As CLIC1 exhibits higher K_m value (2.503mM) compared to CLIC3 (0.9941 mM), this can be interpreted that CLIC1's affinity to the HEDS substrate is lower and therefore, a greater amount of this substrate is required in order to saturate the enzyme's active site. Whereas, the lower K_m in the case of CLIC3 means that this enzyme has a greater affinity to the HEDS substrate and for that reason, a lesser substrate is required in order to saturate its active site. Moreover, the obtained high

reaction rate for the CLIC3 enzyme (3.33 μM/ min) also indicates that the saturation of the enzyme active site occurs very quickly and hence, the chemical reaction is catalysed faster.

To conclude, from these studies important information about the specific control of the redox state of regulatory CLIC proteins could be obtained. This Kinetic information would also be useful in comparative studies between members of this family in order to discover their favourable physiological substrates. In addition, from these kinetic descriptions, the features such as enzyme or substrate availability can be predicted, that can be valuable in controlling the chemical reaction. Finally, by knowing the CLIC kinetic profiles, the index of their catalytic efficiency from different tissues or organisms can also be predicted. Therefore, the significance of obtaining such knowledge could be useful and of interest in discovery and designing inhibitor drugs in future studies and for determining the physiological roles of these interesting, multifunctional proteins.

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