Investigating the Dual Function of the Chloride Intracellular Ion Channel Proteins

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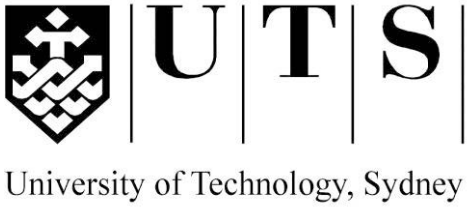

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Dedicated to My Family and all IRAQ with love

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Publications

1) Valenzuela, S. M., Alkhamici, H., Brown, L. J., Almond, O. C., Goodchild, S. C., Carne, S., Curmi, P. M., Holt, S. A., and Cornell, B. A. (2013) Regulation of the membrane insertion and conductance activity of the metamorphic chloride intracellular channel protein CLIC1 by cholesterol. PLoS One 8, e56948.

Alkhamici, H contributed to paper (1) by performing and assisting in the design of all the impedance spectroscopy experiments and all data analysis, providing related figures for the manuscript and proof reading of manuscript.

2) Jiang, L., Phang, J. M., Yu, J., Harrop, S. J., Sokolova, A. V., Duff, A. P., Wilk, K. E., Alkhamici, H., Valenzuela, S. M. Brown. L. J., Curmi. P., Breit, S. N. (2013) CLIC proteins, ezrin, radixin, moesin and the coupling of membranes to the actin cytoskeleton: A smoking gun?. Biochimica et Biophysica Acta (BBA)-Biomembranes 1838, 643-657.

Review paper (2): Alkhamici, H contributed directly to the section regarding membrane interactions of CLIC proteins and the role of sterols and proof reading of the manuscript.

3) Al Khamici, H., Brown, L. J., Khondker. R. H., Hudson. A., Ng, J., Sinclair. A., Hare. J., Cornell, B, A., Curmi. P., Davey. M. D. W. and Valenzuela. S. M. (2014) Members of the Chloride Intracellular Ion Channel Protein Family Demonstrate Glutaredoxin-Like Enzymatic Activity. PLoS One 10(1), e115699.

Al Khamici's contribution to paper (3) included designing and performing experiments, data analysis and writing of the paper.

Conference Oral Presentations

1) Al Khamici. H., Hussain. R. H., Danial. E., Hudson. A., Davey. M., Brown. L. J., Valenzuela. S. M. Chloride Intracellular Channel Proteins Resemble The Glutaredoxin Enzymatic Activity. The New Horizon, Sydney, Australia. 2013.

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Conference Poster Presentations

1) Al Khamici. H., Carne. S., Hare. J., Brown. L. J, Cornell. B. A., Valenzuela. S. M. The Characterisation of Ion channel Proteins using Tethered Bilayer Lipid Membranes and Impedance Spectroscopy. Scientific Research Meeting, Sydney, Australia. 2011.

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Table of Contents

List of Abbreviation

λ	Wavelength
A	Absorbance
AA	Ascorbic acid
A9C	Anthracene-9-carboxylic acid
AβP	Amyloid β-protein
ABS	Ammonium persulfate
ac	Alternating current
Ala	Alanine amino acid
AM199	Zwitterioninc lipids
Arg	Arginine amino acid
Asp	Asparagine amino acid
BCA	Bicinchoninic acid assay
AFM	Atomic Force Microscopy
BLM	Black lipid membrane
BSA	Bovine serum albumin
ºC	Degree celsius
$CaCl_2$	Calcium chloride
CDC	Cholesterol-dependent cytolysin
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)
Cm	Capacitance
CRAC	Identification of cholesterol recognition amino acid consensus
Cs	Counter electrode capacitance
Cu^{+1}	Cuprous cation
Cu^{+2}	Cupric ion
Cys	Cysteine

DDT	Dithiothreitol
DHA	Dehydroascorbate
DHAR	Dehydroascorbic acid reductase
DIDS	4,4`-diisothiocyano-2,2`stilbene-disulfonic acid
*Dm*CLIC	Drosophila- *melanogaster* CLIC protein
DNA	Deoxyribonucleotides acid
E	Glutamic amino acid
E-coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EIS	Electrochemical impedance spectroscopy
ER	Endoplasmic reticulum
ERK7	Extracellular signal-regulated kinase 7
EXC	Excretory canal abnormality
EXL	EXC4-like
f	Frequency
G	Glycine amino acid
G-site	Glutathione binding site
GABA	Gamma-aminobutyric acid
GS-Se-SG	Selenodiglutathione
Grx	Glutaredoxin
Grx-1, 2 to 5	Glutaredoxin-1, 2 to 5
GPx	Glutathione peroxidase
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
GST-π	Glutathione-S-transferase pi class
H-site	Hydrophobic region
HSe^- or RSe^-	Selenide
H_2O_2	Hydrogen peroxide

HCSK	High conductance channels with slow kinetics
HcTrx-5	Thioredoxin-related protein in *Haemonchus contortus*
HEDS	2-hydroxyethyl disulfide
HEPES	N-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid
His	Histidine
ld	Liquid disordered phase
LLO	Listeriolysin-O
lo	Liquid ordered phase
IAA	Indanyloxyacetic acid
ILY	Intermedilysin
IPTG	Isopropyl-β-thiogalactopyranoside
K^+	Potassium ion
KCl	Potassium cholride
kDa	KiloDalton(s)
Km	Dissociation constant of the enzyme-substrate complex
LB	Luria-Bertani medium
M	Molar
MAPK	Mitogen-activated protein kinase
mg	Milligram
min	Minute
MLP	Mobile lipid phase
mM	Millimolar
mV	Millivolt
N	Amino
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NaN_3	Sodium Azide
Na_2SeO_3	Sodium selenite
NCC27	Nuclear chloride channel protein-27kDa
N-domain	Amino terminal domain
NEM	N-Ethylmaleimide
nF	Nano-faraday

NIH	National Institutes of Health
nm	Nanometer
nM	Nanomolar
OD	Optical density
P64	Bovine chloride channel protein -64kDa
PBS	Potassium buffered saline
PC	Phosphotidylcholine
PE	Phosphotidylethanolamine
PFO	Perfringolysin-O
PFT	Pore forming toxin
Phe	Phenylalanine amino acid
POPC	1-palmitoyl-2-oleoylphosphatidylcholine
POPE	1-palmitoyl-2-oleoylphosphatidylethanolamine
POPS	1-palmitoyl-2-oleoylphosphatidylserine
Pro	Proline amino acid
PTMD	Putative transmembrane domain
PtoDHAR2	Dehydroascorbic acid reductase-2 from *Populus tomentosa*
QCM	Quartz Crystal Microbalance
Rm	Resistance
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
RyR	Ryanodine receptor
s	Second
SAM	Self- assembled monolayer
SAXS	Small-angle X-ray scattering
SCSK	Small conductance channels with slow kinetics
S.E	Standard error
Se^0	Metallic selenium
SEC	Size exclusion chromatography
SeCys	Seleno amino acids
SeMet	Selenomethionine
$SeO(OH)_2$	Selenite
$SeO_2(OH)_2$	Selenate

Ser	Serine amino acid
SDS	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLB	Supported lipid bilayer
SLO	Streptolysin-O
so	Solid ordered phase
SOH	Sulfenic acid
SO_2H	Sulfinic acid
SO_3H	Sulfonic acid
SPR	Surface Plasmon Resonance
STOML	Stomatin- Like proteins in mammals
tBLM	Tethered bilayer lipid membrane
TCEP	Tris(2-carboxyethyl)phosphine
TEMED	N,N,N,N',N-tetramethylenediamine
Tris	Tris[hydroxymethyl]aminomethane
Triton-X100	Octylphenyl-nonaoxyethylene
Trp35	Tryptophan residue number 35
Trx-1	Thioredoxins-1
TrxR	Thioredoxin reductase
Trxs	Thioredoxins
Tween-20	Polyoxyethylene-sorbitan monolaurate
Tyr	Tyrosine amino acid
µg	Microgram
µM	Micromolar
µS	Microsiemens
UV	Ultraviolet
Val	Valine amino acid
WT	Wild type
X	Any amino acid
Z	Impedance

List of Figures

List of Tables

Abstract

The Chloride Intracellular Ion Channel (CLIC) family consists of six conserved proteins in humans, CLIC1-CLIC6. These are a group of enigmatic proteins, which adopt both a soluble and membrane bound form. CLIC1 in particular has challenged the widely held view that most proteins adopt one stable native structure essential for their biological function. In contrast, CLIC1 was found to be a metamorphic protein, where under specific environmental triggers it adopts more than one stable soluble structural conformation.

CLIC1 was also found to spontaneously insert into cell membranes and form chloride ion channels. However, factors that control the structural transition of CLIC1 from being soluble into a membrane bound protein have yet to be adequately described. Thus, the first objective of this thesis was to identify factors that are involved in CLIC1's insertion and assembly into membranes using tethered bilayer lipid membranes and impedance spectroscopy as a novel system for the study of ion channel activity.

Our findings demonstrate that CLIC1 ion channel activity is dependent on the type and concentration of sterols in bilayer membranes. These findings suggest that membrane sterols play an essential role in CLIC1's acrobatic switching from a globular soluble form to an integral membrane form, promoting greater ion channel conductance in membranes. What remains unclear is the precise nature of this regulation involving membrane sterols and ultimately determining CLIC1's membrane structure. Furthermore, our impedance spectroscopy results of CLIC1 mutants, suggest that residue Cys24 is not essential for CLIC1's ion channel function however it is important

for its optimal activity in membranes. Therefore oxidation and reduction may not be the only regulators of the ion channel activity of CLIC1.

Structural studies have revealed that, soluble CLIC proteins adopt a glutathione S-transferase fold with a conserved glutaredoxin–like active site motif, similar to the GST-Ω class. Therefore the second aim of this project was to investigate the function of the soluble CLICs.

Using the 2-hydroxyethyl disulfide enzyme assay, we have demonstrated for the first time that CLIC1, CLIC2 and CLIC4 possess "glutaredoxin-like" oxidoreductase activity. CLIC1 was found to catalyse the metabolism of the typical glutaredoxin substrates, sodium selenite and dehydroascrobic acid. As expected, the active site Cys24 was detected to be essential for the enzymatic activity of CLIC1 *in vitro*. Most importantly, indanyloxyacetic acid-94 and anthracene-9-carboxylic acid were found to also inhibit the enzymatic activity of CLIC1.

Members of the CLIC protein family can now be classified as "moonlighting proteins" as they exhibit two independent functions; one as ion channels when in their membrane bound form and the other as oxidoreductase soluble enzymes.

Chapter 1

Literature Review

1.1 Introduction

Chloride ion channels are well known for their important cellular functions and association with cell plasma membranes (6). In contrast, the chloride intracellular ion channel (CLIC) protein family is comprised of members that are found to localise principally with intracellular membranes and to a lesser extent with the cell plasma membrane (7). They have been given the name, chloride intracellular ion channels, however recent studies suggest that they are capable of transporting other anionic and even cationic species making their name somewhat misleading (8). CLIC proteins are unusual in that they exist in both a soluble and membrane bound state. They also possess the unusual, if not unique ability to shift between two stable tertiary protein conformations, an ability described as "metamorphic" (9). Despite extensive experimental study and review, much still remains unknown regarding the role of the membrane and the soluble form of the CLIC proteins and the regulatory mechanisms controlling their transition between these two states.

This chapter provides an overview of the known roles of the CLIC proteins. It introduces the recently identified structural similarities between the CLIC proteins and the glutathione-S-transferase (GST) protein superfamily. This similarity has provided clues to better predict the role and function of the CLICs. A summary is provided of the structural data that indicated the likely ability of the CLIC proteins to function as enzymes and of recent supporting empirical evidence. In addition, this chapter reviews key studies of the membrane associating and functional characteristics of the CLIC proteins (with a focus on CLIC1) confirming their ability to spontaneously insert into and form functional ion channels in the membranes of different cell-lines and in artificial lipid bilayer membranes. Finally, this chapter highlights gaps in our present

understanding of the CLIC family protein function, along with a summary of the main objectives of this project.

1.2 Chloride Ion Channels

The cell plasma and intracellular endomembrane systems are composed of proteins, carbohydrates, cholesterol, glycolipids and phospholipids (10,11) (Figure 1.1). All of these membranes are configured as lipid bilayers, and are largely impermeable to water and solutes. They are arranged with the hydrocarbon tails of the phospholipids facing the inner core of the membrane bilayer, while the phospholipid polar head groups face outwards forming the membrane-water interfaces (12).

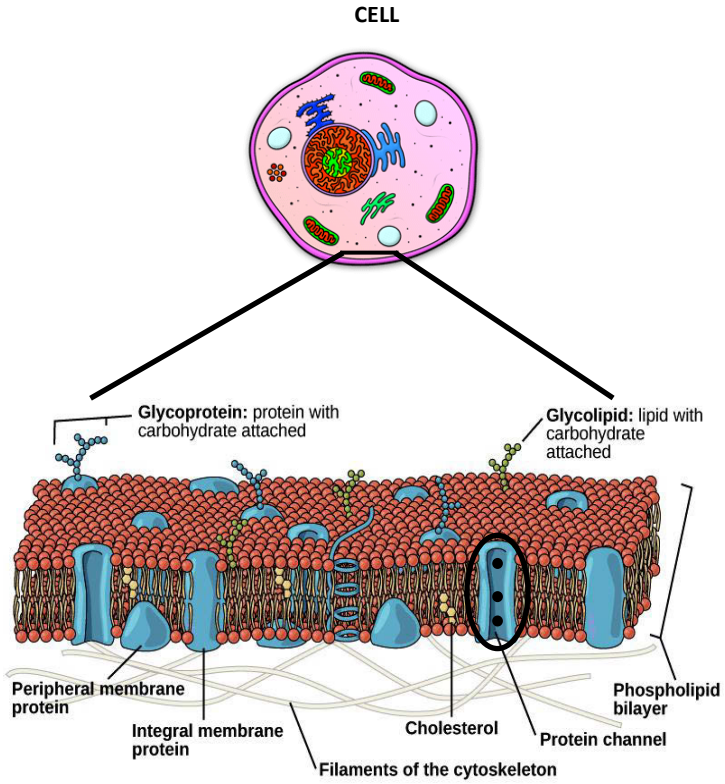


Figure 1.1: Cell plasma membrane. The fluid, mosaic model of the cell plasma membrane - phospholipids self-assemble to form the basis of biological membranes which incorporate proteins, cholesterol, other lipids and carbohydrates. Modified figure was taken from (13).

In order for a cell to carry out its vital functions, it needs to interact with its surroundings (10,11), this occurs largely via its plasma membrane components, while at the same time, passage of both hydrophilic and hydrophobic molecules into and out of the intracellular environment is needed without compromising the integrity of the membrane (11). The presences of peripheral and integral membrane proteins facilitate such functions (10,12,14). Whereas peripheral membrane proteins associate with membrane surfaces through electrostatic interactions and hydrogen bonding (14), integral membrane proteins can span either one leaflet of the membrane or the entire lipid bilayer (12). Ion channels are integral membrane proteins that contain hydrophobic domain/s that interact with the hydrophobic core of the bilayer membrane, and hydrophilic regions that are in contact with the surrounding aqueous environment. In addition, ion channel proteins also create an aqueous pore within their structure that permits ions and small electrolytes to permeate across the hydrophobic region of the lipid bilayer (10,12,14) (Figure 1.1). Ion channels also have the characteristic feature of "gating" (opening and closing) which serves to control the movement of ions across membranes. There are three categories of ion channel gating: voltage gating, where channels open and close due to changes in the membrane potential, ligand gated channels that are activated by the binding of specific molecules to their active sites and ion channels that are gated via mechanical stimuli, for example cell swelling or other mechanical membrane perturbations (15).

Chloride ion channels are proteins that predominantly facilitate the transport of chloride ions across the membrane. Chloride channels are thought to be principally involved in: maintaining homeostasis and the internal pH of cells*;* cellular responses to neurotransmitters*;* bone resorption (16); regulation of action potentials. Chloride ion channels were also found to play an important role in human disease processes and

involved in conditions such as Bartter's syndrome (17), Dents disease (15), cystic fibrosis and kidney disorders (18), macular and retinal degeneration (19) and muscle myotonia (20,21). These findings have highlighted the importance of chloride channels in the regulation of cellular activities. The major classes of chloride ion channels include: the gamma-aminobutyric acid (GABA) and glycine receptor ligand-gated ion channels (22); the chloride ion channels (CIC) family of proteins (22-24); the cystic fibrosis trans-membrane conductance regulators (CFTR) (25) and the most recently discovered chloride intracellular ion channels, the CLIC proteins (7,22,26).

1.2.1 The CLIC Proteins

The human chloride intracellular ion channel (CLIC) proteins are ~240 amino acid residues in length. There are six conserved CLICs in humans: NCC27 or CLIC1 (7), CLIC2 (27), CLIC3 (28), CLIC4 (29,30), CLIC5(~251 amino acid residues) (30,31), CLIC6 (~408 amino acid residues long) (32). The first member of this family, p64 which was isolated from bovine kidney tissues (22,33,34) was also was found to be a splice variant of human CLIC5, while CLIC4 was originally called p64H1 (29,31). In addition, there are also known to be CLIC-like proteins in invertebrates such as the proteins EXL-1 and EXC-4 found in the nematode *Caenorhabditis elegans* (*C. elegans*) (35). These two proteins share a sequence homology of about 42% and 43% when compared with human CLIC1 respectively (36,37). Knockout of the gene encoding the protein EXC-4 resulted in the development of a cystic excretory canal in *C. elegans* (35,38). The *Drosophila melanogaster* fruit fly contains a single CLIC1-like protein (*Dm*CLIC) which shares a sequence homology of about 25% with human CLIC1. *Dm*CLIC knockout flies were found to have a 60-70% shorter life span compared to wild type flies (39,40). In addition, four CLIC-like proteins were found

in the plant *Arabidopsis thaliana* with a sequence identity of about 26% to human CLIC1 (39,40).

The physiological characterisation of the CLIC proteins has been based on a number of different knock-out organisms (35,38). Currently knock-out mouse models exist for CLIC1 (28,41), CLIC4 (41,42) and CLIC5 (43). However a clear picture of the cellular functions of the CLIC proteins in vertebrates has been difficult to obtain due to the presence of the six paralogues, resulting in functional redundancy between the CLIC proteins. Known functions for each of the CLICs are summarized in Table 1.1.

Protein Name and Molecular Mass	Tissue Distribution	Known Functions / Links to pathological conditions	References
CLIC1 26.9 kDa	Expressed in most body tissues. Associates with cytoskeletal proteins and actin microtubules cytoskeletons. It is localised to the nuclear and plasma membranes, including apical membranes of the kidney, as well as to intracellular vesicles, and is also located in the cytoplasm and nucleoplasm.	Ion channel activity; involved in cell division and apoptosis regulation; a biomarker in for colorectal cancer. Alzheimer's disease and in cancer therapy	(24,44-53)
CLIC2 28.2 kDa	Expressed in most body tissues except the brain. Highest expression was found in the spleen and the lungs.	Involved in the regulation of cardiac ion channels by modulating the activity of Ryanodine receptor (RyR)	(27,36,45,54-57)
CLIC3 26.7 kDa	Expressed in the placenta, heart and the lungs with very low expression in the skeletal muscles, kidney and the pancreas. Associates with ERK7, a mitogen-activated protein kinase.	Involved in the regulation of cellular growth	(28,45,58,59)
CLIC4 27 kDa	Found to be highly expressed in the brain, lungs, liver and the skin. Associates with tubulin, dynein intermediate chain (DIC) and dynamin I and actin.	Functions as an ion channel, induces apoptosis in several cells including human keratinocytes and is involved in angiogenesis and acidification of cells	(20,29,45,51, 52,57,60-63)
CLIC5 46 kDa	Localised to the Golgi of human colonic adenocarcinoma cell lines within the cytosol, placenta and post-acrosomal region of the sperm head. Associates with actin and ezrin.	Demonstrates ion channel activity, is involved in ion absorption and secretion, formation of stereocilia and in the development of the organ of Corti	(43,48,64-66)
CLIC6 71 kDa	Associates with the C-terminus of dopamine D2-like receptor. Localized to the plasma membrane and the cytosol of human kidney cells. In rats it was found to be endogenous on the plasma membranes and exists mainly in the cytosol. Expressed in rabbit lacrimal glands, brain, kidneys and chorioretinal epithelium.	Involved in the regulation of body fluid via chloride ions transport and hormone secretion in neuroendocrine cells of the pituitary gland	(32,45,67)

Table 1.1: Summary of human CLIC protein functions. Detailing the molecular mass; tissue expression; localization and known functions of human CLIC proteins

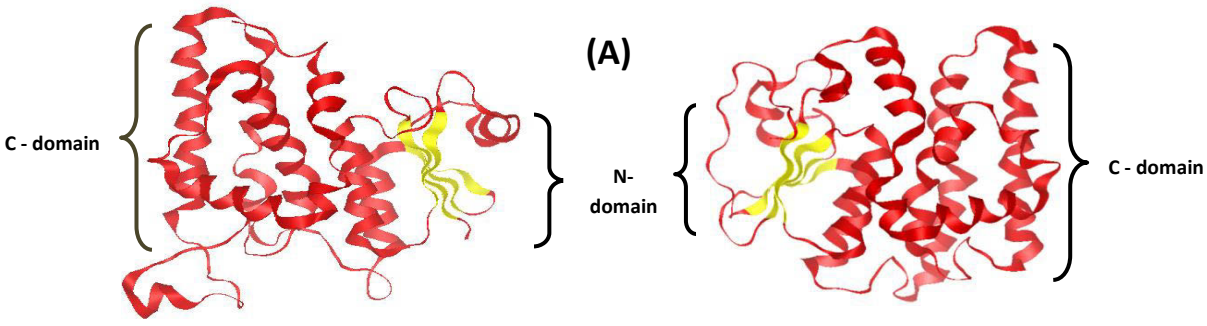
1.2.2 CLIC Proteins are Part of the GST Structural Superfamily

Sequence and structural homology studies by Dulhunty et al, 2000 (55) have revealed significant sequence similarities between members of the GSTs and the CLIC family. Detailed examination of the sequence alignments and subsequent homology modeling of their three dimensional X-ray crystal structures, support inclusion of the CLICs into the GST superfamily of enzymes, and of which they are most closely related to the GST-Ω class members (Figure 1.2).

The classical glutathione-S-transferase enzymes (GSTs) are found as dimers in solution. Their primary structure contains a thioredoxin domain that exhibits an active site motif (Cys-X-X-Cys) capable of non-covalently binding of glutathione (GSH). They also contain conserved residues at the active site (Tyr or Ser) that provide the main chain hydrogen bond donors and hydrogen bond acceptors for GSH (37,55,68). Other residues at the active site are catalytic and comprise two cysteine residues that form a disulfide bond when oxidized and when reduced form a disulphide bond between GSH and the first cysteine residue (37,69).

The structure of soluble CLIC1 has been resolved by X-ray crystallography to 1.4Å resolution (70). CLIC1 is monomeric in solution and adopts a fold similar to the GST superfamily (Figure 1.2 A). In particular it contains two domains: (i) a C-terminal domain, consisting of all α-helical strands closely resembling the canonical fold of the GST-Ω group and (ii) an N-terminal domain that consists of four beta strands sandwiched between three α-helices that exhibit the thioredoxin active site motif and a glutathione binding site that covalently binds glutathione, due to the presence of a redox active cysteine residue near the N-terminal (specifically Cys24 in CLIC1) (Figure 1.2 B) (37,70,71).

There are four key residues conserved in all the GSTs and CLIC proteins: 1) a *Cis*-proline residue that provides the active site for binding GSH in the GSTs, 2) an aspartic acid residue and two glycine residues that play structural roles. In addition, all CLIC proteins contain a Cys-Pro-Phe motif that includes the cysteine active site of the GST-Ω class which was also found in the CLIC proteins (24,28,68,72).



(B)

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CLIC1 MAEEQPQVELFVKAGSDGAKIG--------NCPFSQRLFMVLWLKGVTFN 42
GST-Ω MSGESARSLGKGSAPPGPVPEGSIRIYSMRFCPFAERTRLVLKAKGIRHE 50
      *: *  :      *       *         ***::*  :**  **:  :

CLIC1 VTTVDTKRRTETVQKLCPGGQLPFLLYG-TEVHTDTNKIEEFLEAVLCPP 91
GST-Ω VININLKNKPEWFFKKNPFGLVPVLENSQGQLIYESAITCEYLDEAYPGK 100
      *  :: * : *   *  * * :* *     ::  ::    *:*:

CLIC1 RYPKLAALNPESNTAGLDIFAKFS----AYIKNSNPALNDNLEKGLLKAL 137
GST-Ω KLLPDDPYEKACQKMILELFSKVPSLVGSFIRSQNKEDYAGLKEEFRKEF 150
      :       :   :   *::*:*      ::*:  *      *:: : * :

CLIC1 KVLDNYLTSPLPEEVDETSAEDEGVSQRKFLDGNELTLADCNLLPKLHIV 187
GST-Ω TKLEEVLTN----------------KKTTFFGGNSISMIDYLIWPWFERL 184
        *:: **                 :  *: ** ::: *  : * :  :

CLIC1 QVVCKKYRGFTIPEAFRGVHRYLSNAYAREEFASTCPDDEEIELAYEQVA 237
GST-Ω EAMKLNECVDHTPKLKLWMAAMKEDPTVSALLTSEKDWQGFLELYLQNSP 234
      : :  :      *:    :     :      ::*    :  :**  ::

CLIC1 KALK--- 241
GST-Ω EACDYGL 241
      :*
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Figure 1.2: CLIC proteins are structurally related to GSTs. *A)* The three dimensional structure of human CLIC1 (left) and human GST-Ω1 (right). Indicated in *red* the C- terminal domain and *yellow* is the N-terminal domain. Structures were created using amino acid alignments and the program DNA Star. *B)* Sequence alignment of human CLIC1 (accession number CAG46868.1) and GST-Ω (accession number AAF73376.1). *Asterisks* indicate the conserved amino acid residues in GST-Ω and CLIC1 and the *two vertical dots* indicate conservative replacements of amino acids. The alignment was produced using Clustalw.

1.3 Putative Enzymatic Function of the CLIC Proteins

The Glutathione S-Transferase (GSTs), are a superfamily of multifunctional enzymes that were first discovered in 1961 (73). They function as mediators in cellular oxidative stress responses (74), are involved in the synthesis of prostaglandins, and facilitate the intracellular transport of hydrophobic compounds (75). These enzymes catalyse nucleophilic attack by reduced glutathione on non-polar compounds that contain an electrophilic carbon, nitrogen or sulphur atoms (76). GSTs are reported to have additional functions including the binding of bilirubin and carcinogens, and their over expression in tumor cells was found to contribute to anticancer drug resistance in these cells (77).

To date, the GST enzymes have been found in all organisms including fungi, insects, plants, animals and some bacteria (68). The GSTs are divided into classes that include: alpha, beta, delta, epsilon, theta, zeta, mu, pi, tau and omega with a molecular mass of about 24-25 kDa for each subunit (69,74,77)

The most common theme of the characterized clades of GST fold proteins is that they contain a Cys-X-X-Cys/X active site motif (78) and are found to function as enzymes that couple GSH to a redox reaction where the target substrate maybe either a small molecule or a biological macromolecule (72). Structural studies of the CLIC proteins demonstrate that their N-terminal domain contains a glutaredoxin- like motif (Cys-X-X-Ser) with a redox-active cysteine residue (Cys24 in CLIC1) which can form a disulphide bond with GSH, as is the case for the GST-Ω class of proteins (36,37,70,78) (Figure 1.3 A).

Detailed studies revealing the crystal structure of the complex of CLIC1/GSH complex, showed that the GSH group is poorly ordered and only makes few contacts with CLIC1

(low affinity binding) (37), This also led to the observation of the existence of an opening or an "empty slot" located near the GSH binding site of the CLIC proteins (as in Figure 1.3 B) (70,71,78). For this reason it was speculated that this empty site could be the binding site for an extended macromolecular chain, perhaps a polypeptide molecule that may function as a substrate. However, the crystal structure of invertebrate CLIC-like proteins e.g. EXC-4 and *Dm*CLIC showed that there is a metal ion (calcium or potassium ion) bound to the protein near the GSH binding site (40). This feature suggests that the CLIC-like proteins may not have the ability to function as enzymes.

In summary, the amino acid sequence homology and the structural similarities between the GSTs and the vertebrate CLIC proteins, support the hypothesis that the CLICs may also have enzymatic functions. However, no catalytic activity has been reported in the literature to date (37,70,78).

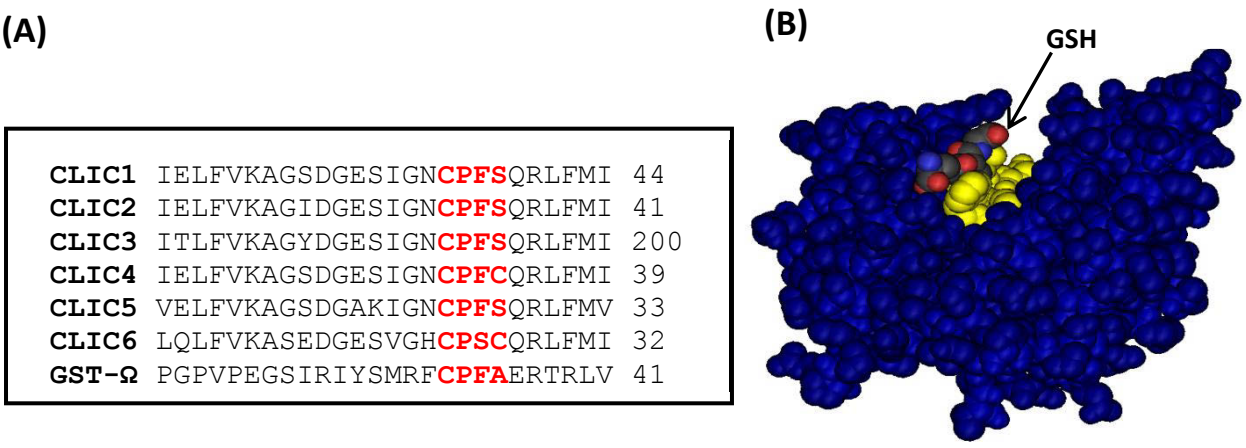


Figure 1.3: G-site or GSH binding site of CLIC proteins. *A)* Amino acid sequence alignment of the secondary structure of all six human CLIC proteins and human GST-Ω. In *red* the conserved glutaredoxin/thioredoxin like motif that contains a redox-active cysteine residue. Alignment was produced in Clustalw using amino acids and accession numbers of CLIC1 (CAG46868); CLIC2 (CAG03948); CLIC3 (NP_004660.2); CLIC4 (CAG38532); CLIC5 (AAF66928); CLIC6 (NP_444507); GST-Ω (AAF73376). *B)* GSH-CLIC1 complex indicates that glutathione-binding site is located at the edge of another possible binding site in CLIC1 structure as shown in *yellow*. CLIC1 structure was generated using the amino acid sequence of CLIC1 and DNA star.

1.4 CLIC Proteins are Metamorphic and Form Chloride Ion Channels

Classical integral membrane proteins can be single or multi-pass transmembrane proteins, depending on the number of hydrophobic membrane spanning segments in their structure (79). Therefore it has been argued by some, that the CLIC proteins are most likely modulators or regulators of ion channels because they exist largely as soluble proteins and their primary structure lacks a typical hydrophobic transmembrane domain like the other classical ion channel proteins (37,70).

The soluble recombinant form of CLIC proteins members has been expressed in *E-coli* and purified in order to study their electrophysiological properties and investigate the functions of these proteins. Some of these techniques have included: chloride efflux studies (8,57), patch clamp electrophysiology (7,44,49), black lipid membranes (BLM) and surface plasmon resonance methods (59,80).

The first member of the CLIC family to be identified due to its affinity to the chloride ion channel inhibitor, indanyloxyacetic acid-94 (IAA-94), was the bovine protein p64 (22,26,34,64). Biochemical and electrophysiological studies later confirmed that p64 was able to form chloride ion channels in planar lipid bilayer membranes (26,64,65).

Subsequently channel activity was also reported for human CLIC1 (7,8,44,49,81), CLIC2 (36,54), CLIC4 (8,47,48,71) and CLIC5 (82); and in addition, the invertebrate orthologous EXC-4 from *Caenorhabditis elegans* (35,40), *Dm*CLIC from *Drosophila melanogaster* (40) as well as the CLIC-like proteins from *Arabidopsis thaliana* plant that have demonstration ion channel activity (39).

Despite the similar ionic conditions of many of these experiments, the single channel conductance reported for the CLIC proteins was highly variable from one experiment to

the other. The reported single channel conductance of CLIC1 in planar lipid membranes containing asolectin or phosphatidylethanolamine / phosphotidylserine (PE/PS) of 60-120pS (8,83) was higher than the channel conductance from cells and tip-dip experiments of only 8-16pS (49,80). Also the conductance of recombinant CLIC4 in membranes containing phosphatidylcholine (PC) lipids was 30-86pS (71), whereas CLIC5 conductance in membranes with asolectin was 26-400pS (34,50). CLIC4 and CLIC5 also showed higher conductance in planar membranes than the single channel recordings from Chinese Hamster Ovary (CHO-K1) cell membranes that were very similar to ion channel characteristics of CLIC1 (80). It was speculated that this difference in CLIC protein conductance is due to the ability of proteins to form aggregates in planar membranes (49).

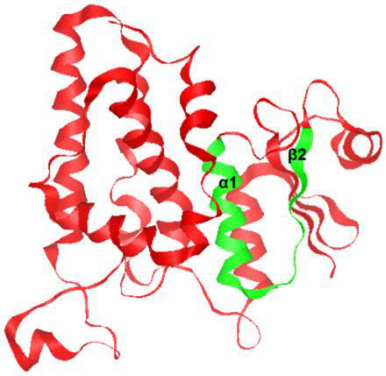
Further electrophysiological studies using an antibody to a FLAG epitope-tag on CLIC1 expressed protein; have indicated that CLIC1 spanned the plasma membrane an odd number of times in order to form ion channels (84). Furthermore, it was demonstrated that CLIC1 forms non-selective anion channels and the channel activity was highly dependent on the anion concentrations in solution (8), whereas CLIC4 and CLIC5 proteins were found to form ion channels that were equally permeable to K^+ and Cl^- ions (39,47,48,81). Studies of the reconstitution of CLIC1 into bilayer membranes with resultant conductance changes, confirm that the protein forms functional ion channels in the absence of ancillary proteins (8,48,49,81) and CLIC4 has been localized to specific cholesterol rich domains of the membrane (85). It has been suggested that the CLIC proteins may form functional ion channels when reconstituted to specific cholesterol rich micro-domains in membranes and it was speculated that these domains facilitate the unfolding and insertion of CLIC proteins (85). However there are currently no reports

of significant interactions or associations of CLIC4 with cholesterol molecules in cell membranes.

As mentioned previously, CLIC proteins lack a prominent hydrophobic trans-membrane domains (55,56,70,78,86); instead CLIC1 was found to contain two moderately hydrophobic regions: the first located at the N-terminus forming the putative trans-membrane domain (PTMD comprised of an α helix 1 and a β-strand 2) (refer to Figure 1.4 A) which was found to be conserved amongst all the CLIC proteins (Figure 1.4 B).The second hydrophobic region is in the C-terminal domain (comprising most of α helix 6 and some parts of the loop preceding it) (Figure 1.4 C) (70).

It is predicted that in order for the CLIC proteins to form ion channels in membranes, they require a large-scale conformational change in structure to allow them to span the membrane and utilize their PTMD region and anchor it into the lipid bilayer (84). The ability of CLICs to transit between two stable structures and convert from being soluble in solution to membrane bound proteins is similar to a number of other proteins now called "metamorphic proteins". These include the spindle assembly checkpoint protein Mad2, viral glycoprotein, RNA polymerase and the chemokine lymphotactin (Ltn) (9). All these proteins were found to undergo structural transitions between their alternative conformations, where these changes occur as a result of specific environmental cues (9,40,87).

(A)



(B)

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                    24                    46
CLIC1 AGSDGAKIGNCPFSQRLFMVLWLKGVTFNVTTVDT
CLIC2 AGSDGESIGNCPFCQRLFMILWLKGVKFNVTTVDM
CLIC3 ASEDGESVGHCPSCQRLFMVLLLKGVPFTLTTVDT
CLIC4 AGSDGESIGNCPFSQRLFMILWLKGVVFSVTTVDL
CLIC5 AGIDGESIGNCPFSQRLFMILWLKGVVFNVTTVDL
CLIC6 AGYDGESIGNCPFSQRLFMILWLKGVIFNVTTVDL
```

(C)

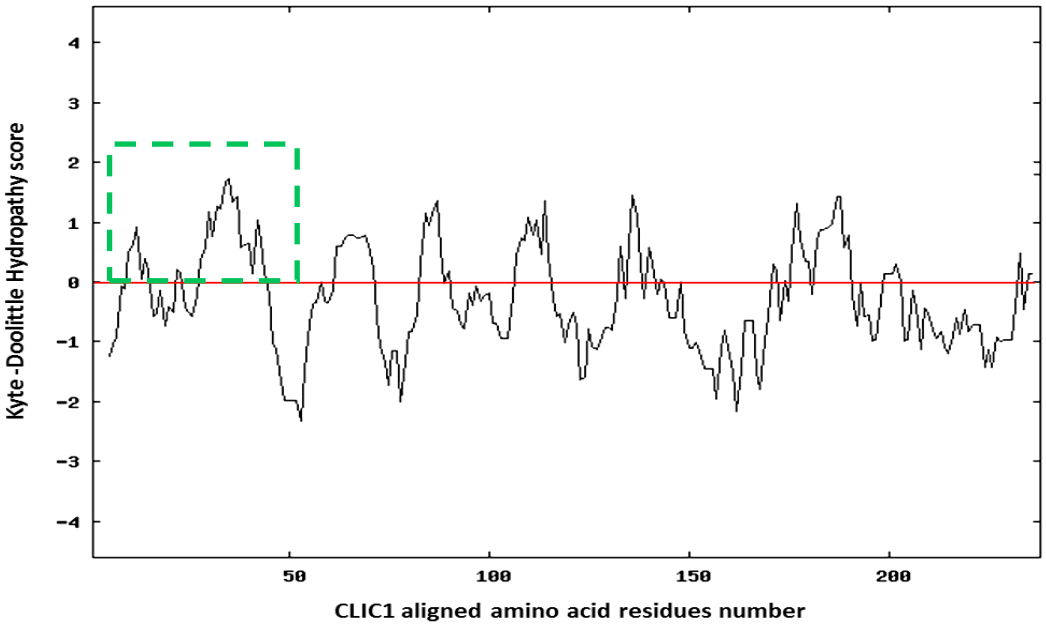


Figure 1.4: Putative transmembrane domain region of the CLIC proteins. *A)* Three-dimensional structure of CLIC1 shows the locations of the PTMD (*shaded in green* α1-β2). *B)* Conservation of the hydrophobic region (PTMD) as shown in *green* at the N-terminal domain of all the human CLIC proteins. In CLIC1 the PTMD span from Cys24 to Val46. *C)* Kyte-Doolittle Hydrophobicity plot for CLIC1 amino acid residues shows the hydrophobic regions (*green dotted rectangle indicated most likely the transmembrane domains*).

1.4.1 Structural Changes of CLIC1 under Redox Control

Due to the existence of a glutaredoxin-like active site located around a critical cysteine residue (Cys24 in human CLIC1), it is postulated that CLIC function may be under redox control via reactive oxygen and nitrogen species (84). Littler et al (84) have further proposed that the oxidation state catalyses the insertion of monomeric CLIC proteins into lipid membranes.

It was reported that oxidation of CLIC1 with 2mM H_2O_2 results in its reversible conversion from a soluble monomer into a non-covalent dimer (70,88). The dimer is stabilized via a non-covalent intramolecular disulphide bond formed between Cys24 and Cys59 (Figure 1.5). The 3D crystal structure reveals that the C-terminal domain of the CLIC1 dimer was identical to the CLIC1 monomer, whereas the N-terminal domain showed major structural rearrangements, including loss of the four β-strands and extension of the α-helix1 segment towards the C-terminus by two α-helical turns, predicted as part of the Putative Transmembrane Domain (PTMD) (47,71,78,81,84) (Figure 1.6). In the presence of a strong reducing agent, such as 5mM DDT, this conformational change can be completely reversed, with the soluble dimer shifting back to its monomeric state (84).

The physiological significance of oxidation and CLIC1 dimer formation is still not fully understood. One possible function is to regulate the transition between soluble and membrane form, where the half-dimeric state (oxidised monomer) represents the membrane docking form of the protein (84). However there is no structural evidence of CLIC1 membrane binding intermediates that are involved in the formation of membrane docking state (84,88,89). This will only be revealed, once the structure of the membrane form of the protein is solved.

Further studies have suggested certain structural features of the CLIC proteins are involved in ion channel formation. Cys24 in CLIC1 was proposed to be a critical pore forming residue in the protein, as it was essential for ion channel formation in artificial bilayer membranes (47,81). This was supported by observations that alanine mutants of Cys24 in CLIC1 (C24A) had the ability to insert into membranes, but failed to form conductive ion channels. In addition, the CLIC1-C24A mutant showed no redox sensitivity, further suggesting Cys24 is essential for the functional ion channel form of CLIC1 (47,81,84).

Even though oxidation of CLIC1 was thought to be essential for the formation of functional chloride ion channels, the related nematode protein EXC-4 formed functional ion channels in artificial lipid bilayers in a non-redox dependent process. This was likely possible due to the lack of an equivalent critical cysteine residue in the EXC-4 protein. It has, instead an aspartic acid residue that is not redox regulated (Figure 1.7) (35,38). Therefore this would suggest that redox control may not be the only mechanism underpinning the structural transitions of the CLIC proteins although such alternative mechanisms have yet to be proposed.

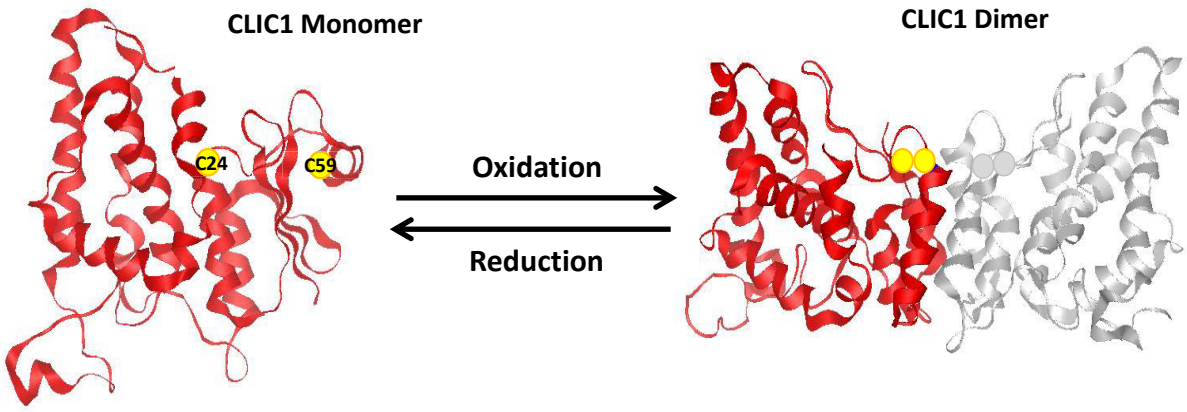


Figure 1.5: Structural rearrangement of CLIC1. Under oxidative conditions, Cys24 forms a disulphide bond with Cys59 stabilising CLIC1 dimer form. This process is fully reversible under reducing condition, in which the CLIC1 dimer shifts back to a monomeric state. Structures were produced using DNA Star.

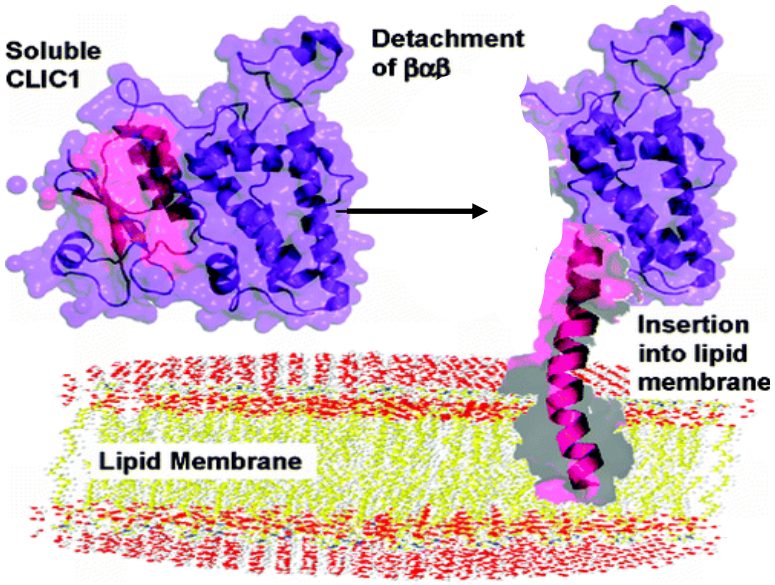


Figure 1.6: Proposed membrane-docking structure of CLIC1. Conversion of soluble CLIC1 into its membrane bound form causes the disappearance or transformation of theβ strands and α-helix1 (highlighted in *pink and purple* in soluble CLIC1 crystal structure) and the extension of α-helix1 segment towards the C-terminus creating PTMD anchoring region (highlighted in *pink*) which is predicted to insert into the membrane. Modified figure was taken from (90).

```
CLIC1  IELFVKAGSDGESIGNCPFSQRLFM 44
CLIC2  IELFVKAGIDGESIGNCPFSQRLFM 41
CLIC3  ITLFVKAGYDGESIGNCPFSQRLFM 200
CLIC4  IELFVKAGSDGESIGNCPFCQRLFM 39
CLIC5  VELFVKAGSDGAKIGNCPFSQRLFM 33
CLIC6  LQLFVKASEDGESVGHCPSCQRLFM 32
EXC-4  LELYVKASGDARRIGADLFCQEFWM 43
DmCLIC MCVDQASTIDGRRKGACLFCQEYFM 58
```

Figure 1.7: Amino acid sequence alignment of human CLIC proteins and EXC-4 from the nematode, *Caenorhabditis elegans*. The aligned protein sequences show the conservation of the cysteine active site residue of human CLICs (*bold blue*), *Dm*CLIC (highlighted in green) and the active site of EXC-4 with an aspartic acid residue (*highlighted in yellow*). Alignment was achieved using Clustal W of EXC-4 (accession number AAQ75554.1), *Dm*CLIC (accession number (AGB95379.1) and human CLICs (accession numbers as in figure 1.3).

1.4.2 CLICs are Spontaneous Membrane Inserting Proteins

Chloride ion channel activity is enhanced at low pH levels for a number of CLIC proteins (8,49). Observations suggest that channel activity was highest at pH 5 and was found to decrease at pH 6 and further diminish at pH 7 (8,36,37,40,49,71,90). This characteristic of spontaneous membrane insertion of CLIC proteins make them similar to other human proteins that self-assemble into lipid membranes in response to specific environmental conditions. One such group is the annexin family of proteins that spontaneously bind to membranes as a result of changes in the calcium concentration in cells (91). Also annexin B12 was found to reversibly insert into membranes in response to low pH levels (19).

Pore-forming proteins or toxins (PFTs) that were found to undergo structural transformations from a soluble monomeric form into a membrane embedded pore within a lipid bilayer membrane were found to be controlled by specific environmental conditions that aid their transitions and insertion into cellular membranes. An example of a PFT is colicin-A from *E-coli,* which exists as a monomer in solution with the ability to bind to a receptor on a cell membrane in order to oligomerise and form an ion-conducting pore. The process of toxin insertion into lipid bilayers is achieved by the formation of a hydrophobic helical hairpin which is fusogenic and triggers entry into the membrane. Following fusion with the membrane the monomers aggregate to form a transmembrane pore (92). A decrease in pH levels was found to induce insertion of colicin-A toxin into membranes and it was proposed that low pH disrupts the hydrogen bonding network involving three aspartate residues causing an unfolding of the toxin secondary structure catalysing its fusogenic properties (93,94).

Diphtheria toxin produced by *Corynebacterium diphtheria* (C. *diphtheria*) is another example of PFTs. It contains two negatively charged regions and in particular six acidic residues on the loops located on one face of a helical barrel (95,96). It was found to form pores in endosomal membranes where the insertion of a hydrophobic hairpin into the membrane is followed by the insertion of the remaining pore forming helices. The acidification of the endosomes was found to be essential for insertion of diphtheria toxin as the low pH triggers it's unfolding of toxin by neutralising the acidic loops and exposing the hydrophobic surfaces of the protein and leading to a membrane pore (90,97,98). This process is the same as that of Exotoxin-A from *Pseudomonas aeruginosa*; in which low pH was found to neutralize the charge of an aspartate residue which in turn triggers the unfolding of protein and the exposure of a tryptophan residue which in turn leads to the formation of membrane pores (81,90,99). The pore-forming toxins are a large family of proteins that include anthrax, α-Hemolysin and many others that all possess the common feature of spontaneous membrane insertion. In all cases the insertion processes is triggered by low pH (87,94,99,100).

There are no significant sequence similarities between the different classes of PFTs however; the C-terminal domain of CLIC proteins bears some resemblance to a PFT as it consists of alpha helical bundles with long hydrophobic helix (h6) and two negatively charged regions at the base of CLIC1 and acidic alpha helix (α-h9) which makes CLIC1 proteins structurally similar to the diphtheria toxin (37,81). It is currently proposed that the protonation of these highly negatively charged residues by low pH may lead to increase their hydrophobicity which then leads to protein unfolding and insertion into the membrane (78). Also the acidic environment was thought to enhance the aggregation of proteins and increase their interaction and insertion of the N-terminal domain into the cell membranes (8,49). A further proposal is that the decreased pH

promotes membrane insertion by neutralizing the more acidic residues of the protein in order to increase their hydrophobicity (as occurs with diphtheria and botulinum toxins) (8,49,87,96,101,102).

To date, the physiological significance of the link between the channel activity or the CLIC proteins' insertion and increased proton concentrations at low pH is still not clear. It was hypothesised by Littler et al, 2010 (78) that CLIC channel activity can be linked with the vesicle acidification by a proton pump or that the chloride ion channels formed by the CLIC proteins can be linked with conditions within cytoplasmic vesicles such as the endosomes (78,103).

1.5 Aims of This Research Project

Elucidate Mechanisms Controlling the Translocation between the Soluble and Membrane Forms of the Protein, CLIC1

Electrophysiological studies have confirmed that purified recombinant CLIC1 forms ion channels in artificial lipid bilayers, with its channel properties showing similar characteristics to similar channels observed in native cell membranes. CLIC1 also demonstrates properties similar to the spontaneously membrane inserting and pore-forming proteins that require specific triggers to induce their structural transitions, for membrane insertion. Redox control, pH and lipid composition have been found to be factors that trigger the structural conformation of the soluble CLIC1 protein to insert into membranes. However the question still remains un-answered regarding the physiological triggers and mechanisms that are involved in regulating its structural

conformation; membrane insertion; oligomerisation and arrangement within the membrane. Therefore, this project also aims to:

- Establish a robust methodology using tethered bilayer lipid membranes and impedance spectroscopy, in order to study the conductance properties and function of membrane inserting proteins or compounds to elucidate the factors regulating these activities. Chapter 2 of this thesis covers the findings of this aim.

- Investigate the conditions that control the insertion of CLIC1 into lipid bilayer membranes, including the effects of sterols, redox and specifically the interactions of CLIC1 with different lipid compositions and types of biomimetic cell membranes. As will be covered in Chapter 3.

These studies will be undertaken using a tethered bilayer lipid membrane (tBLM) system and impedance spectroscopy measurements (104) using purified recombinant CLIC1 (wild type protein and mutant forms). The tBLM and impedance spectroscopy system will provide direct information on the insertion and functional activity of the membrane form of CLIC1, and will allow a systematic characterization of factors regulating the membrane channel activity and membrane insertion of CLIC1, along with critical structural features involved in these processes.

Establish whether CLIC Proteins Possess Oxidoreductase Enzymatic Activity

As it will be covered in Chapter 4, members of the CLIC protein family exist in two stable forms under differing redox conditions, and they also localise to distinct cellular regions, as soluble and as membrane-associated forms. According to structural analysis of the CLIC proteins it was found that they adopt the same canonical fold as the glutathione-S-transferase (GSTs) and glutaredoxins (Grxs) by having a redox active-site, which is conserved amongst all vertebrate CLIC proteins. For this reason it has been hypothesised that CLIC proteins may also have intrinsic oxidoreductase enzymatic activity. However no empirical evidence of catalytic activity has been presented to date.

Therefore, this research project aims to:

- Determine whether members of the CLIC protein family have intrinsic oxidoreductase enzymatic activity typical of the GST and glutaredoxin families.

  This will be undertaken by using purified recombinant CLIC protein members assessed using standard GST and Glutaredoxin enzyme assay systems.

- Identify the main structural residues that are involved in any enzymatic function demonstrated by the CLIC proteins, in particular the critical active site Cysteine residue and associated residues within the GST conserved GSH binding site motif.

  This will be undertaken by using site directed mutagenesis studies of CLIC proteins.

- Determine potential substrates for any demonstrated CLIC protein enzymatic activity.

Selected substrates will be assessed dependent upon the results obtained from the former 2 studies.

Chapter 2

Tethered Bilayer Lipid Membranes and Impedance Spectroscopy to Characterise Functions of Membrane Inserting Drugs and Proteins

2.1 Membrane Lipids and Sterols Affect the Function and Activity of Membrane Proteins

Cell plasma and intracellular membranes contain a large variety of proteins (105,106). This is not surprising given membrane proteins are considered to be the most active components within membranes and hence play key roles in regulating cellular functions (105). Cell bilayer membranes also contain sterols and a great diversity of lipids as exemplified by the plasma membranes of red blood cells, which alone contains over 200 different lipids (107,108). These lipids differ in type and size of head groups, length of the acyl chains and also degree of saturation (109,110)

In order for membrane proteins to function normally within cells, they are required to interact physically or molecularly with the bilayer lipid membrane components (110). Previous studies have shown that the zwitterionic phospholipid, phosphatidylcholine (PC) makes up more than 50% of the lipids in the plasma membranes of most vertebral cells (111,112). An important steric or morphological property of PC lipids is that they are cylindrical in shape where the cross sectional area of the head group is similar to the cross sectional area of the acyl chains (112,113). This morphological property of the PC lipids has allowed them to spontaneously assemble to form lipid bilayers with a curvature near zero (113,114) and at their optimal transition temperatures they give raise to the liquid crystalline lamellar phase of biological membranes (112,113,115). Membranes are also known to contain non-bilayer forming phospholipids (113,116), where the cross sectional area of the head group and the acyl chains of such lipids are very different (they have a conical shape rather than cylindrical shape) causing the formation of aggregates, as in the case of detergent molecules that form micellar structures, resulting in positive curvature (116,117) in membranes.

Phosphatidylethanolamine (PE) lipids whose structure contains smaller head groups, are examples of such non-bilayer forming lipids that were found to give raise to conical shapes as in the inverted hexagonal phases (111,116,117) and cause negative curvatures in membranes (117). The curvature stress of the bilayer lipid membranes and the presence of non-bilayer forming lipids were in turn, found to affect the folding and conformational changes of peripheral and integral membrane proteins and their binding affinity to bilayer lipid membranes (118). Non-bilayer forming lipids modulate the flexibility and the general properties of bilayer lipid membranes (110,117). Previous reports have proposed a correlation between membrane curvature and the function of some membrane proteins (119-121) and it has been shown that the function of some membrane proteins is highly influenced by the presence of non-bilayer forming lipids as indicated by the increased activity of protein kinase C (122,123), integral membrane protein Rhodopsin (124) and calcium activated potassium channels (125).

The sterols in cell membranes are also found to affect function and structural conformation of membrane proteins (2). Vertebrate cell bilayer lipid membranes contain cholesterol, which is a small molecule with 27 carbon atoms containing a rigid steroid ring with a hydroxyl group that gives the molecule an amphiphilic nature (Figure 2.1). Cholesterol also contains a short hydrocarbon tail that intrudes into the membrane, while the hydroxyl group will be positioned at the interfacial region of the bilayer membranes (3,110,126,127). Fungal and yeast membranes on the other hand, contain ergosterol which is similar to cholesterol however it contains an additional double bond in its B ring structure as shown in Figure 2.1. Plant cell membranes predominantly contain sitosterol as their predominant membrane sterol (2).

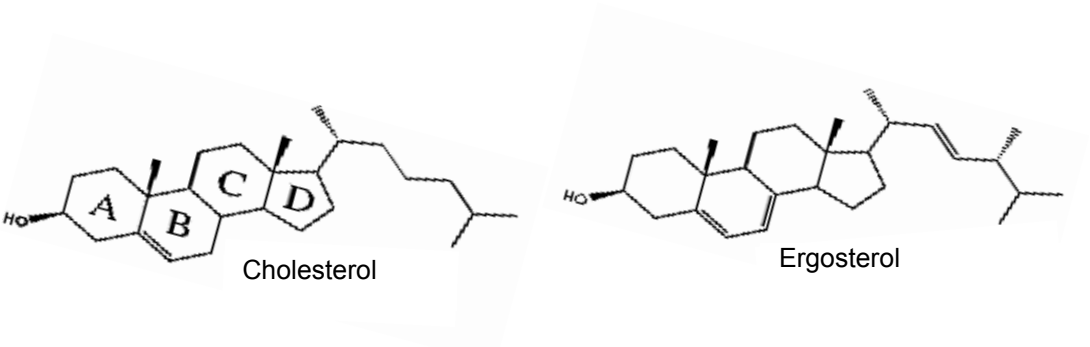


Figure 2.1: Structures of cholesterol and ergosterol. Both sterols contain 4 hydrocarbon rings with one hydroxyl (OH) group. Ergosterol contains one additional carbon, the hydrogen in the side chain was replaced with methyl group and it also contains 2 additional double bonds instead of one as in cholesterol. Structures taken from (2).

The high content ratio of sterols to phospholipids in biological membranes, (~25-50 mol %) suggest they play an important function in cells in which they are found (110,128-131). Various studies have indicated that membrane sterols are involved in regulating the bilayer fluidity and stiffness (132,133); bilayer deformation energy and thickness (107,134); bilayer free volume (135) and lateral pressure profile of membranes (136). They have also been cited as being involved in protective roles, in the regulation of membrane properties and dynamics (2,137). Membrane sterols and in particular cholesterol were found to contribute to the formation of specific membrane structures such as caveolae which are lipid rafts containing caveolin integral membrane proteins that are involved in receptor-independent endocytosis (138). They have also been identified as receptors for proteins/toxins to bind and insert into membrane bilayers in order to form pores or channels (110,139-141). The polyene antibiotics Amphotericin B produced from a strain of *Streptomyces nodosus* and Nystatin A from *Streptomyces noursei* were found to mediate changes in fungal cell membranes by interacting directly with the ergosterol component of membranes in order to form antibiotic-sterol complexes that are barrel-like membrane spanning channels that cause leakage of ions and cell molecules resulting in fungal cell destruction and death (2,142,143). The cholesterol dependent-cytolysins (CDCs) are a large family of pore-forming proteins

produced from at least 20 species from Gram positive bacteria such as *Clostridium, Streptococcus, Listeria bacillus* and *Arcanobacterium,* and include listeriolysin, perfringolysin, streptolysin and pneumolysin (96,144) that were all found to exist as soluble monomers with the ability to undergo structural transformations in order to insert into membranes (96). CDCs are commonly thought to interact with membranes via a two-step process, the first involves binding to cholesterol within the membrane followed by insertion into and oligomerisation resulting in final pore structure typically 20-30nm diameter (96,101).

In this Chapter we describe the establishment of robust method allowing application of tethered bilayer lipid membrane and impedance spectroscopy technology in the study of membrane protein regulation and effects of membrane lipid composition on their activity. We have chosen to include in the study two well-described spontaneously membrane inserting proteins (α-Hemolysin, Listeriolysin-O) in addition to CLIC1. We also have included Amphotericin B and Nystatin A, as other membrane disrupting agents.

2.2 Membrane Models: Tethered Bilayer Lipid Membrane and Impedance Spectroscopy System

Understanding protein-membrane interactions and the study of ion channel function in lipid bilayer membranes is central to our understanding most aspects of sensing, signalling and metabolism in plants and animals (145). Membrane proteins are known to play essential roles in cellular functions and metabolism such as cell-cell communication, signal transductions processes, transport of nutrients, metabolites and ions across the cell membranes (145). Due to their fundamentally important roles, they have become prime targets for medical applications and pharmaceutical therapeutics (4,146,147). Hence the importance of studying and understanding, transmembrane proteins with a particular focus on ion channel proteins.

Monitoring and investigating ion channel functions can be achieved by different methods that include electrophysiological techniques such as whole cell patch-clamp, liposome transport and black lipid membranes (BLMs) (148). However not all ion channels and membrane proteins are accessible to the extracellular patch electrodes because they are located on intracellular membranes (148). Furthermore techniques such as BLM and patch clamp are unstable and require significant skill to perform successfully. Liposomal release techniques are poorly defined and difficult to perform in order to obtain quantitative data. Therefore the establishment of artificial membrane models with defined lipid compositions, into which purified membrane proteins can be incorporated, is becoming increasingly important in order to improve the medical and biophysical characterization of integral membrane proteins and ion channels (145,149).

In isolated lipid vesicles or unilamellar liposomes, the lipids form an enclosed volume which can make it experimentally difficult to control the concentration of solutes inside

the inner compartments (146,150). The application of a transmembrane potential is also limited to the application of osmotic gradients and the facilitated diffusion of ions using ions-specific ionophores (146,150). This severely limits the precision with which measures of ion permeation may be obtained from studies using liposomes (146). Artificial planar phospholipid bilayer membrane models including, black lipid membranes (BLMs), and supported lipid bilayers (SLBs) (146,147) provide some advantages over studies using liposomes.

The BLMs or painted solvent base bilayers (151) have been found to act as one of the more useful mimics in the study of the physiological functions of membrane proteins. They also provide access to solutions on both sides of a lipid bilayer which makes them suitable for many electrophysiological studies of membranes involving transmembrane potentials (3,146,148,150,151). In BLMs (as demonstrated in Figure 2.2), a film-like membrane is formed by painting a 1-2% solutions of phospholipids dissolved in an organic solvent, containing a mixture of n-decane or squalene over an opening, typically 100μm to 1.0mm in diameter, assembled on hydrophobic materials such as Teflon, polycarbonate, polystyrene or styrene co-polymers. The film is painted under water across the hole separating the two compartments filled with aqueous solutions (3,146). Following the application of the lipid-solvent solution across the opening, the less dense solvent rises to the top of the opening causing the remaining lipid and a small quantity of solvent to collapse and thus form a molecular film far thinner than the wavelength of light. Because the film is far less thick than the wavelength of visible white light, the light reflected from the front and rear of the film cancel each other, causing the film to appear as black on the reflected light, hence the name black lipid membranes. A significant annulus of solvent forms between the bilayer and the sheet or material in which the aperture is made (152). The annulus and residual solvent are believed to be

necessary for preventing immediate rupture, extending the lifetime and stability of the membranes, however, the solvent, decane also causes a doubling in the thickness of the bilayer, seriously interfering with the interpretation of ion channel transport properties of the added proteins (152,153).

The incorporation of ion channels into BLMs can be monitored or observed by measuring the current flow through the membranes (3). When ion channels are inserted into phospholipid bilayers, the membrane current increases (3). The main limitations of the BLMs include: limited stability and lifetime; they can be easily ruptured when subjected to high flow rates or vibration and they are very sensitive to mechanical damage which will catastrophically rupture the membrane (3,110,150,154,155).

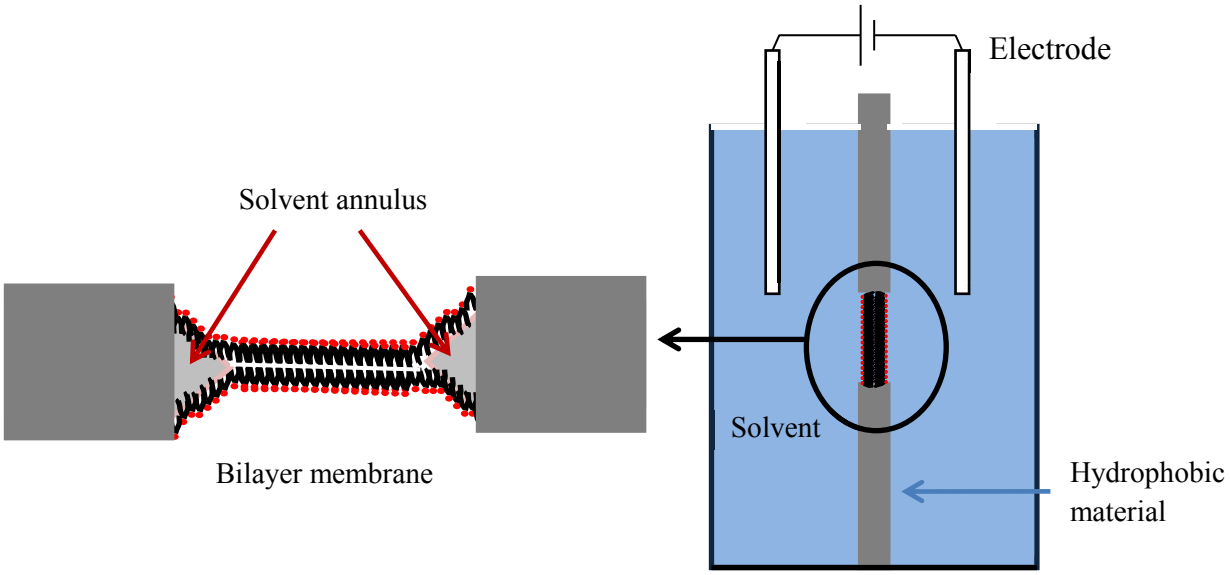


Figure 2.2: A Schematic of Black Lipid Membrane (BLM) System. Bilayer membranes form by painting a phospholipid solution containing hexane like solvent such as n-decane or squalene over a small aperture made in Teflon material separating two champers filled with aqueous solutions containing electrodes in order to test the electrical properties of the bilayer membrane. A significant annulus forms as a result of residual organic solvent that remains at the perimeter which is required to maintain stability of the membrane. This modified Figure was taken from (152).

To alleviate the problems and instability associated with BLMs, the supported lipid bilayers (SLBs) were established, which are as a planar lipid bilayer membrane floating on a solid support. This format has over the years been employed in many sensor applications (3,156,157). The formation of a solid supported lipid bilayer membrane can be achieved by a number of different methods including: Langmuir-Blodgett or Schafer technique (158); the adsorption and fusion of vesicles (159,160); the formation of liquid surfactant micelles (161) and the use of the "solvent exchange technique" (96,162,163) which in particular, permits the use of a particularly wide range of lipid compositions in forming the membrane with minimal residual solvent being retained in the resultant bilayer. In all these methods, a membrane of ~4nm thickness is formed by the constituent phospholipids forming a bilayer in which the inner single lipid leaflet orients towards the solid substrate that can be for example, glass, silicon oxide or mica (3,158). In SLBs, the hydrophobic acyl chains of the inner phospholipid layer interacts with the second leaflet of lipids which orient with their hydrophilic heads directed to the bulk aqueous solution and their hydrocarbon chains to the acyl groups of the inner leaflet. This geometry results in a stable bilayer membrane exposed to the bulk bathing electrolyte over the membrane permitting the addition of proteins and nanoparticle samples (3,156,164) (Figure 2.3).

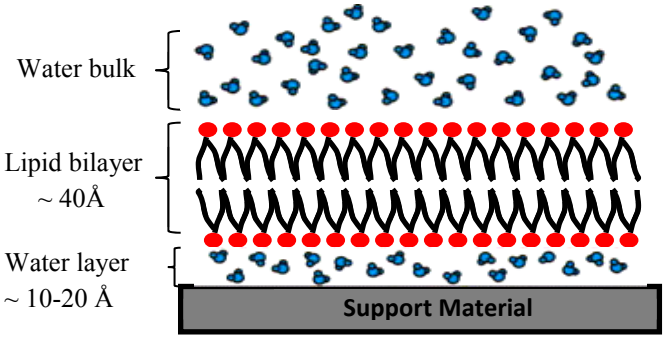


Figure 2.3: Representation of Supported Lipid Bilayer (SLB) Membrane. A bilayer membrane formed on a solid substrate. A water layer separates the inner phospholipid monolayer from the substrate. Also water is excluded from the hydrophobic group of the inner phospholipid leaflet and those out the outer phospholipid leaflet, forming a bilayer membrane that is exposed at its outer surface to the bulk solution. Modified figure was taken from (3).

In SLBs, the hydrophilic head group of the phospholipids will be facing the substrate and will be separated from the support material by an ~1nm thick layer of water (3). However, at such close spacing the inner layer phospholipids are seriously constrained in their diffusion and the geometry of the inner lipid leaflet is substantially altered from that found in a free unsupported bilayer. Also the small volume between the membrane and the supporting substrate limits the diffusion of ions laterally in this space and makes this geometry unsuitable for the measurement of ion fluxes across the membrane resulting from the inclusion of ion channels. Monitoring the formation of these membranes can be achieved by many methods that include quartz crystal microbalance (QCM), surface plasmon resonance (SPR) and atomic force microscopy (AFM) (160)

To achieve a mimetic system that reflects the native biological function of proteins and ion channels in membranes, it is highly desirable to form membranes with a more complex lipid mix containing neutral, charged and ionisable lipids. However, successful SLB membrane formation employing the vesicle fusion technique was reported to be highly dependent on the lipid composition. Even small amounts of cholesterol incorporated into lipid vesicles was found to prevent vesicle fusion and subsequent formation of SLB membranes (165,166). An additional limitation of SLBs is the lack of a well-defined ionic reservoir on the inner side of the membrane and in particular the small gap between the bilayer and the substrate (3,5,149,160,167). Some trans-membrane proteins may extend beyond the inner face of the bilayer, resulting in steric interference with the substrate, leading to protein denaturation and loss of protein functionality (158,164).

Tethered bilayer lipid membranes (tBLMs) were proposed as an improved artificial biomimetic structure to provide a sub-membrane space serving as both an ionic reservoir and to provide space for the incorporation of proteins that project beyond both

surfaces of the membrane. These improved characteristics address many of the problems encountered by SLB membranes (96,101,144,145).

In the tBLM system as in Figure 2.4, a self-assembled monolayer (SAM) of tethering lipids is employed by using terminal disulfide groups as part of the covalent lipid structure to chemically attach the lipids to a gold substrate. Unlike the SLB structure however, the tBLMs incorporate a hydrophilic spacer between the chemical anchoring group and a hydrophobic group that tethers a subsequently formed lipid bilayer. This hydrophilic linker creates both an ionic reservoir between the gold electrode and the bilayer membrane and also obliges the lipid bilayer to form at a greater distance from the gold substrate. At the distal end of these molecules, away from the gold electrode, they have hydrophobic phytanyl groups covalently attached that act to recruit a subsequently formed lipid bilayer (5,96,101,144,145,149,168). Additional reservoir lipids are included as spacers between the tethering molecules. These possess the same benzyl disulphide group that attach the tethering molecules to the gold, however they are far shorter, possessing typically only four oxygen ethylene glycol groups and are terminated with an hydroxyl. They perform two functions, the first being to prevent the tethering phytanyls from becoming adsorbed to the bare gold at the time of deposition, and also to provide a space between the tethering phytanyls groups into which other proteins or peptides may be incorporated. The formation of the actual tethered membrane occurs after the tether/spacer groups have been coated onto the gold-coated slide.

The bilayer forming lipids (mobile lipid phase or MLP) that are dissolved in ethanol are subsequently incubated for 1-2 minutes in direct contact with the preformed tethering monolayer. Following this incubation period the self-assembly of the bilayer lipid membrane is achieved by rinsing with buffer, which drives the process through the

hydrophobic/hydrophilic interactions. Further rinsing with an aqueous buffer solution removes residual solvent leaving a fully formed tethered lipid bilayer structure (5,144,149).

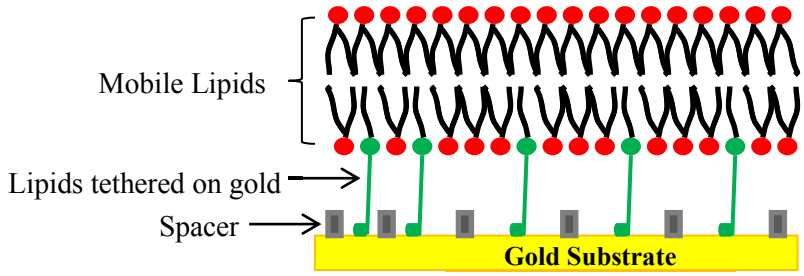


Figure 2.4: A Schematic representation of tethered bilayer lipid membrane (tBLM). Membrane forms by adding mobile lipids onto a monolayer of lipids tethered on a gold substrate that also make the ionic reservoir between the gold and the bilayer membrane. The spacers are hydrophilic disulfide containing molecules that control the lateral spaces between the lipid reservoirs in membranes. Modified Figure was taken from (5).

In a tBLM the inner leaflet of membrane is facing the inner cavity that contains constrained volumes of electrolytes that have different physical properties to the bathing solution that surrounds the outer leaflet of the membranes (169-171). Control over the structural properties of these membranes is possible by varying the length of the hydrophilic reservoir linkers, or of the hydrophilic groups of the spacer molecules between the tethers and in the length of the phytanyl tethers. It is also possible to use tether groups that traverse the entire membrane or only penetrate the inner leaflet.

Care must be taken when measuring transmembrane currents of ions through channels incorporated into tBLMs, that the potential gradient and duration of an applied voltage does not drive a current that exceeds the charge available within the restricted reservoir space. Conditions under which such distortions occur are well outside the operating conditions employed here. Also the tether densities employed in the tBLMs reported here, of between 1-10% and the polyelectrolyte content of the reservoir space is not

very different to that found in natural cell systems such as the spectrin-ankyrin-band 3 network in red blood cells (172).

A useful tool in describing the membrane lipid distribution and reporting the biological events that occur across tBLMs such as ion channel activity and bonding events is Electrical Impedance Spectroscopy (EIS). By applying a small alternating current or potential (20-25mV) across a tBLM system and sweeping the excitation frequency from typically 1000Hz to 0.1Hz an impedance spectrum is generated that may be modelled to yield such measures as the average membrane conductance and capacitance (5,144-147,149,173). At high frequencies the series capacitance of the membrane capacitance and the Cs capacitance can be calculated in order to determine the membrane thickness as in the equation below (2.1):

$$\mathbf{Z} = \frac{\mathbf{1}}{\mathbf{2\pi fC}} \qquad (4,5)$$

Where Z is the impedance in ohms, f is the frequency in Hz and C series capacitances of Cm and Cdl in f which is the frequency.

The electrical equivalent circuit of a tBLM is given with reasonable precision by the circuit shown in Figure 2.5 (147). The conductance arising from the insertion of an ion channel is modelled by the resistance (Rm) an the bilayer membrane capacitance is modelled by Cm, the capacitance at the supporting gold surface in series with the counter electrode capacitance Cs and the series resistance of the bathing ionic buffer solution by Rs. As proteins insert into membranes to form active ion channels, they cause the ions to flow across the membrane causing the Rm values to decrease (5,145,149). Thus changes in Rm values can be used to characterise the activity of an ion channel in a tBLM.

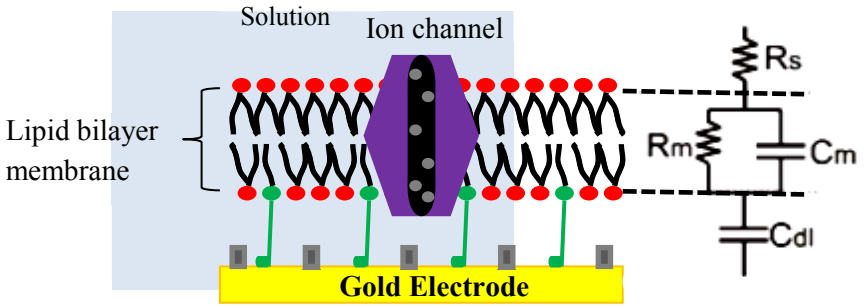


Figure 2.5: Representation of tBLM and impedance spectroscopy as a circuit-like model. Ions movement causes electricity flow across the membranes and Rs represents the resistance to ion movements in the outside solution of membrane. Cm is the capacitance between the outside solution and the solution below the membrane which is created by the membrane itself that acts as an insulator in the system. Rm is the resistance across the bilayer membranes due to membrane proteins. C_{dl} or Cs is the effective series capacitance representing the build-up of charge created by ions on the surface of the gold electrode. Figure was taken from (147).

Chapter 2

Materials and Methods

2. 3 Chemicals

All the chemicals used to perform the experiments in this research project are of high analytical grades. The water was purified by reverse osmosis and ion exchange or organic filtration (MilliQ water). Antibiotics used were sterilised by passing them in solution through sterile filters (0.2μM in diameter) and were stored in -20ºC until needed.

2.4 2xYT Media for Bacterial Growth

2xYT media for growing *E-coli* cultures is comprised of 15g of yeast extract, 24g of bacteriological tryptone and 7.5g of NaCl, dissolved in a final volume of 2 Litres of sterile deionised water and autoclaved for 40 minutes at 1.5kg f/cm^2 at 122°C.

2.5 CLIC1-Transformed Bacterial Glycerol Stocks

Glycerol stocks of *E-coli* bacteria containing BL21 (DE3) with the His-tagged pET28a vector (Novagen) to express CLIC1 WT were prepared by picking a single colony of transformed bacteria from an agar streak plate using a sterile pipette tip. The bacterial cells are then grown overnight in 5mL of 2xYT medium containing 50μg/mL kanamycin antibiotic (Sigma Aldrich). This culture then was reseeded in fresh 2xYT media and grown at 37ºC, 200 rpm shaking until mid-exponential phase (OD 600= 0.6). 60% (v/v) final concentration of sterile glycerol was added, and cells were aliquoted into sterile 2 ml cryo-tubes, placed on dry ice and stored long-term in a -80ºC freezer.

2.6 Preparation of Recombinant Monomeric CLIC1 Protein

2.6.1 Small Scale Cultures

The glycerol stock (prepared as described above) was inoculated in 100mLs of 2xYT media (1g yeast extract, 1.6g tryptone and 0.5g NaCl; pH 7.0) containing 50µg/mL kanamycin (Sigma Aldrich) and left to grow overnight at 37°C, 200rpm.

2.6.2 Large Scale Culture and Induction of Protein Expression

The overnight small scale cultures were added to 1.5L of 2xYT medium containing 30mg/mL kanamycin antibiotic (Sigma Aldrich) and left to grow with shaking at 37°C, 200rpm for about 1.5 hours until an OD of 600 is reached. The bacterial cultures were then induced with 1mM IPTG, returned to shaking incubator and left to grow overnight (no longer than 16 hours) at 20°C, 200rpm.

2.6.3 Harvesting *E-coli* CLIC1-transformed Bacterial Cells

The IPTG induced cells were centrifuged using Sigma 3-18K, rotor 11133/13104, 5500 rpm, 15 minutes at 6°C and the resultant pellets were scraped into a falcon tube and resuspended in approximately 30mL of His-tag binding buffer (300mM NaCl, 50mM sodium phosphate buffer pH 8.0 and 10mM Imidazole).

2.6.4 Lysing of *E-coli* Cells

Ice cold resuspended *E-coli* cells were homogenised using a French Press Homogeniser, by at least 3 passes or until a less viscous solution to be obtained at 800 psi. 1% of TritonX-100 was added to every 100mL of cell lysates that were then incubated in ice for 20 minutes. Cell lysate was then centrifuged using Sigma 3-18K, rotor 12156, 13800rpm for 20 minutes at 4°C in order to remove cells debris. The soluble fraction

supernatant was then decanted carefully and kept on ice to be used in the subsequent purification steps.

2.7 Purification of Monomeric CLIC1 Protein

2.7.1 His-tagged Protein Purification using Ni^{2+}-NTA Resin

2.5mL of Ni^{2+}-NTA resin slurry (Qiagen) was used for each 1L of induced cells. The Ni-NTA beads were washed and equilibrated with at least 75mL of the His-tag binding buffer (300mM NaCl, 50mM sodium phosphate buffer pH 8.0, 1mM DTT or 0.5mM TCEP and 10mM Imidazole) followed by incubating the supernatant with resin beads for 1 hour at +4°C on a rotating shaker in order to allow the His tagged proteins to bind onto the nickel beads. The protein- resin slurry was then packed into disposable chromatography columns volume of 25mL (Biorad). Unbound proteins were washed through by gravity flow by washing the resin with at least 150mL of the His-tag binding buffer until the clear blue colour of the beads retained. The resin bound proteins were then incubated overnight at +4°C with bovine plasma thrombin enzyme (Sigma Aldrich) [30 NIH units per 1L of original bacterial cell culture] resulting in cleavage of the His-tag from the recombinant fusion protein. The cleaved proteins were then eluted as 5 x 2.5 mL fractions with PBS buffer (10mM phosphate buffer, 2.7mM KCl, 140mM NaCl, pH 7.4) containing 0.05% Tween20, 1mM DTT or 0.5mM TCEP.

2.8 Size Exclusion Chromatography (SEC)

In order to achieve high purity reduced recombinant CLIC proteins, the pooled fractions eluted from the affinity Ni-NTA were injected onto a Superdex75 high performance chromatography column (GE Healthcare, Piscatawy, USA) at +4ºC and the resultant largest single peak corresponding to monomeric CLIC proteins (Figure 2.1 and 2.2). The eluted protein from the size exclusion chromatography column was stored in column sizing buffer (100mM KCl, 1mM NaN_3, and 20mM HEPES pH 7.5; containing 1mM DTT or 0.5mM TCEP in order to keep the protein in its reduced monomeric form).

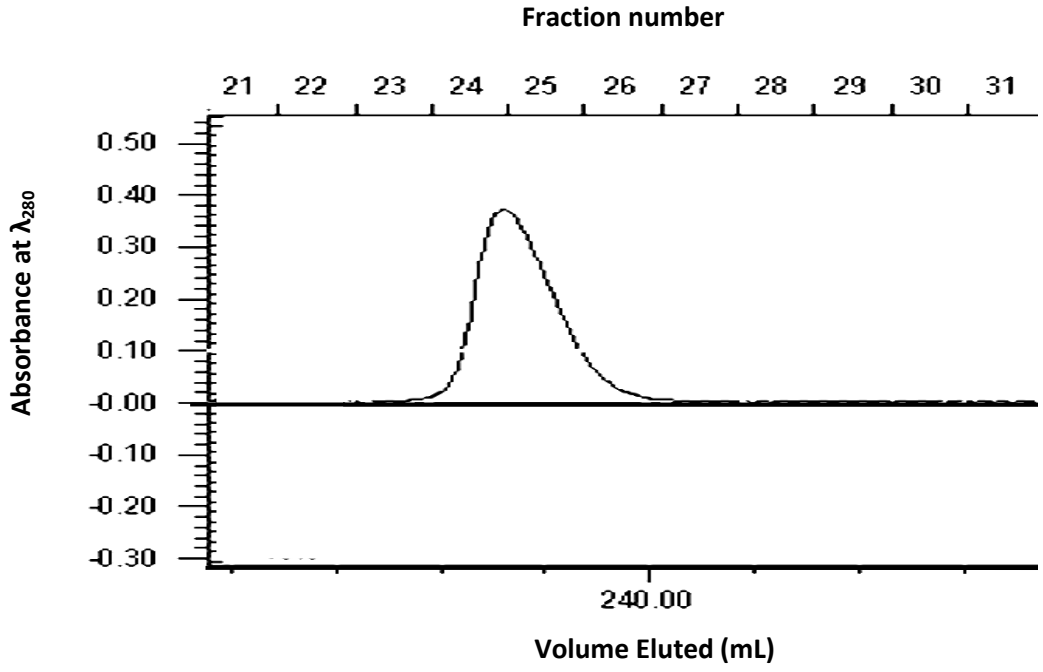


Figure 2.6: Representative profile of the eluted monomeric CLIC1 (WT) protein purified by Size Exclusion Chromatography (SEC). At an absorbance of 280nm CLIC1 WT monomeric protein eluted in fractions 23 to 27 as indicated by the single peak profile of the SEC recording.

2.9 Protein Quantification

2.9.1 UV-Vis Spectrophotometer

Nanodrop with UV-Vis spectrophotometer (Thermo Scientific) or a Varian Cary machines were used as quick methods to determine the protein concentration of the eluted fractions from the high affinity chromatography columns (pooled fractions) and before injecting the proteins into the size exclusion chromatography for final purification. Protein concentration was determined at 280nm wavelength which corresponds to the electronic transition of the tryptophan residue as an aromatic amino acid. The Nanodrop and Varian Cary spectrophotometer were blanked against the protein diluent buffer (1x PBS buffer containing 1mM DTT or 0.5mM TCEP, pH 7.4), then 1.5µL of protein/fraction (using the Nanodrop) or 1mL of protein solution in a quartz cuvette (when using the Varian Cary UV-Vis) were assessed. The concentration of protein was calculated using the Beer-Lambert law according to the equation below (2.2):

$$c = \frac{\lambda_{280}}{\varepsilon_{280 \times l}} \qquad (1)$$

Where c is the protein concentration in mg/mL, λ_{280} is the absorbance of the proteins at 280nm wavelength; ε is the extension coefficient 0.6 cm-1 .mg-1. mL and l is the path length in (cm).

2.9.2 BCA Protein Assay

The Bicinchoninic acid assay is a colorimetric method for the detection and quantification of total protein concentration in a solution. The detection of cuprous cation (Cu^{+1} which is the reduced form of Cu^{+2}) occurs in an alkaline environment using

a reagent with bicinchoninic acid (BCA) and as a result of this reaction a purple coloured water-soluble complex forms due to chelating two molecules of BCA with one cuprous ion which can be detected at an absorbance of 562nm. The structural components of the proteins that will cause the colour change in the BCA assay include the number of peptide bonds as well as the presence of the four amino acid residues (cysteine, cysteine, tryptophan and tyrosine). The assay was performed according to the manufacturer's instructions (Thermo Scientific), and the concentration of protein was determined by generating a standard curve of a series of dilutions of known protein concentrations (BSA, bovine serum albumin) against which the absorbance of the unknown (protein solution whom concentration to be determined) was compared at 280nm using Varian Cary UV-Vis spectrophotometer.

2.10 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is an effective method to determine the purity and oligomeric state of purified proteins. In this method, SDS, which is anionic detergent, provides a negative charge to the proteins dissolved in the detergent solution. The SDS also acts to unfold the protein structure, after which an electric field applied that forces the proteins to migrate through a gel matrix towards a positively charged electrode in order for the proteins to be separated according to their molecular weight. According to the manufacturer's instructions, polymerisation of gel was achieved by using 10% gel of NEXT-Gels^{TM} and (Amresco, Astral Scientific) (10mL) was mixed with 10% ammonium persulphate (60 µL of APB) and 6µL of N,N,N',N'-Tetramethylethylenediamine (TEMED). Gel was left to set and polymerise for around 20 minutes. Prior to loading the protein samples onto gel, 10µL of each sample was

mixed with 5µL of 5x sample buffer (2.5% w/v SDS, 10% w/v glycerol, 5% (v/v) β-mercaptoethanol, 0.01% w/v bromophenol blue and 50 mM Tris-HCl pH 6.8) and boiled for 2 minutes. Then protein samples and standard molecular weight marker sample (Invitrogen Protein Benchmarker Ladder) were loaded to the gel that were electrophoresed in 1x NEXT-Gel running buffer at 170 V using a Biorad PowerPC 300 for 60-90 minutes.

Once run, the gels were removed from the apparatus and stained for 1 hour with Coomassie blue (0.1% Coomassie brilliant blue, 40% methanol and 10% acetic acid) and then de-stained overnight with de-staining buffer (40% methanol and 10% acetic acid). Gels and protein bands were then imaged with Epson perfection 3490 flatbed scanner.

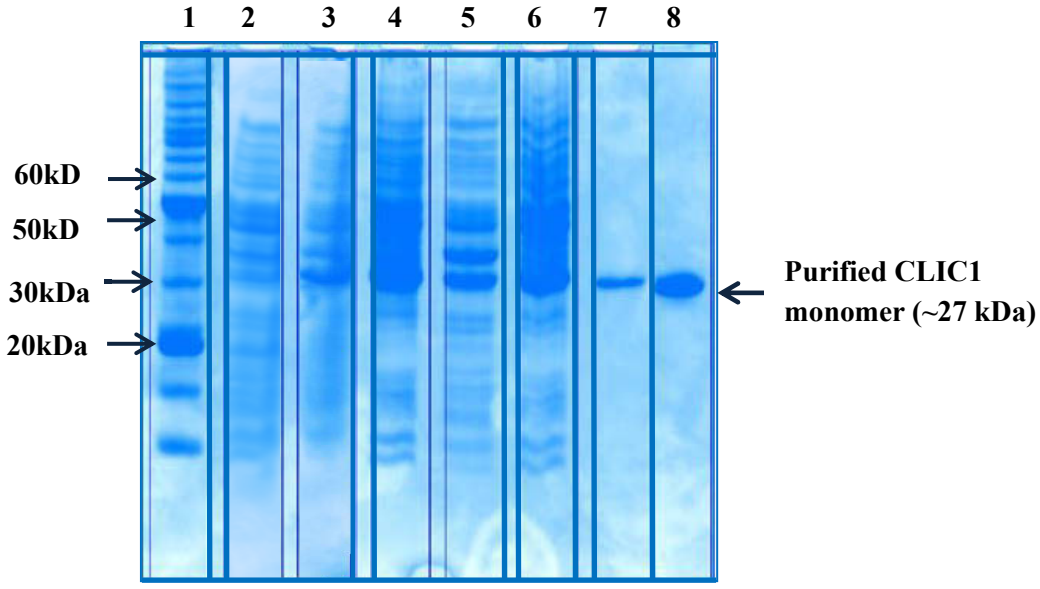


Figure 2.7: SDS-PAGE of CLIC1 (WT) protein expression and purification. *Lane 1*- Ladder with estimated molecular weights of proteins, *Lane 2*- sample of un-induced *E-coli* bacterial cells, *Lane 3*- sample of induced *E-coli* cells with 1mM IPTG, *Lane 4*- sample of the soluble proteins following cell lysis, *Lane-5* sample of pellet, *Lane 6*- eluted sample from the His-tag high affinity chromatography column prior incubation with thrombin sample of supernatant, *Lane 7*- sample of wash and *Lane 8*- Purified CLIC1 by size exclusion chromatography.

2.11 Measuring the Conductance of CLIC Proteins with tBLMs and Impedance Spectroscopy

2.11.1 Formation of Tethered Bilayer Lipid Membranes (tBLM)

Artificial membranes of ~4nm thickness capable of incorporating proteins of up to 40kDa were formed using methods reported in (96,144,174). The monolayer tethering coating was prepared by coating freshly deposited, 100nm pattern gold electrodes, on 25mm x 75mm x 1mm polycarbonate slides, with two benzyl disulphide families, one being a spacer molecule containing a four oxygen-ethylene glycol spacer, terminated with an OH group (90%), and the second being a tethering group comprising an eleven oxygen–ethylene glycol linker group with a single C20 hydrophobic phytanyl chain (10%) as the hydrophobic tether (Figure 2.8). The gold electrode was then assembled onto a 6 well polyethylene cartridge, possessing a $2mm^2$ active area and a flow cell chamber of 100μm in height. 8μL of 3mM mobile lipid phase (MLP) containing 70% zwitterionic C20 diphytanyl-ether-glycero-phosphatidylcholine: 30% C20 diphytanyl-diglyceride ether lipids that were dissolved in 99% (v/v) ethanol (figure 2.8) and added into each of the 6 wells and allowed to incubate at room temperature for ~ 2 minutes. This process was then followed by rinsing (3 x 100μL of HEPES/KCl buffer: 0.1M KCl, 0.1mM HEPES and 0.01mM $CaCl_2$, pH 6.5). Rinsing away the ethanol solvent and replacement with an aqueous buffer drives the rearrangement of the dissolved lipids to form a lipid bilayer, which is detected by the changes in impedance spectroscopy measurements of Rm and Cs.

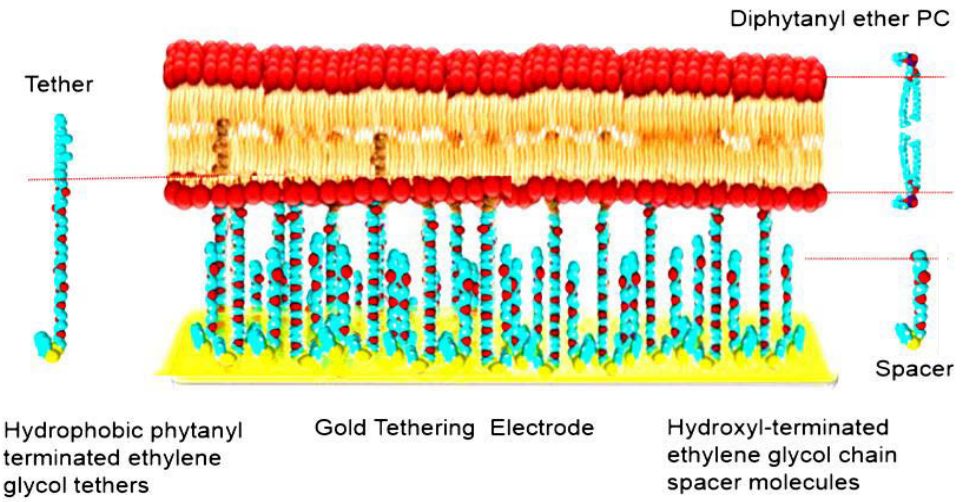


Figure 2.8: Tethered Bilayer Membrane (tBLM) structure. Two benzyl disulfide families attached to a gold electrode containing tethers (ethylene glycol groups terminated with hydrophobic phytanyl chain) and spacers (ethylene glycol chains terminated with hydroxyl group). Diphytanyl ether PC lipids then were incubated with the first layer of lipids for ~2 minutes to form lipid bilayer of membranes.

2.11.2 Formation of tBLM using Yeast and Bacterial Lipids

Lipid extracts of yeast (*saccharomyces cerevisiae*- provided from BioAustralis Pty Ltd, Australia) and *E-coli* bacterial cells (provided by Victor Chang Institute for medical research, Australia), were dissolved separately in 95% (v/v) ethanol with the aid of heating in a 50°C water bath followed by continuous vortexing for at least 15 minutes. 3mM of yeast or *E-coli* lipid solution was added to the first layer of membrane in the coated gold electrode in place of the mobile lipid solution and tBLM formation was as described in section 2.11.1.

2.11.3 Alternating Current (ac) Impedance Spectroscopy

The EIS characteristics, Rm and Cm for membranes formed with or without cholesterol or ergosterol, were measured using a *tethaPod*^{TM} conductance reader (SDx Tethered Membranes Pty Ltd, Australia) as described by Canfield et al, 2014 (96) and in the introduction section, 2.2. Briefly, swept frequency impedance spectra were recorded using an alternating excitation potential of 25mVp-p typically over a frequency range of 0.1Hz - 2.0 kHz. The reader acquires measures of the impedance and presents them as a bode plot of magnitude and phase (dots in Figure 2.9). From this data a modelled curve is fitted (continuous line in Figure 2.9).

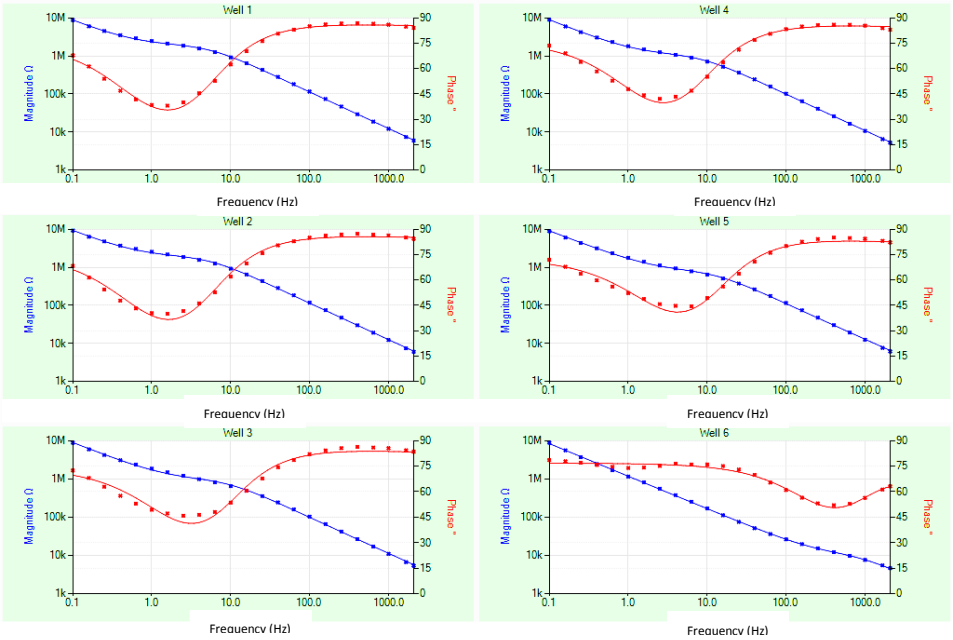


Figure 2.9: Traces of impedance magnitude. (Z which is impedance in Ohm) and phase (ϕ, in degrees) against frequency (f, is frequency in Hz) presented as a bode plot i.e. {Log_{10} (Z) vs Log_{10} (f)} and {ϕ vs Log_{10} (f)}. Figure was taken from (Surgical Diagnostics, Pty, Ltd, Sydney, Australia)

According to the electrical equivalent circuit shown in Figure 2.10, the values of Gm and Cm reported throughout this thesis were obtained from the estimates based on this modelling procedure which is performed automatically by the reader.

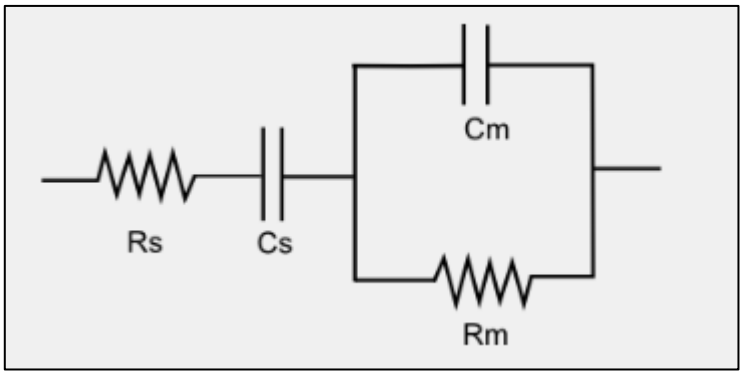


Figure 2.10: Electrical equivalent circuit used to interpret the impedance spectroscopy data. Cm (membrane capacitance in Farad), Rm (membrane resistance in Ohms, 1/Gm=Rm, where the Gm is the membrane conductance measured in Siemen), Cs (gold interfacial capacitance in Farad) and Rs (the electrolyte solution resistance in Ohms). Modified Figure was taken from (96).

The quality of the agreement between the data and the model is by the "Goodness of Fit" (GOF) which has a cut-off of 0.2 according to modelling the raw data as in the equation below:

$$GOF = SUM \sqrt{\{(data\ value - fit\ value) / (data\ value)\}} \qquad (96)$$

The conductance (Gm= 1/Rm) of well-sealed membranes (containing 100µL of Hepes/KCl buffer: 0.1M KCl, 0.1mM HEPES and 0.01mM $CaCl_2$ and 0.5mM TCEP, pH 6.5) was typically in the range 0.2 - 0.4µS for the 2.1 mm^2 electrode area and a

capacitance of 18-22nF. The formed membranes were left to stabilize for up to 30 minutes until Rm appeared constant before the addition of proteins.

2.11.4 Incorporation of CLIC1 Reduced Monomeric Protein into tBLMs

Recombinant wild type (WT) monomeric CLIC1 was diluted to a concentration of 20µg/100µL (7.4µM) in HEPES/KCl buffer (0.1M KCl, 0.1mM Hepes and 0.01mM $CaCl_2$ of pH 6.5). The protein sample was then incubated with fresh 0.5mM TCEP for ~1 hour in order to reduce any disulphide bonds and prevent dimerisation of proteins that were then applied to prepared tethered bilayer lipid membranes with or without cholesterol or ergosterol that had been equilibrated for ~1 hour with 100µL of Hepes/KCl buffer containing 0.5mM TCEP. Changes in conductance across the membrane were monitored by impedance spectroscopy.

2.11.5 Incorporation of α-Hemolysin into tBLMs

The bacterial toxin α-Hemolysin from *Staphylococcus aureus* is a pore forming protein that causes cell death when binds to the outer plasma membranes of cells (164). Lyophilized α-Hemolysin was purchased from Sigma Aldrich that was dissolved in Hepes/KCl buffer (0.1M KCl, 0.1mM HEPES and 0.01mM $CaCl_2$ of pH 6.5) and stored in -80°C as 20µL aliquots. α-Hemolysin spontaneously inserts into membranes and causes an increase in conductance and was used as a control protein. It was added to the tBLM at concentrations of 10 to 400nM per 100µL Hepes/KCl buffer and the resulting conductances were recorded.

2.11.6 Incorporation of Listeriolysin-O into tBLMs

Listeriolysin-O from *Listeria monocytogenes*, is a well-known cholesterol-dependent cytolysin (CDC) that requires cholesterol in order to insert into membranes to form pores (175,176). It was used in this research as a control protein in order to detect the change in conductance due to the incorporation of proteins in tBLMs with different cholesterol content. Listeriolysin-O (Sapphire biosciences) was diluted in HEPES/KCl buffer (0.1M KCl, 0.1mM HEPES and 0.01mM $CaCl_2$ of pH 6.5), aliquoted and placed at -80°C freezer for long term storage. Listeriolysin-O was then serially diluted to make a final concentration of 2µM/100µL in HEPES/KCl buffer (pH 6.5) and added to the pre-formed tBLMs where the conductance was monitored by impedance spectroscopy as described in 2.11.3.

2.11.7 Incorporation of Antifungal Drugs into tBLMs

Nystatin A and Amphotericin B (BioAustralis Pty Ltd, Australia) were used as controls in this research to characterise and test our tethered bilayer lipid membranes and impedance spectroscopy as a novel system to study the activity of metabolic drugs or integral membrane proteins. Nystatin A and Amphotericin B are highly hydrophobic antifungal drugs that were detected to bind to ergosterol in fungal cell membranes in order to form pores that increase leakage of ions resulting in cell death (177). Nystatin A and Amphotericin B were provided as lyophilized powders that were dissolved in 95% (v/v) methanol to make a final concentration of 1mM solution that was stored in -20°C until required. For measuring the conductance of these antibiotics, they were first diluted to 100µM in 1mL of HEPES/KCl buffer that was further diluted to 100nM/1mL buffer followed by series of dilutions to make final concentrations of 3, 6, 12 and 25nM in 100µL HEPES/KCl buffer. These concentrations then were applied to tBLMs

(formed by mobile lipid solution containing different ergosterol content or tBLMs containing yeast or *E-coli* natural lipid extracts as per method 2.12.4) and membrane conductance was measured by impedance spectroscopy as described previously.

2.12 Dialysing DTT from CLIC1 Protein in Solution

DTT was removed from the buffer or the solution containing CLIC1 by dialysis with large volumes of HEPES/KCl buffer (pH 6.5), 1mL of CLIC1 containing 1mM DTT was dialysed placed in 500mL where this buffer was changed at least 2 times. The DTT was dialysed by using cellulose dialysis tubing (Sigma Aldrich) retaining proteins larger than 12,000Da, the dialysis process was carried out in at +4ºC, with continuous stirring overnight.

Chapter 2 Results

2.13 Characterisation of Varying Lipid Sterol Composition in tBLMs

Given that the optimal functioning of many membrane proteins requires specific lipid composition such as the presence of sterols in membranes, it was important to investigate the effect of different sterols on the integrity and property of the tethered bilayer lipid membranes containing phosphatidylcholine (PC) or zwitterionic lipids, in order to establish a model biological membrane system for testing the CLIC proteins (117).

As discussed previously, cholesterol is the major sterol in bilayer lipid membranes of higher eukaryotic cells such as in animals, while ergosterol is the main sterol in membranes of lower eukaryotic cells such as those in plants, yeast, fungi and insects (178-181). These two sterols were the main sterols of interest in this study where the conductance and capacitance of membranes containing different concentrations of these two sterols was measured using impedance spectroscopy as described in the materials and methods.

Initial studies undertaken looked at the effect of incorporating different concentrations of cholesterol or ergosterol (0mol% to 50mol%). This resulted in no significant observable changes to the tethered bilayer lipid membranes, and two ways ANOVA followed by Benferroni's multiple comparison test for the impedance spectroscopy measurements of membranes containing cholesterol or ergosterol also confirm there were insignificant effects on membrane conductance and capacitance (p values obtained are greater than 0.5 for both) when compared to membranes containing zwitterionic lipids only with no sterols as seen in Figures 2.11 A and B, below.

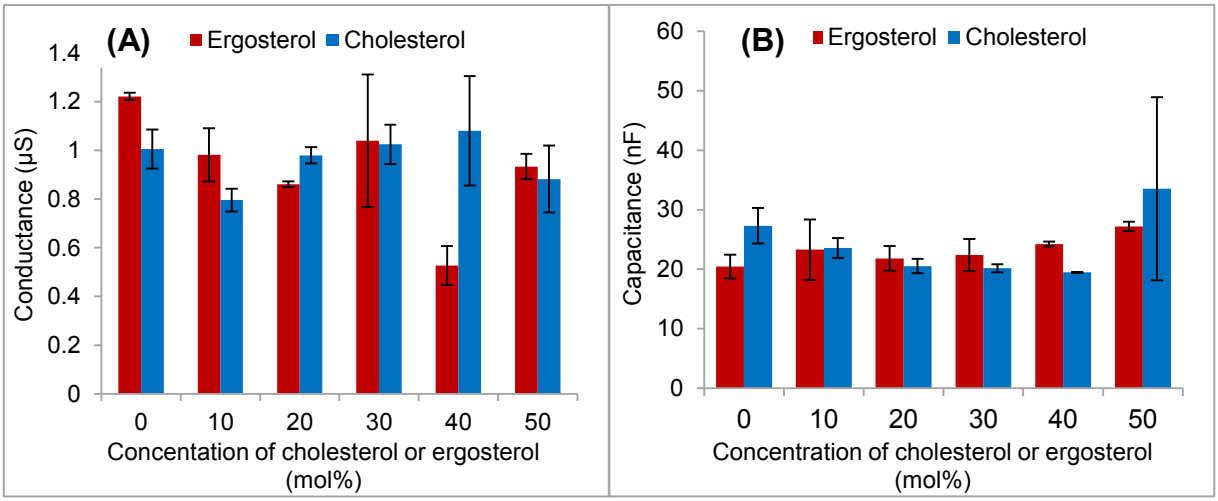


Figure 2.11: Conductance and capacitance of membranes containing different concentrations of cholesterol or ergosterol. Bilayer lipid membranes were formed using 10% tethered lipids on a gold electrode representing the monolayer of membrane and zwitterionic lipids dissolved in ethanol containing different concentrations of cholesterol or ergosterol was used as the second layer for the membrane. Membranes were then rapidly flushed with HEPES/KCl buffer (pH 6.5) in order to remove the ethanol by solvent exchange method and enhance the formation of the bilayer lipid membranes. The conductance and the capacitance of membranes were measured using impedance spectroscopy. *(A)* Represents membrane conductance at different cholesterol or ergosterol concentrations and *(B)* Capacitance of membrane with different cholesterol or ergosterol concentrations. Analysis of results was generated using Microsoft Excel 2010 and the error bars represent the standard error of three independent impedance spectroscopy conductance measurements.

2.14 Characterising the Function of Antifungal Drugs Using tBLM and Impedance Spectroscopy System

To further characterise our tethered bilayer lipid membranes and impedance spectroscopy system and the effects of membrane sterols on membrane conductance, we tested the activity of two well-known antifungal drugs (Amphotericin B and Nystatin A) that require the presence of sterols in membranes in order to exert their antifungal function. The antifungal drugs Amphotericin B and Nystatin A are insoluble in polar solvents such as water; therefore they were dissolved and stored in methanol. Therefore it was important to test the effect of methanol on membranes and determine the maximum concentration of methanol that when added onto the tethered bilayer lipid membranes do not cause disturbances to membrane conductance.

According to our results as seen in Figure 2.12, increasing amounts of methanol in the solution above 2% to a maximum of 10% causes the membranes to become leaky as indicated by the increase in membranes conductance. A final concentration of <2% was considered the maximal concentration of methanol that the membranes would tolerate. Therefore using a final concentration of 1% methanol with the tBLMs was considered to be well within the acceptable level to ensure a minimal to no disturbance effects may result to the tBLMs.

In order to determine whether this leakiness caused by methanol was reversible, test the degree of alcohol elimination from the membranes and to demonstrate that the, various concentrations of methanol were added to the membranes and then subsequently washed out with (HEPES/KCl buffer, pH 6.5) buffer. As seen in Figure 2.12 that washing the membranes with the buffer, caused a dramatic increase in membrane conductance, which then returned to the original baseline, equivalent to that observed

pre- the addition of methanol. This indicated that the methanol induced leakiness was totally reversible following washout of methanol from the membranes.

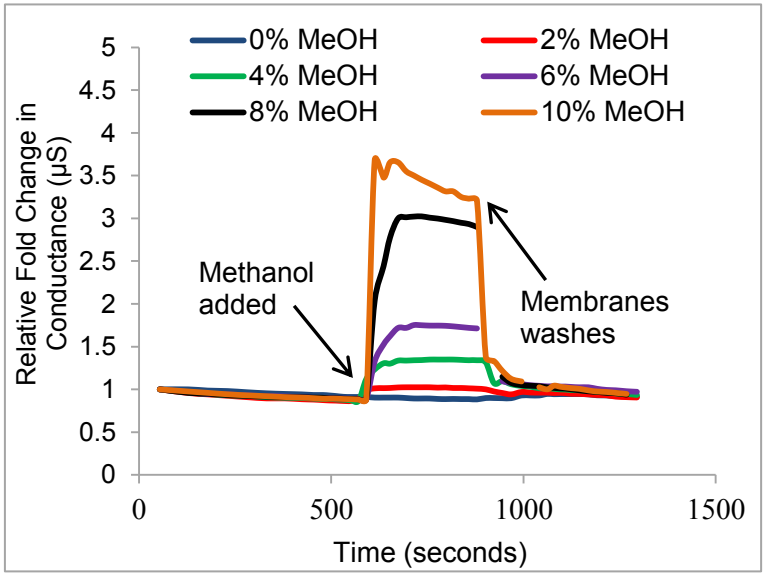


Figure 2.12: A Representative impedance spectroscopy recording of the effect of methanol on the conductance of tBLMs. Tethered bilayer lipid membranes were formed using 10% tethered lipids on a gold electrode and zwitterionic lipids dissolved in ethanol to form the second layer of membranes. By using the solvent exchange method using HEPES/KCl buffer (pH 6.5) ethanol was removed out of the membranes. Different concentrations of methanol were added into the membranes and the conductance was measured using impedance spectroscopy. Following the addition of methanol into membranes and the stabilisation of conductances, the membranes were rinsed or washed out with HEPES/KCl buffer (pH 6.5).

As part of the process to establish a robust method for the testing of the CLIC proteins using the tBLMs, Amphotericin B and Nystatin A are two well-known antifungal drugs were employed in this research and used as positive controls to test our tBLMs and impedance spectroscopy system that we are using for this research. Experimental evidence suggests that these two polyene antibiotics, Amphotericin B and Nystain A, induce changes in membrane permeability as a result of their interaction with the membrane ergosterol found in fungal and yeast membranes (127,182-184). Our results (Figure 2.13 A and B) show that the addition of increasing concentrations of

Amphotericin B and Nystatin A onto tBLMs containing zwitterionic lipids and 20 mol% ergosterol result in an increase in membranes conductance compared to membranes without ergosterol.

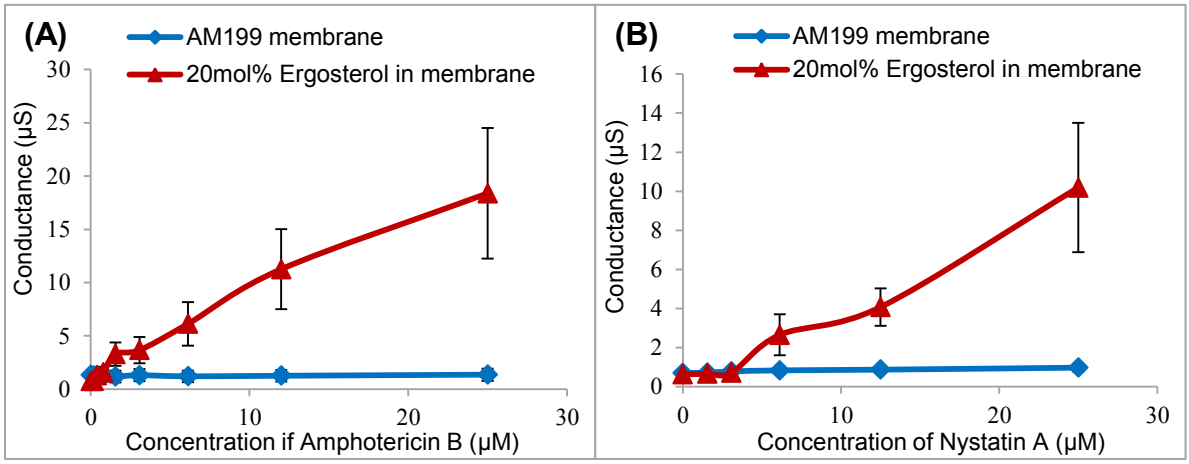


Figure 2.13: Conductance of Amphotericin B and Nystatin A in tBLM containing 20mol% ergosterol. Figures *(A)* and *(B)* represent membranes that were formed using 10% tethered lipids on a gold electrode representing the monolayer of the membranes and AM199 (zwitterionic lipids) only or with 20mol% ergosterol as the second layer for forming the membranes. The membranes formation was induced by rinsing them with HEPES/KCl buffer (pH 6.5). Different concentrations of Amphotericin B *(A)* and Nystatin A *(B)* were added into the membranes after being stabilised for at least 1 hour. Analysis of conductance measurements was carried out using Microsoft Excel 2010. The error bars represent the standard error of three independent experimental repeats.

Using yeast and *E-coli* lipid extracts to form the tBLMs again we confirm the functional-dependency of Amphotericin B and Nystatin A on the presence sterols in membranes in order to bind and increase membrane permeability. As shown in Figure 2.14 A and B that both metabolites were active in membranes formed from yeast lipid extracts due presumably, to the presence of ergosterol, however no activity or increase in membrane conductance was detected in *E-coli* membranes, given that *E-coli* membranes do not contain sterols (185-188).

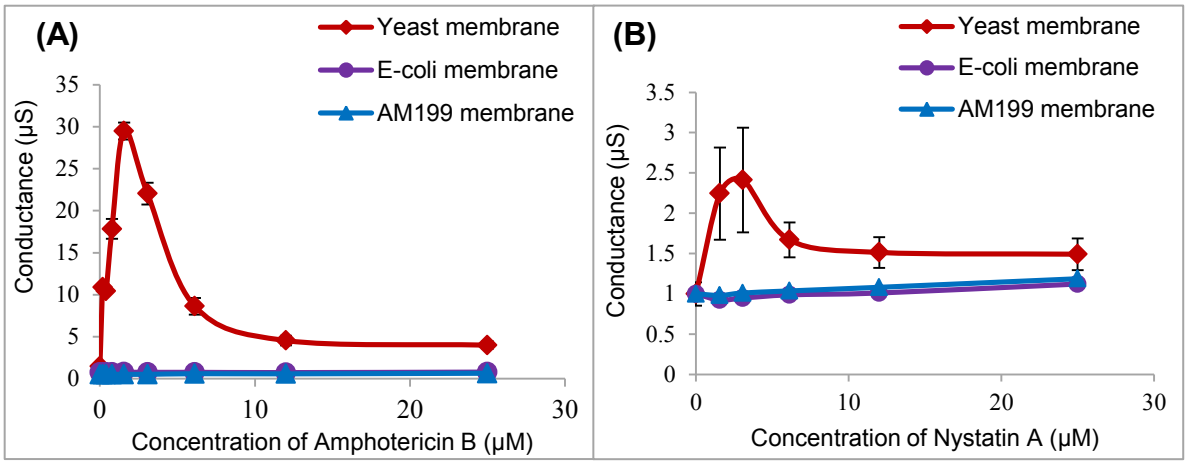


Figure 2.14: Conductance of Amphotericin B and Nystatin A in tBLM containing biological lipid extracts. Figures *(A)* and *(B)* represent membranes that were formed using 10% tethered lipids on gold electrode and yeast or *E-coli* lipid extracts as the second layer for making the bilayer lipid membranes that were also formed using HEPES/KCl buffer (pH 6.5), where Amphotericin B was added to membranes in figure *(A)* and Nystatin A was added to membranes in figure *(B)*. Analysis of conductance measurements was carried out using Microsoft Excel 2010. The error bars represent the standard error of three independent experimental repeats.

According to Figure 2.14, it can be shown that Amphotericin B demonstrates higher activity in tBLMs containing yeast lipid extracts than Nystatin A, however high concentrations of both metabolites (3.07µM for Amphotericin B and 6.125µM for Nystain A) in membranes caused decrease in membrane conductance. Further investigations were preformed where the time-dependent conductance measurements of membranes containing yeast lipid extracts were obtained as shown in Figure 2.15 A and B. Interestingly, addition of higher concentration of Amphotericin B added into membranes containing yeast lipid extracts cause higher initial conductance with subsequent decrease in membrane conductance measurements. This finding has not been previously reported and it may provide suggested mechanisms of action or

aggregation and insertion of Amphotericin B and Nystatin A in membranes containing ergosterol.

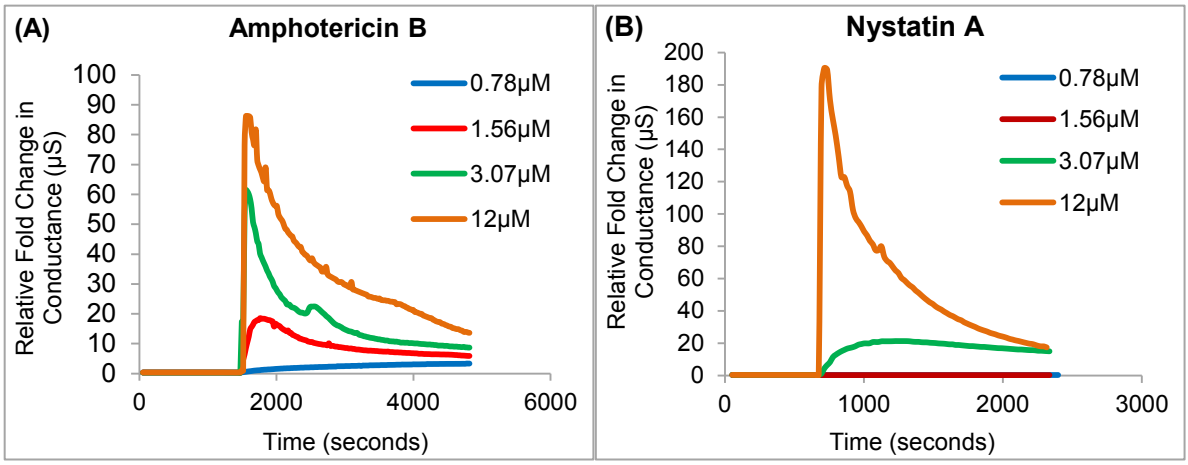


Figure 2.15: Representative impedance spectroscopy recording of Amphotericin B and Nystatin A activity in tBLM containing yeast lipid extracts. *(A)* and *(B)* represent membranes that were formed using 10% tethered lipids on a gold electrode representing the monolayer of the membranes and yeast lipid extracts were used as the second layer for forming the bilayer lipid membranes. The membranes formation was induced by rinsing them with HEPES/KCl buffer (pH 6.5). Different concentrations of Amphotericin B *(A)* and Nystatin A *(B)* were added into the membranes after being stabilised for at least 1 hour. Conductance recordings were obtained by Impedance Spectroscopy and data analysis was performed using Microsoft Excel 2010.

2.15 Conductance Properties of the Bacterial Toxins α-Hemolysin and Listeriolysin-O using tBLMs and Impedance Spectroscopy System

Alpha Hemolysin (α-Hemolysin) from *Staphylococcus aureus* is a well-known spontaneous membrane inserting protein that is not defined as requiring the presence of any specific binding receptors or membrane component (189) such as ergosterol in the case of the polyene antifungal drugs tested in the previous section in order to bind and insert into membranes. α-Hemolysin exists as a monomer in aqueous solution that oligomerises to form a heptamer in membranes (149). It has also been shown to have high binding affinity to membranes from rabbit erythrocytes, at concentrations less than 50nM (5). Cholesterol has however been shown to affect the interaction of α-Hemolysin with membranes; as the insertion and formation of the heptamer by the toxin was triggered by high cholesterol concentrations (50mol%) (3,169,190). Other studies have demonstrated a 90% decrease in its haemolytic activity when incubated with membranes containing only 5-20mol% cholesterol (169,189).

In the current study α-Hemolysin was incorporated into tBLMs containing zwitterioninc lipids with varying cholesterol concentrations. Our results showed a consistent trend where α-Hemolysin had higher activity/conductance in membranes lacking cholesterol, with conductance seen to decrease as cholesterol concentration increased, with the lowest conductance recorded in membranes containing 50mol% cholesterol. However a linear curve regression analysis for α-Hemolysin conductance (R^2 *is 0.697*) indicates that the p value is 0.078 which is greater 0.05 suggesting that the change in α-Hemolysin activity in response to changing cholesterol concentration in membranes is insignificant (Figure 2.16).

In contrast, the bacterial toxin Listeriolysin-O from *Listeria monocytogenes* is a member of the cholesterol dependent cytolysins (CDC). It's interaction with bilayer membranes is well-known to be highly dependent on the presence of membrane. As seen in Figure 2.16, Listeriolysin-O showed higher activity or conductance in tethered bilayer membranes containing 50mol% of cholesterol and a decrease in the conductance observed when cholesterol concentrations were decreased to 25, 12.5, 6.25 and 0mol% cholesterol. Also regression analysis of the curve (with an R^2 *of 0.974*) confirm the significant difference in the results as the obtained p value of Listeriolysin-O activity in membranes containing different cholesterol concentrations is 0.0018 which is less than 0.05.

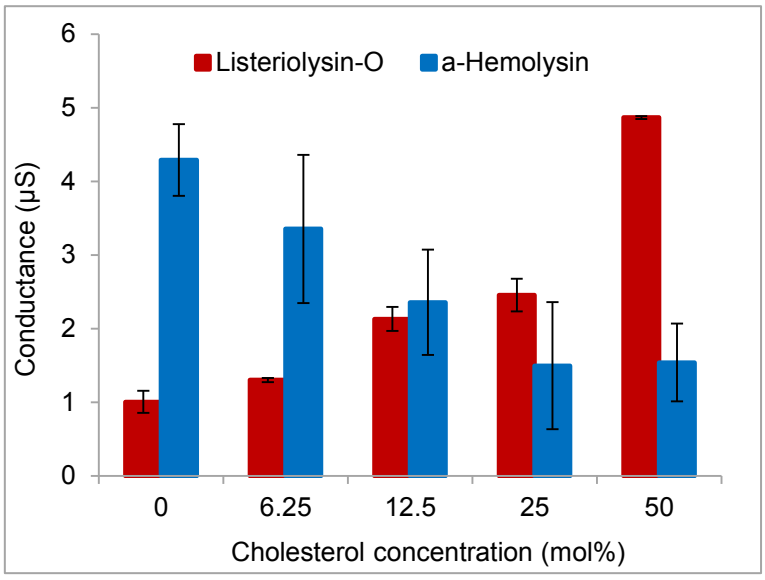


Figure 2.16: Conductance of α-Hemolysin and Listeriolysin-O in tethered bilayer lipid membranes and impedance spectroscopy system. 50nM of α-Hemolysin or 2µM of Listeriolysin-O in 100µL of HEPES/KCl buffer (pH 6.5) were added into membranes made from neutral lipids containing 0, 6.25, 12.5, 25 and 50mol% cholesterol. The conductance of toxins was measured using impedance spectroscopy. Analysis of conductance measurements was carried out using Microsoft Excel 2010. The error bars represent the standard error of three independent experimental repeats.

2.16 Conductance Properties of CLIC1 Monomeric (WT) Protein in tBLMs

In order to ensure the CLIC1 protein to be used was in its reduced monomeric form and to prevent its conversion into its dimeric form, the reducing agents 1mM DTT or 0.5mM TCEP were added to the protein samples during protein purification and in their storage buffer. When preparing oxidised/dimeric CLIC1, the protein was incubated with 2mM H_2O_2 as the oxidizing agent.

To test the effect of the reducing or oxidizing agents on the tBLMs, 100µL of HEPES/KCl buffer containing 1mM DTT or 0.5mM TCEP were added onto membranes made from zwitterionic lipids with the conductance changes measured using impedance spectroscopy as mentioned by the materials and methods section 2.11.3.

From Figure 2.17, it can be seen that addition of buffer containing 1mM DTT caused the membranes to become leaky as indicated by the high conductance recording. On the other hand the addition of buffer containing 0.5mM TCEP or 2mM H_2O_2 resulted in a decrease in conductance, indicating that the membranes are becoming more sealed. Of note, we also determined that the addition of 0.5mM TCEP in HEPES/KCl buffer changes the pH of the buffer by dropping it from 6.5 to 3.86. Similarly, H_2O_2 which is classified as a weak acid also reduces the pH of the buffer from 6.5 to 5.

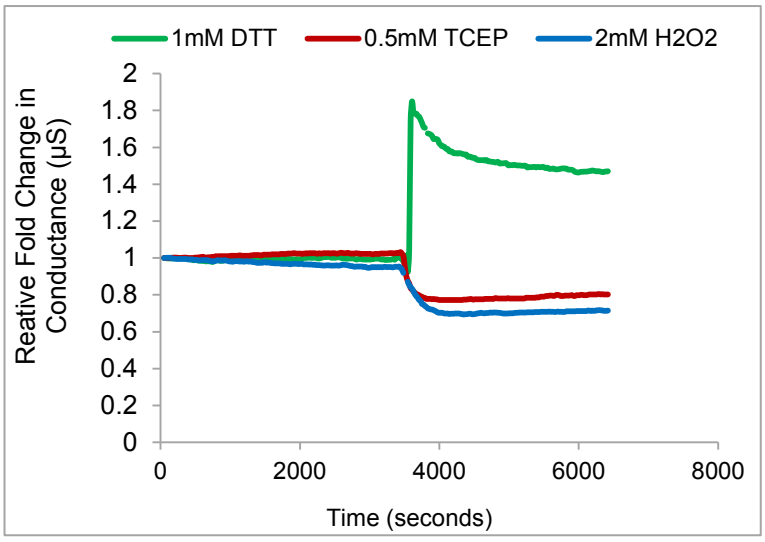


Figure 2.17: A representative impedance spectroscopy recording of DTT, TCEP or H_2O_2 effect on tBLMs. Membranes were formed using 10% tethered lipids on gold electrodes as the monolayer where zwitterionic lipids were added as the second layer to form bilayer lipid membranes. HEPES/KCl buffer (pH 6.5) was used to remove the excess ethanol and induce the formation of the bilayer lipid membranes. 100µL of HEPES/KCl buffer containing 1mM DTT, 0.5mM TCEP or 2mM H_2O_2 were added into the membranes where conductance was measured using impedance spectroscopy. Analysis of conductance measurements was carried out using Microsoft Excel 2010.

CLIC1 reduced monomer protein prepared and stored in the presence of 0.5mM TCEP was added to tBLMs containing zwitterionic lipids only that were equilibrated with HEPES/KCl buffer containing 0.5mM TCEP (pH 6.5). Figure 2.18, shows that adding increasing concentrations of CLIC1 monomeric protein onto the membranes did not affect the membrane conductance significantly. However CLIC1 monomeric protein that was pre-incubated with 2mM H_2O_2 was more conductive in membranes containing zwitterionic lipids only.

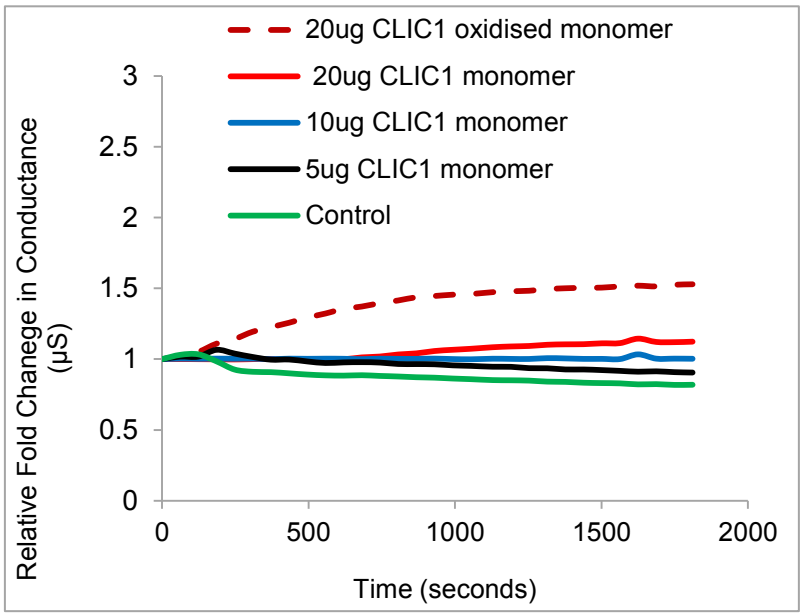


Figure 2.18: A representative impedance spectroscopy recording of CLIC1 monomeric protein conductance in tBLMs containing zwitterioninc lipids. 5, 10 and 20µg of CLIC1 monomer was pre-incubated with reducing agent (0.5mM TCEP) in 100µL HEPES/KCl buffer (pH 6.5) or 20µg CLIC1 monomeric protein was pre-incubated with 2mM H_2O_2 in HEPES/KCl buffer (pH 6.5) for ~1 hour before addition to membrane. The conductance of a representative impedance spectroscopy recording of CLIC1 against time was measured after the addition of monomeric wild type protein into tethered bilayer lipid membranes containing zwitterionic lipids only. The Control represents a membrane containing membranes containing 100µL buffer only with no protein added.

Chapter 2 Discussion

2.17 Cholesterol and Ergosterol Affect the Conductance and Capacitance of tBLMs

Membrane sterols are known to affect the structure, function and microfluidity of lipid bilayer membranes as they alter the packing of the hydrocarbon chains of the phospholipid molecules making the membranes (2,110). Changes in temperature also impact on the state or the phase of membranes via increasing or decreasing the molecular motion and rotation of phopholipids within a membrane, which then cause the bilayer lipid membranes to transient into different phases. At low temperatures the membranes transient into the solid ordered phase (*so*) where the hydrophobic chains of the lipidsare arranged in a crystalline order, however at high tempreature the membranes become more fluid- like as in the the liquid disordered (*ld*) phase where the distand between molecules is larger and the hydrophobic chains of the lipids become disordered (2). In membranes containing sterol, the high ordered phase at low temprerature was found to decrease and the low ordered phase at high temperature was increased. In this manner, sterols act to maintain the stability of membrane microfluidity across a range of temperatures. In membranes contianing 20-25mol% cholesterol or ergosterol in comaprison to neutral lipid concentrations in membranes (115,191,192), the liquid ordered (*lo*) phase of the bilayer would normally be maintained, where the molecules within a membrane are able to undergo small lateral and rotational diffusions with minimal disruption or disordering of the acyl chains of the PC lipids and minimal effect to the thickness of the membranes.

Overall our impedance spectroscopy measurments of tBLMs made from zwitterioninc lipids either with or without cholesterol and ergosterol, showed little effect on membrane conductance or capascitance. There were however some slight variations noted (even though not statistically significant) that indicate membranes containing

cholesterol become slightly more leaky as cholesterol concentrations increase in membranes. Interestingly membranes containing higher ergosterol concentration become slightly better sealed when compard to membranes with no ergosterol (Figure 2.11 A). Again these results were not found to be statistically significant, however these observations support previous findings, whereby membranes containing ergosterol were found to be more rigid and tighly packed compared to cholesterol containing membranes. Also it was previously reported that cholesterol increases the acyl chain order of phospholipids and therefore was found to increase the thickness of membranes (185,193). Our capacitance measurements of membranes containing 0 to 40mol% cholesterol, indicate a slight increase in membrane thickness with an increase in cholesterol concentration. However ergosterol did not show this effect, as seen in Figure 2.11 B.

Published studies by others have shown that cholesterol concentrations higher than 20 mol% cause an increase in fluidity of artificial membranes made from PC lipids (192). This could explain the high fluctuations or the variations in our results and the lack of correlation between the membrane conductance and sterols concentration as shown in the impedance recordings of tBLMs containing 30 to 50mol% cholesterol or ergosterol. Given the results from these experiments, it was decided that a final concentration of 20 to 25mol% cholesterol or ergosterol was to be routinely employed in subsequent tBLM experiments in order to characterise the function of the CLIC proteins in this study. This was based on the fact that, these concentrations represent an appropriate sterol concentrations for the detection of protein conductance, that resulted in consistent conductance and capacitancce measurements (hence the small error bars obtained from experimental repeats as in Figure 2.11 A and B) and also correlates with the

physiological sterol concent found in animal cells, where these proteins are normally expressed.

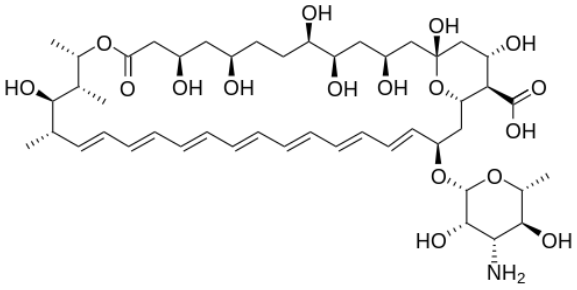
2.18 Conductance of Antifungal Drugs and Toxins in tBLMs

As part of the initial process to establish the use of the tBLM and impedance spectroscopy system for CLIC protein studies, we used Amphotericin B and Nystatin A as drugs that were readily available and are well-known antifungal agents that exert their activity by binding to and changing membrane permeability. Amphotericin B and Nystatin A were dissolved in less than 1% methanol, and then added to tBLMs with/without ergosterol. It had previously been shown that up to 1% methanol caused minimal disturbance to the membrane conductance (Figure 2.12).

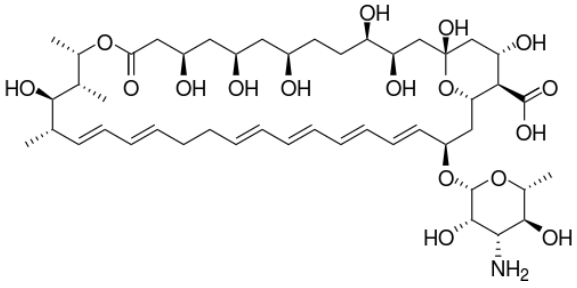
The conduction of both drugs was higher when they were incorporated into tBLMs containing ergosterol (25mol% ergosterol with zwitterionic lipids only, or in membranes containing yeast lipid extracts) as in Figures 2.13 A and B. These findings are similar to previously reported results, which suggests that both Nystain A and Amphotericin B induce changes in membrane permeability as a result of interacting directly with ergosterol in fungal and yeast membranes (194).

Both antifungal drugs were also found to interact with other sterols such as cholesterol, however, the type of sterol was found to influence the stability, structure and also the kinetics of the membrane conductance resulting from the creation of membrane ion channels formed by these drugs (195-197). Studies have shown that these antibiotics are more selective to ergosterol than cholesterol as the double bond of ergosterol aids the formation of tightly packed antibiotic-sterols complex or aggregates that help to maintain the stability of the formed channels in fungal membranes (143,198).

Polyene antifungal drugs such as Amphotericin B and Nystatin A are structurally similar (Figure 2.19) in that both drugs are composed of large cyclic ester ring, that on one side it contains multiple conjugated carbon-carbon double bonds (7 double bonds in Amphotericin B and 6 double bonds in Nystatin A – hence the name polyene), and the other side of the ring consisting of multiple hydroxyl groups. Both structures contain an amino glycoside or mycosamine group. Extensive studies have been carried out to understand the mechanisms of action and interaction of these antibiotics with cell membranes and in particular with the sterol component. Earlier reports proposed that the mycosamine group at C19 in Amphotericin B is critical for binding to ergosterol, and the functional formation of ion channels that induce cation leakage in the fungal cells and hence the fungicidal activity of Amphotericin B (127,196,198,199)



Amphotericin B



Nystatin A

Figure 2.19: Chemical structures of Amphotericin B and Nystatin A. Both drugs contain a cyclic ester ring with mycosamine group. One side of the ester ring contains 7 carbon-carbon double bonds as for Amphotericin B or 6 double bonds as for Nystatin A. whereas the second side of the ester ring contains the hydroxyl groups.

Also recent studies show that the channel formation by Amphotericin B is not important for the fungicidal activity of the antibiotic however ergosterol binding was found to be essential for fungal cell death (200). Our results show that the conductance of

Amphotericin B in tBLMs containing yeast lipid extracts, reached a peak conductance, followed by a decrease beyond a certain concentration of antibiotic as seen in Figure 2.14 A and B. The rapid drop in conductance arising from the addition of high concentrations of Amphotericin B against time (Figure 2.15 A and B) which can be due to the formation of aggregates or domains that prevent or block the formation of stable pores or channels by the antifungal drug in membranes as was seen in a previously published study with the loss of PGLa peptide conductance in tBLMs (96). It was also proposed by Anderson et al, 2014 (200), that Amphotericin B exists in membranes containing ergosterol as large extramembranous complexes or aggregates that sequester ergosterol from fungal cell bilayer membranes leading to cellular function disruptions, as ergosterol was believed to be involved in the regulation of membrane proteins, endocytosis, micro-domain formation, cell signalling and many other important functions required for fungal cells maintenance and sustainability. So the newly defined model of Amphotericin B interaction with ergosterol in membranes, the "sterol sponge model" is a novel approach to explain the mechanism of action of the polyene antifungal drug Amphotericin B and by extrapolation to Nystatin A. Our results are consistent with models in which the Amphotericin B inserts into membranes containing ergosterol forming ion channels. However, at high concentrations of Amphotericin B, the effect becomes inhibitory likely due to the formation of non-conducting aggregates.

2.19 Conductance and Properties of Pore Forming Toxins in tBLMs

Further confirmation that the tBLM system was suitable for the purposes of studying membrane protein insertion, protein-lipid-sterol interactions and conductance properties, came from our studies using the two bacterial toxin proteins α-Hemolysin and Listeriolysin-O.

Listeriolysin-O is a well-known cholesterol-dependent cytolysin (CDC) whose function is highly dependent on cholesterol in membranes (201-203). First it binds to membranes containing cholesterol then it inserts and oligomerises in order to form membrane pores (201). As shown in Figure 2.16, Listeriolysin-O conductance levels are positively correlated to the membrane cholesterol concentrations. Conversely, the water soluble α-Hemolysin toxin showed distinct sterol requirements for optimal membrane interaction and conductance. The toxin α-Hemolysin exists as monomer in solution that can bind to cell membranes, then oligomerises and form heptameric pores (204-207). It is characterised as spontaneous membrane inserting toxin that demonstrates higher rates of multimerisation and structural assembly in fluidized membranes comprising of unsaturated lipids and greater than 20mol% cholesterol. However, under the conditions used in our impedance spectroscopy studies, we did not see any significant effect by cholesterol concentration in the bilayer membranes, on the conductance levels or activity of α-Hemolysin (Figure 2.16).

2.20 Conductance of CLIC1 in tBLMs

The pH of intracellular and extracellular fluids affect numerous biological processes including cholesterol domain formation (208-210), membrane fusion (211) and membrane phase transition (212-215), membrane mechanical and electrostatic interactions have also been detected to change in response to changes in pH (210). It was hypothesized that changing the ionic strength of the surrounding milieu, can also affect membrane mechanical properties by changing their lipid packing (210,216,217); the electrical charge of zwitterionic phospholipids, such as PC lipids, containing phosphate and choline groups, are altered according to the bound protons or hydroxide ions which then results in changes in the interfacial electrical properties of membranes (210,217-219). From our results, rinsing the tBLMs (that contain zwitterionic lipids) with buffer containing either 0.5mM TCEP or 2mM H_2O_2 caused the membranes to further seal (Figure 2.17). According to a study by Zhu et al, 2005 (220), it was found that increasing the concentration of H_2O_2 affect vesicles made from brain lipids by making them liquid crystalline, whereas the bilayer lipid membranes of astrocytes were found to transient into the gel-like phase which means the membrane fluidity of the astrocytes decreased in response to the increased H_2O_2 concentrations. The effect of H_2O_2 on astrocytes membranes is supported by our results, with the tBLMs becoming less fluid-like, resulting in decreased conductance and hence more sealed membranes.

We also propose that the slight decrease in conductance or the membrane sealing effect of TCEP and H_2O_2 could be due to increased lipid packing of our membranes as a result of lowering the pH of the HEPES/KCl buffer used in these experiments. TCEP (which contains HCl) makes the pH of the HEPES/KCl buffer drop by 2.64 pH units and H_2O_2 which is a weak acid also decreases the pH slightly and drops it by 1.14 pH units. On

the other hand, the addition of buffer containing 1mM DTT caused the membrane conductance to increase suggesting that membranes are becoming leaky as shown in Figure 2.17. The effects of DTT on the tBLMs were not further pursued however they also warrant further investigations.

Removing the DTT from protein solution by dialysis using cellulose tubing (materials methods section 2.12) resulted in protein losses via the formation of some visible protein aggregates and precipitate as well as, loss via protein adhesion to the cellulose tubing. Therefore in this research project, TCEP was used in placed of DTT as the main reducing agent for inclusion in the preparation and storage buffers of the CLIC and other protein samples. The problem of increased membrane resistance/sealing of the membranes post-TCEP or H_2O_2 addition was alleviated by re-adjusting the pH of HEPES/KCl buffer containing TCEP or H_2O_2 to 6.5, by using a small volume of a concentrated alkali solution, such as potassium hydroxide.

Incubating CLIC1 monomer with 2mM H_2O_2 causes the formation of non-covalent intramolecular disulphide bond between Cys24 and Cys59 that results in the dimer formation of CLIC1 (84). According to previous results using unilamellar liposomes made from soybean phosphatidylcholine (PC) lipids and cholesterol (ratio 9:1), it was shown that the CLIC1 dimer had higher chloride efflux measurements than CLIC1 monomer. Tip-dip experiments showed that treating the monomeric or dimeric protein with 5mM DTT resulted in the full suppression of CLIC1 ion channel activity (84). In a similar experiment by Goodchild et al, 2009 (88), they used lipid vesicles and Stern-Volmer quenching with acrylamide to study the effect of oxidation on CLIC1 interactions with membranes via measuring the relative accessibility of Trp35 in CLIC1. It was reported that there was no change in the accessibility of Trp35 in CLIC1 monomer under reducing conditions, which suggests that there was no interaction

occurring between CLIC1 and the bilayer membranes of lipid vesicles, which also means that reduced CLIC1 did not insert into the membrane. Conversely, a decrease in Trp35 accessibility was observed when testing CLIC1 dimer and CLIC1 oxidised monomer exposed to lipid membranes.

According to our initial impedance spectroscopy measurements of CLIC1 reduced monomeric protein (pre-incubated with 0.5Mm TCEP) in tBLMs formed from PC or zwitterionic lipids lacking any sterols (Figure 2.18), it is seen that CLIC1 has failed to show any significant conductance in these membranes at low protein concentrations. While at higher concentration there was a small level of conductance recorded, following a relatively long lag time, post addition of the protein to the tBLMs. However addition of CLIC1 monomer that was oxidised with 2mM H_2O_2 prior to its addition to the tBLM, demonstrated greater conductance activity and had much faster kinetics compared to the reduced sample. These results are in concordance with the previous published findings from both the tip-dip electrophysiology measurements of CLIC1 conductance in bilayer lipid membranes containing pure PC lipids and the chloride efflux studies.

Studies by Warton et al, 2002, reported that upon addition of CLIC1 to artificial membranes, two distinct ionic conductance events were observed (49). Firstly, there were variable lag periods of null events that were then followed by small increases in conductance with slow kinetics (SCSK: Small conductance channels with slow kinetics). These were proposed to be due to CLIC1 proteins that have not yet been fully assembled to form channels as in the case of the CLIC1 reduced monomer. The second observed event occurred later in time, when the small and slow conductances turned into larger channel conductance events that had faster kinetics (HCSK: High conductance channels with slow kinetics) which was suggestive of final state

multimeric channel formation. This correlates with the data seen for the CLIC1 oxidised monomeric protein via our impedance spectroscopy and tBLM studies.

Conclusion

Overall, the results obtained from these experiments demonstrate the feasibility of using impedance spectroscopy in combination with tBLMs is a suitable assay system for the study of membrane inserting and ion conducting proteins, such as the CLIC proteins. It also clearly demonstrated the importance of controlling the membrane lipid and sterol components, along with the surrounding bathing milieu in terms of pH and redox state in ensuring robustness, reliability and repeatability of the system.

Chapter 3

Sterols are Required for the Optimal Conductance of CLIC1 in Tethered Bilayer Lipid Membranes

3.1 Introduction

CLIC family members contain no obvious transmembrane domain in their protein structure; nevertheless, they are capable of inserting into phospholipid membranes directly from their soluble state, where they can function as ion channels.

As discussed in Chapter 1, single channel recordings of CLIC1 *in vitro* were found to be highly inconsistent (47), where conductance recordings of CLIC1 in bilayer lipid membranes containing asolectin with phosphatidylethanolamine (PE) and phosphotidylserine (PS) lipids were calculated as 60pS and 120pS (7,44,46,47,49,53,70,80,84). CLIC1 channel activity in membranes containing PC lipids was 28pS (8,49,221) while the single channel conductance in CLIC1-transfected CHO-K1 cells was 8pS and 16pS (29,48,71).

Similar discrepancies were also reported in patch clamp measurements for CLIC4. It was demonstrated that CLIC4 ion channels have high conductance measurements in bilayer lipid membranes containing reconstituted brain microsomes compared to other bilayer lipid membranes with neutral lipids only (29,221). Therefore it was speculated that CLIC proteins may require specific conditions such as specific lipid composition in membranes besides other external factors, including oxidation/reduction state and low pH in order to insert and form functional and stable chloride ion channels in membranes (8,47,81). Given that CLIC4 was found to be localized in the Cholesterol rich micro domains called caveolae (85), it was proposed that CLIC proteins may only be functional in membranes containing cholesterol. In a study by Tulk et al, 2002 (8), it was found that CLIC1 demonstrated no ion channel activity in membranes containing neutral lipid mixtures, while adding 10% negatively charged lipids such as phosphatidylethanolamine (PE) or phosphotidylserine (PS) supported channel activity

of CLIC1. Increasing cholesterol concentration to 30mol% in membranes caused the channel activity of CLIC1 to be suppressed (8). However this study was performed using phospholipid vesicles and chloride efflux assays where the results obtained may suffer from high levels of inconsistency and inaccuracy as in another study by Singh, et al (47) where Langmuir-Blodgett membrane monolayers and patch clamping techniques have been used, it was reported that membranes containing POPE, POPS and cholesterol in the molar ratio of 4:1:1 have induced the conduction of ion channels formed by CLIC1. However in both studies it was not elucidated whether the conduction of CLIC1 in membranes is dependent on specific biophysical properties of the bilayer membranes such as a requirement for PE or PS lipids, which change the charge and also the flexibility of the membranes via altering its curvature. Similarly, the presence of cholesterol that promotes the protein insertion or ion channel assembly in membranes was not fully considered. Here-in, we investigate the effect of sterols (cholesterol and ergosterol) on the conductance/activity of the protein CLIC1 using our custom tethered bilayer lipid membranes (tBLMs) and impedance spectroscopy system.

Chapter 3
Materials and Methods

3.2 Recombinant CLIC1 Dimeric Protein

Dimeric CLIC1 was provided by Dr Louise Brown from Macquarie University, Australia. It was reported prepared as previously described (89).

3.3 Preparation of Recombinant CLIC1-C24A and C59A

Procedures for the expression, purification and quantification of His-tagged CLIC1-C24A and CLIC1-C59A mutants are as described in the previous chapter (Materials and Methods Sections 2.6.1-2.10)

3.4 Preparation of Recombinant EXC-4 and CLIC1-C24S by GST Gene Fusion System

Glutathione S-Transferase (GST) Gene fusion system (AMRAD-Pharmacia) was used for the expression and purification of fusion proteins in *E-coli* bacteria (7,70).

Glycerol stocks of *E-Coli* bacteria strain, BL21 (DE3) containing pGEX-4T-1 vector (Novagen) was inoculated in 100mL LB medium containing 100µg/mL Carbenicillin and left to grow as in the small scale culture of His-tagged proteins (section 2.4.1). Then the grown bacterial cells were transferred into 1.5L of 2xYT medium also containing Carbenicillin in order to scale up the culture of cells that were left to grow on a shaker of 180rpm, at 37°C for 2.5 hours or until an OD of 600 was achieved. Cells then were induced with 1mM IPTG and returned to incubation for another 4.5 hours at 37°C with 180rpm shaking.

he induced cells were harvested and pelleted by centrifugation (Sigma 3-18K, rotor 11133/13104, 5500rom for 15 minutes at 6°C), the supernatant was poured off and cells

pellet was resuspended in ~30mLs of PBS buffer (0.01M phosphate buffer, 0.0027M KCl, 0.14M NaCl, 0.05% Tween containing 1mM DTT or 0.5mM TCEP, pH 7.4). The resuspended cells were homogenised as for the His-tagged proteins as described in Chapter 2, materials and methods, section 2.4.4. Then purification of the supernatant fraction was prepared first by equilibrating 3mL of glutathione-sepharose 4B resin (Amersham Biosciences) with ~300mL of PBS buffer (containing 1mM DTT or 0.5mM TCEP); and second by incubating the supernatant with the resin beads for 1 hour at 4°C while rocking. Then the mixture of supernatant and resin was poured into disposable chromatography column with a frit (Biorad) and washed with ice cold 300mL of PBS buffer (containing 1mM DTT or 0.5mM TCEP), then column containing resin and GST-tagged proteins was further equilibrated with 50mL of thrombin cleavage buffer (150mM NaCl, 2.5mM $CaCl_2$, 1mM sodium azide, 0.5mM DTT or 0.5mM TCEP and 20mM Tris-HCl, pH 8). Cleavage of GST-tagged proteins from resin beads was then achieved by incubating them with 30 NIH units per 1L of cells culture of bovine plasma thrombin (Sigma Aldrich) and left overnight at 4°C. Eluting the proteins from the high affinity chromatography column was achieved in a similar way to the His-tagged proteins purification steps, sections 2.5.1.

In order to use the chromatography column for several times, the GST-tag was removed from the sepharose resin. This was achieved by washing the column with 50mL of glutathione wash buffer (10mM L-glutathione reduced (Sigma Aldrich), 50mM Tris-Cl, pH 8.0) to enable the GST-tag to be eluted off the column. The column was then washed out or equilibrated with 200mL PBS buffer (pH 7.4) and stored at 4°C.

Proteins were then further purified by size exclusion chromatography as previously described in Chapter 2, Materials and Methods, section 2.8. The size exclusion chromatography profile was found to contain one single peak of the protein representing

the purified monomeric proteins. To quantify the amount of purified proteins obtained UV-Vis spectrophotometer and BCA Protein Assays were performed and also in order to confirm the molecular weight and the structural state of the purified proteins (whether they are monomeric or dimeric or exist as aggregates) and they were run through SDS-PAGE gel, as described in Chapter 2, materials and methods, section (2.9.2 and 2.10)

3.5 Incorporation of CLIC1 and EXC-4 into tBLMs Containing Cholesterol

Recombinant CLIC1 (WT) monomeric and dimeric proteins; CLIC1-C24A; CLIC1-C59A and EXC-4 (WT) were diluted to a concentration of 20µg/100µL (7.4µM) in HEPES/KCl buffer (0.1M KCl, 0.1mM HEPES and 0.01mM $CaCl_2$ of pH 6.5). Each protein was incubated with 0.5mM TCEP for ~1 hour in order to reduce the disulphide bonds and prevent the dimerisation of proteins. They were then applied to pre-prepared tethered bilayer lipid membranes with or without cholesterol or ergosterol that were equilibrated for ~ 1 hour with 100µL of HEPES/KCl buffer containing 0.5mM TCEP when measuring the conductance of monomeric reduced CLIC1 or membranes were equilibrated with 2mM H_2O_2 when conducting experiments with oxidized or dimeric CLIC1.

3.6 Addition of Boiled CLIC1 to Membranes with 25mol% Cholesterol

20µg and 40µg of CLIC1 monomeric protein were boiled in a hot water bath for 2 minutes and added to 100µL of HEPES/KCl buffer (pH 6.5) prior addition to tethered bilayer lipid membranes containing 25mol% cholesterol. Conductance was measured using impedance spectroscopy as described in Chapter 2 sections (2.11.3 and 2.11.4).

3.7 Pre-incubation of CLIC1 with Cholesterol or Ergosterol

CLIC1 (WT) monomeric protein (20µg in 100µL of HEPES/KCl buffer) was incubated for approximately 1 hour with 2µL of 13.3mg of cholesterol or ergosterol dissolved in 1mL of 99% (v/v) ethanol prior addition to tBLMs with or without sterols.

3.8 Pre-incubation of Listeriolysin-O with Cholesterol

Listeriolysin-O (Sigma Aldrich), 2µM in 100µL as a final volume of HEPES/KCl buffer was incubated with 2µL of 13.3mg of cholesterol dissolved in 99% (v/v) ethanol before application to membranes with or without cholesterol

Chapter 3 Results

3.9 CLIC1 Conductance in tBLM Containing Cholesterol

In order to characterise the ion channel activity of CLIC1 in membranes and the effect of different sterols on the ion channel function of the protein, CLIC1 was reconstituted into tethered bilayer lipid membranes containing different concentrations of cholesterol where the conductance and capacitance of the membranes were measured by impedance spectroscopy, as previously described.

Our tBLMs and impedance spectroscopy results show that the conductance of CLIC1 is highly dependent on the cholesterol concentration in membranes (Figure 3.1). CLIC1 reduced monomer (reduced protein incubated with 0.5mM TCEP), CLIC1 dimer (oxidised protein incubated with 2mM H_2O_2) showed low conductance in membranes with 0mol% cholesterol which is similar to the control (no added protein- buffer only in membranes with 50mol% cholesterol).

In membranes containing 6.25mol% cholesterol CLIC1 monomer and dimer showed similar conductance to each other. Their conductance was then increased in membranes containing 12.5mol%, 25mol% cholesterol and the conductance was highest in membranes containing 50mol% cholesterol. Regression analysis of the conductance curves of CLIC1 reduced monomer and CLIC1 oxidised dimer by prism pad 6 statistical analysis program indicate that the p value for CLIC1 reduced monomer is 0.0048 with an $R^2 = 0.950$ and CLIC1 oxidised dimer has got a p value of 0.028, $R^2 = 0.841$. Both p values are less than 0.05 which indicate significant difference in conductance of both proteins (CLIC1 monomer and CLIC1 dimer) in membranes with different cholesterol concentrations.

Further statistical analysis suggests that there is no significant difference between the activities of the two proteins (CLIC1 monomer and CLIC1 dimer) in membranes as the p value is 0.388 which is greater than 0.05.

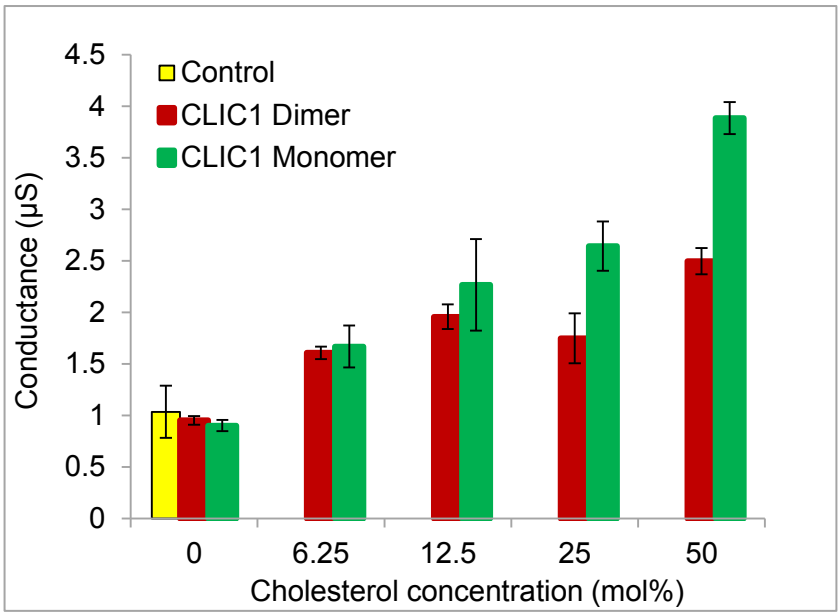


Figure 3.1: Conductance of CLIC1 in tBLMs containing cholesterol. 20µg of CLIC1 oxidised dimer was pre-incubated with 2mM H_2O_2; 20µg of CLIC1 reduced monomer was pre-incubated with 0.5mM TCEP in 100µL of HEPES/KCl buffer (pH 6.5) prior addition into tethered bilayer membranes containing 0, 6.25, 12.5, 25 and 50mol% cholesterol concentrations. Control is membrane with 0mol% cholesterol containing 100µL HEPES/KCl buffer (pH 6.5) with no protein added. Analyses of results were generated using Microsoft Excel 2010 and the error bars represent the standard error of three independent impedance spectroscopy conductance measurements. (n=3)

CLIC1 reduced monomer demonstrates a linear relationship between cholesterol concentration and conductance along with faster initial conductance in membranes, as the slope of conductance measured is 0.0003µS/s (R^2 *value is 0.9853*) which was found to be slightly higher than the CLIC1 oxidised dimer (slope is 0.0001 µS/s and R^2 = *0.9757*) see Figure 3.2 below.

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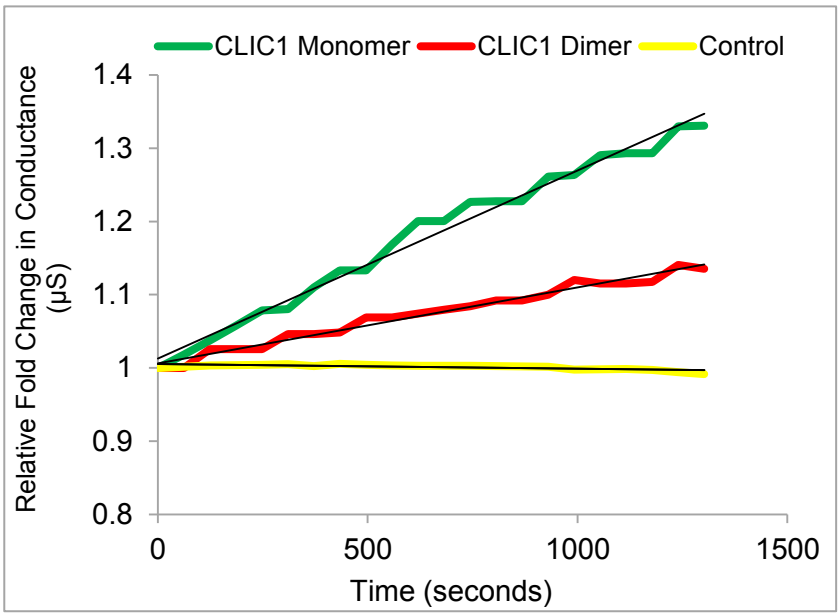

Figure 3.2: Representative impedance spectroscopy recording of CLIC1 conductance in tBLMs containing 25mol% cholesterol. 20µg of CLIC1 monomer was pre-incubated with reducing agent (0.5mM TCEP) or an oxidising agent (2mM H_2O_2) as for CLIC1 dimer in 100µL of HEPES/KCl buffer for ~1 hour before addition to membrane. Linear least squares fitting was performed on the conductance of a representative impedance spectroscopy recording of CLIC1 against time after the addition of CLIC1 monomer, CLIC1dimer into tethered bilayer lipid membranes containing 25mol% cholesterol. Control is membrane with 25mol% cholesterol containing 100µL buffer only with no protein added. Plot, linear trend lines and slops were generated using Microsoft Excel 2010 (***$y= 0.0003x + 1.0129, R^2= 0.9853$*** for CLIC1 monomer and ***$y= 0.0001x + 1.0063, R^2= 0.9757$*** for CLIC1 dimer).

Furthermore, addition of different concentrations of CLIC1 reduced monomer in membranes containing 25mol% cholesterol shows that as the concentration of CLIC1 increases, the conductance of protein in membrane also increases. As indicated in Figure 3.3, that there is a linear relationship between conductance and low concentrations of CLIC1 reduced monomer (10µg and 20µg) where it has a faster rate of conduction (slope is 0.0769µS/s and R^2 *of 0.9978*) than CLIC1 dimer (slope = 0.0487 µS/s and R^2 *of 0.9664*). However at higher concentrations CLIC1 monomer (40, 60 and

80µg in 100µL buffer) the conductance against concentration plot has a quadratic curve. That is as the protein concentration increases the conductance also increases proportionally to the square root of the protein concentration as in a horizontal parabola-like behaviour.

In contrast, CLIC1 oxidised dimer was found to have linear relationship between high protein concentration and conductance. The initial conductance rate for CLIC1 reduced monomer was higher (slope is 0.0924µS/s with $R^2 = 0.9985$) than CLIC1 oxidised dimer (slope = 0.0399µS/s and $R^2 = 0.9857$).

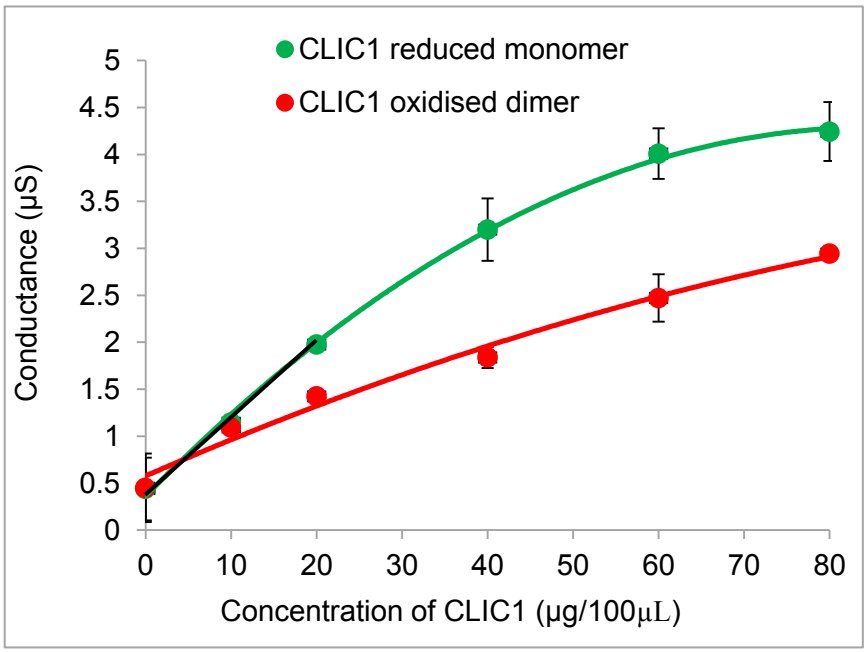

Figure 3.3: Conductance of different concentrations of CLIC1 in tBLMs containing 25mol% cholesterol. Concentrations of 0, 10, 20, 40 and 60µg of CLIC1 reduced monomer (pre-incubated with 0.5mM TCEP) and CLIC1 oxidised dimer (pre-incubated with H_2O_2) in 100µL of HEPES/KCl buffer (pH 6.5) were added into membranes containing 25mol% cholesterol where the conductance was measured and linear fitting (as indicated in black for CLIC1 reduced monomer and red for oxidised dimeric CLIC1) and quadratic polynomial fits were generated using Microsoft Excel 2010 ($y= -0.0005x^2 + 0.0924x + 0.3639$, $R^2= 0.9985$ for CLIC1 monomer and $y= -0.0001x^2 + 0.0399x + 0.5761$, $R^2= 0.9857$). The error bars represent the standard error of three experimental repeats. (n=3)

In order to examine the effect of CLIC1 protein addition to bilayer lipid membranes themselves, membrane capacitance was measured following the addition of CLIC1 (reduced monomer or oxidised dimer) to tBLMs containing 25mol% cholesterol. As shown in Figure 3.4, the capacitance across the bilayer membranes increased slightly with time (slope or rate of capacitance change ranges from $1e^{-5}$ for control to $4e^{-5}$ and $7e^{-5}$ nF/s for CLIC1 reduced monomer, oxidised dimer, respectively). This slight increase in capacitance indicates that membranes become slightly thinner following addition of CLIC1 protein to the tBLMs however overall membrane integrity remains.

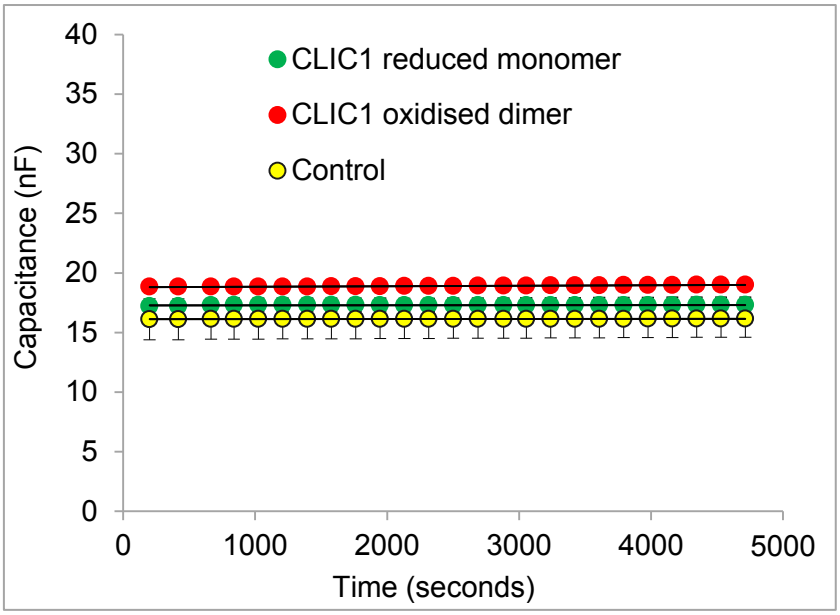

Figure 3.4: Capacitance of tBLMs containing 25mol% cholesterol and CLIC1. Capacitance of membranes containing 25mol% cholesterol and 20µg of CLIC1 reduced monomer (pre-incubated with 0.5mM TCEP) and oxidised dimer (pre-incubated with H_2O_2) in 100µL of HEPES/KCl buffer (pH 6.5) were recorded every 3-5 minutes after CLIC proteins addition to membranes. Linear least squares fits, trend lines and slopes were generated using Microsoft Excel 2010 and the changes in membranes containing CLIC1were compared to control (membrane containing 100µL of buffer only with no protein added) ($y= 7E^{-06}x + 17.274$, $R^2= 0.1167$ for CLIC1 monomer, $y= 4E^{-05}x + 18.811$, $R^2= 0.9697$ for CLIC1 dimer and $y= 1E^{-05}x + 16.106$, $R^2= 0.8048$ for control). The error bars represent the standard error of three repeats of capacitance measurements. (n=3)

As a negative control, adding denatured-boiled CLIC1 onto membranes made with 25mol% cholesterol did not cause any change in membrane conductance, as shown in Figure 3.5.

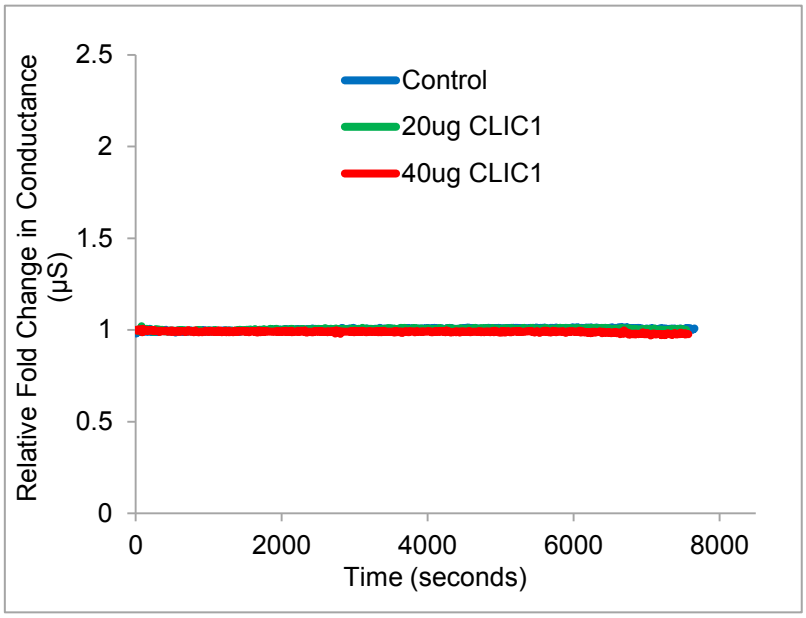

Figure 3.5: Representative impedance spectroscopy recording of boiled CLIC1 in tBLMs containing 25mol% cholesterol. 20µg or 40µg of CLIC1 monomeric protein in 100µL of HEPES/KCl buffer containing 0.5mM TCEP (pH 6.5) was added to tBLMs containing 25mol% cholesterol. Control is membrane with 25mol% cholesterol containing 100µL HEPES/KCl buffer (pH 6.5) only with no protein added. This graph was generated using Microsoft Excel 2010.

3.10 Conductance of CLIC1 in tBLMs Containing Ergosterol

Our results from Figure 3.6 indicate the maximum conductance measurements of CLIC1 protein are achieved in membranes containing either cholesterol or ergosterol. It can also be seen that CLIC1 monomer and CLIC1 dimer were both conductive in bilayer membranes containing 25mol% ergosterol. Also the activity or conductance of CLIC1 protein in membranes with ergosterol was detected to be higher than the conductance measured for CLIC1 monomer in membranes containing cholesterol; as was indicated by the 3.7 fold increase in conductance for CLIC1 monomer and 2.8 fold

increase in conductance for CLIC1 dimer when they were incorporated into tBLMs containing 25mol% ergosterol in comparison to membranes containing 25mol% cholesterol.

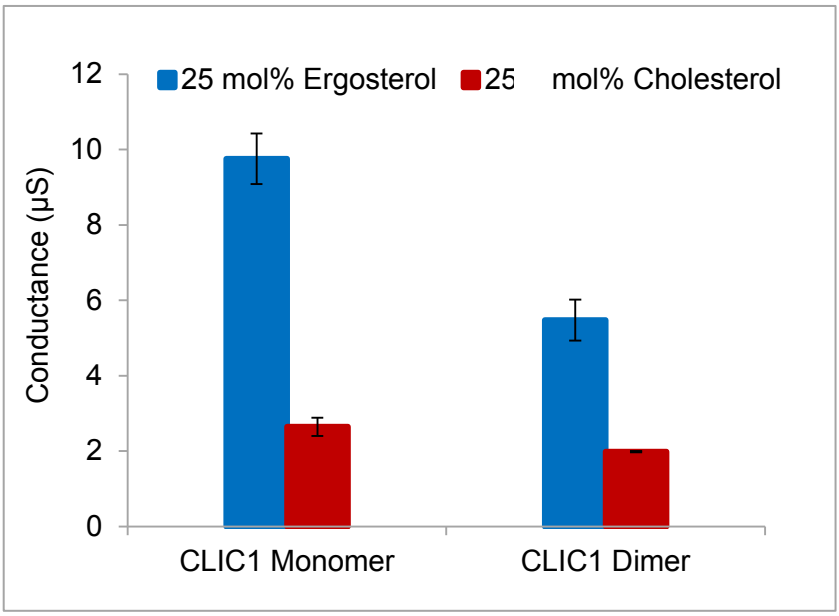

Figure 3.6: Conductance of CLIC1 in tBLMs containing 25mol% ergosterol. 20µg of CLIC1 monomer (pre-incubated with 0.5mM TCEP) and dimer (pre-incubated with 2mM H_2O_2) in 100µL of HEPES/KCl buffer (pH 6.5) were incorporated into membranes containing zwitterionic lipids and 25mol% ergosterol. Analyses of conductance measurements were carried out using Excel 2010. The error bars represent the standard error of three independent experimental repeats. (n=3)

Following the addition of the reduced CLIC1 monomer to tBLMs containing 25mol% ergosterol, it was noted that CLIC1 reduced monomer conductance has a linear relationship with time which indicates that the rate of change in conductance is proportional to the rate of change in time and has a slope of 0.0036µS/s with $R^2 = 0.9949$. However CLIC1 oxidised dimer showed a quadratic or horizontal parabola-like curve (slope is 0.0036 µS/s and $R^2 = 0.996$) which indicates that the conductance of CLIC1 oxidised dimer is proportional to the square root of time as shown in Figure 3.7.

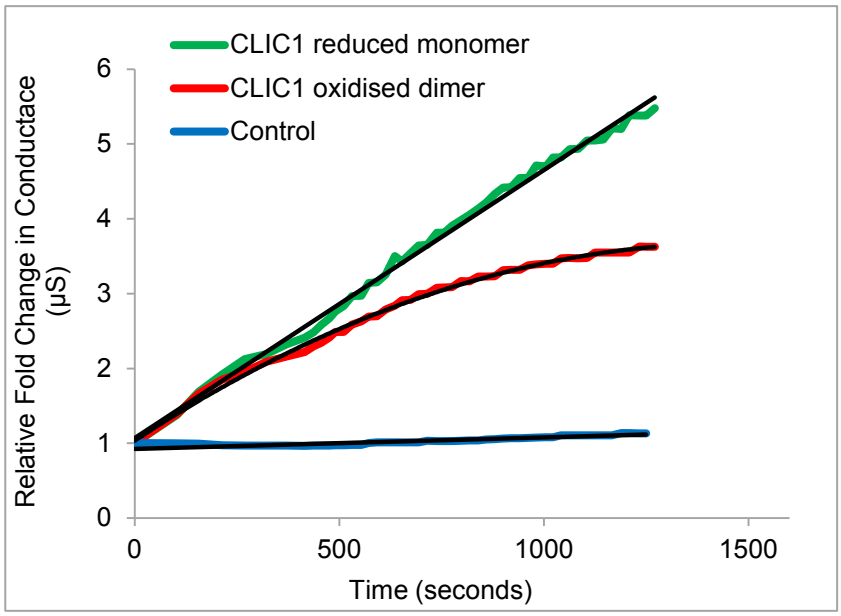

Figure 3.7: Representative impedance spectroscopy recording of CLIC1 added to tBLMs with 25mol% ergosterol. 20µg of CLIC1 reduced monomer (pre-incubated with 0.5mM TCEP) or oxidised dimer (pre-incubated with 2mM H_2O_2) in 100µL HEPES/KCl buffer (pH 6.5) was added to tethered bilayer lipid membranes containing 25mol% ergosterol. Conductance graph, trend lines and slops were obtained using Microsoft Excel 2010 (***y= 0.0036x + 1.0676, R^2= 0.9949*** for CLIC1 monomer and ***y= -1E$^{-0.6}x^2$ + 0.0036x + 1.0365, R^2= 0.996*** for CLIC1 dimer).

Bacterial membranes contain about 40% phospholipids and 60% membrane proteins and importantly they do not contain sterols except for mycoplasma and methylotrophic bacterial species (185-188). CLIC1 monomer added to tBLMs made from yeast lipid extracts showed higher conductance than CLIC1 monomer in membranes with bacterial lipid extracts (Figure 3.8).

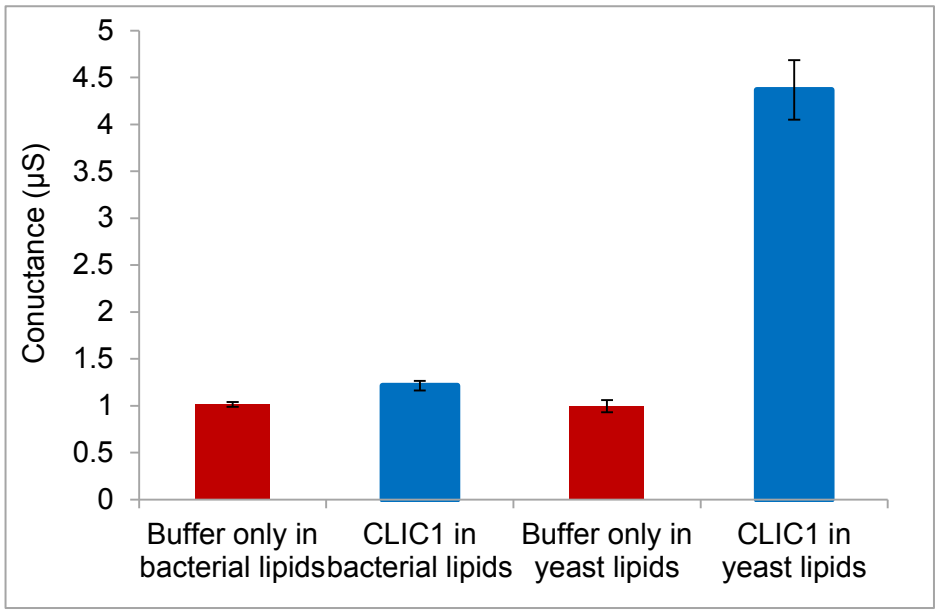

Figure 3.8: Conductance of CLIC1 monomer in tBLMs containing yeast (*saccharomyces cerevisiae*) or bacterial (*E-coli*) lipid extracts. 20µg of CLIC1 reduced monomer protein in 100µL HEPES/KCl buffer (pH 6.5) was pre-incubated with 0.5mM TCEP prior addition to membranes containing bacterial or yeast lipid extracts. The controls used in this experiment are yeast or bacterial membranes containing buffer with 0.5mM TCEP and no CLIC1 added. Conductance analyses were generated using Microsoft Excel 2010 where the error bars represent the standard error of three repeats of experimental measures. (n=3)

3.11 Pre-incubated with Sterols Inhibits the Conductance of CLIC1

Others have published studies that more directly investigate the interaction between proteins and sterols in membranes. These were performed via hemolysis assays where CDC pore forming proteins were incubated with free cholesterol prior to addition to the cells. The result was an inhibition of the cytolytic activity of the toxins against erythrocytes (146,147). Using a similar experimental set-up, CLIC1 was pre-incubated with 1% free-cholesterol or ergosterol prior to addition to tBLMs containing 50mol% cholesterol or ergosterol.

Figure 3.9 shows that CLIC1 monomer pre-incubated with cholesterol had a low conductance similar to the activity of Listeriolysin-O (LLO) and buffer only that were also pre-incubated with 1% cholesterol. Interestingly, CLIC1 monomer that was pre-incubated with 1% ergosterol also showed low conductance when compared to CLIC1 monomer in 1% ethanol not pre-incubated with ergosterol or cholesterol in membranes containing 50mol% cholesterol or ergosterol. These results suggest that the pre-incubation of CLIC1 with cholesterol or ergosterol result in inhibition of chloride ion channel activity in membranes.

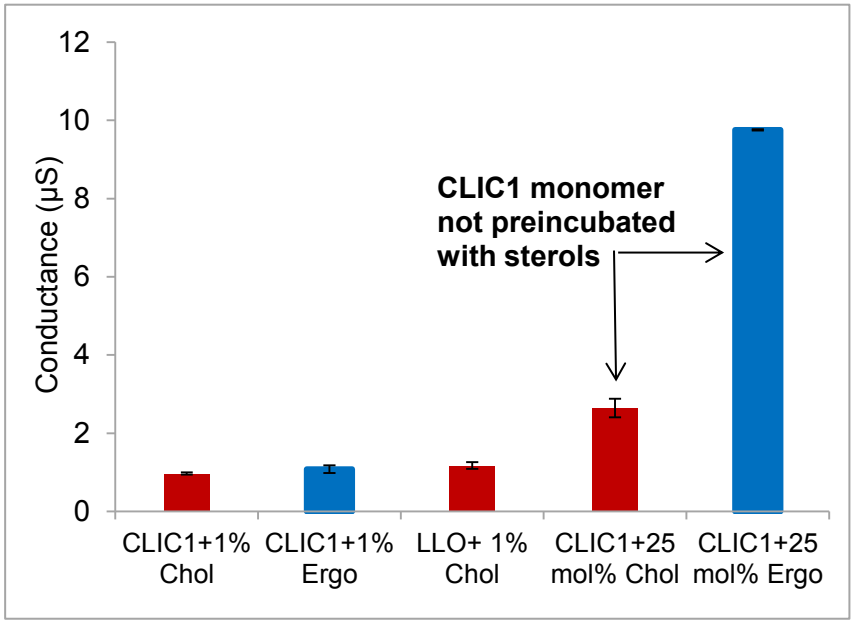

Figure 3.9: Conduction of pre-incubated CLIC1 monomer with sterols in tBLMs containing 50mol% cholesterol or ergosterol. CLIC1 (WT) monomeric protein (20µg in 100µL of HEPES/KCl buffer, pH 6.5) was incubated with 1% cholesterol or 1% ergosterol for ~ 1 hour prior addition to tethered bilayer lipid membranes containing 25mol% cholesterol or ergosterol. Conductance of pre-incubated CLIC1 monomer with sterols was then measured with impedance spectroscopy and compared to Controls: CLIC1 monomer not pre-incubated with sterols added into membranes containing 1% of cholesterol or ergosterol, listeriolysin-O (20µg of LLO in 100µL of HEPES/KCl buffer, pH 6.5) was also incubated with 1 mol% cholesterol under the same conditions as CLIC1 monomer followed by addition to membranes with 50mol% cholesterol. Conductance analyses were generated using Microsoft Excel 2010 where the error bars represent the standard error of three repeats of experimental measures. (n=3)

Membrane capacitance measurements were also recorded – see Figure 3.10. There were no discernible changes or alterations to membrane thickness following the addition of pre-incubated CLIC1 with sterols or control (buffer pre-incubated with 1% of either cholesterol or ergosterol). However it was noted that the rate of change in capacitance of membranes containing CLIC1 pre-incubated with cholesterol or ergosterol has a negative slope ($-5e^{-5}$nF/s) compared to control that had a positive slope of (1^{e-5}nF/s for membranes containing buffer pre-incubated with cholesterol and $4e^{-5}$nF/s for membrane contained buffer pre-incubated with ergosterol). The difference between the positive and negative slopes suggests that addition of pre-incubated CLIC1 to membranes containing cholesterol or ergosterol cause the membranes to become fractionally thicker over time.

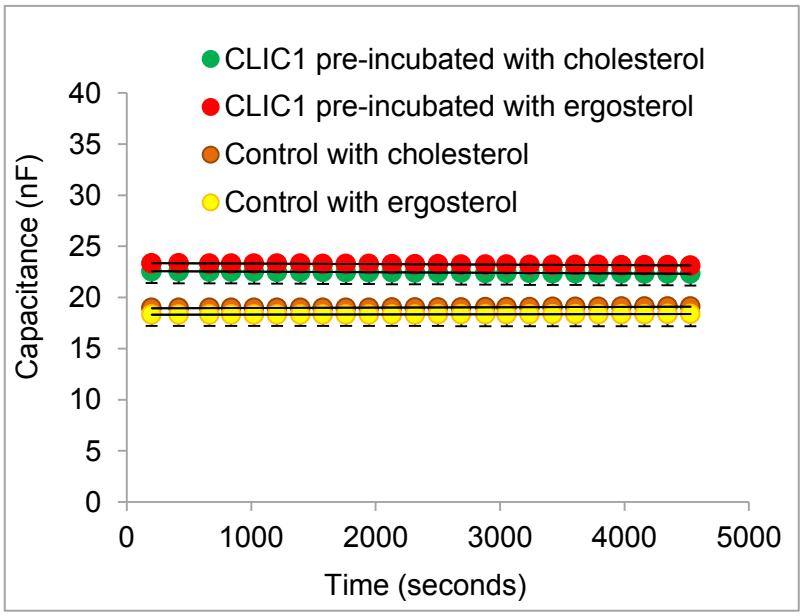

Figure 3.10: Capacitance of tBLMs containing 25mol% cholesterol or ergosterol with CLIC1 pre-incubated with sterols. Capacitance of membranes containing 25mol% cholesterol or ergosterol and 20µg of CLIC1 reduced monomer (pre-incubated with 0.5mM TCEP) in 100µL of HEPES/KCl buffer (pH 6.5) were recorded every 3-5 minutes after CLIC1 addition to membranes. Linear least squares fits were generated on the capacitance plots using Microsoft Excel 2010 and the change in slope of membranes containing CLIC1were compared to control (membrane containing 100µL of buffer pre-incubated with 0.5mM TCEP and 1mol%

cholesterol or 1mol% ergosterol). ($y= -5E^{-05}x + 22.57$, $R^2= 0.9998$ for CLIC1 pre-incubated with cholesterol, $y= -5E^{-05}x + 23.36$, $R^2= 0.9922$ for CLIC1 pre-incubated with ergosterol, $y= 1E^{-05}x + 18.324$, $R^2= 0.9464$ for control pre-incubated with cholesterol and $y= 4E^{-05}x + 18.949$, $R^2= 0.9394$ for control pre-incubated with ergosterol). The error bars represent the standard error of three repeats of capacitance measurements. (n=3)

3.12 Conductance of CLIC1 Mutants and EXC-4 in tBLMs Containing Cholesterol

In order to investigate the role of the critical cysteine residues in CLIC1 (Cys24 and Cys59) in the membrane insertion functional formation of chloride ion channels by CLIC1 in membranes, experiments measuring the conduction of CLIC1 mutants have been carried out where these critical cysteine residues were substituted by alanine or serine. The following annotations will be used to refer to each mutant, with each containing a single amino acid substitution (CLIC1-C24A, CLIC1-C24S and CLIC1-C59A). Furthermore, EXC-4 and CLIC1 wild type monomeric proteins were examined via the tethered bilayer lipid membranes containing 25mol% cholesterol. According to the results in Figure 3.11, it is seen that all proteins including the three CLIC mutants and EXC-4 were more conductive in membranes containing cholesterol, compared to the control which is a sample containing buffer only- no protein added to membrane containing 25mol% cholesterol.

Further statistical analysis was carried out using Graph pad prism 6 excel, according to ANOVA statistical test analysis comparing the conductance values obtained for CLIC1 wild type monomer, CLIC1-C24A, CLIC1-C24S and CLIC1-C59A. The analysis reveals that there is a significant difference in the conductance of the tested proteins in membranes as indicated by one way ANOVA test where the p value obtained is <0.0001 (well below 0.05). Further statistical analysis were performed by carrying out

the Tukey's multiple comparisons statistical test (results as shown in table 3.1) and it was found that CLIC1-C24A and CLIC1-C24S mutants have no significant difference between their conductances; these results are similar to t-test results obtained for the two proteins, CLIC1 wild type monomer and EXC-4.

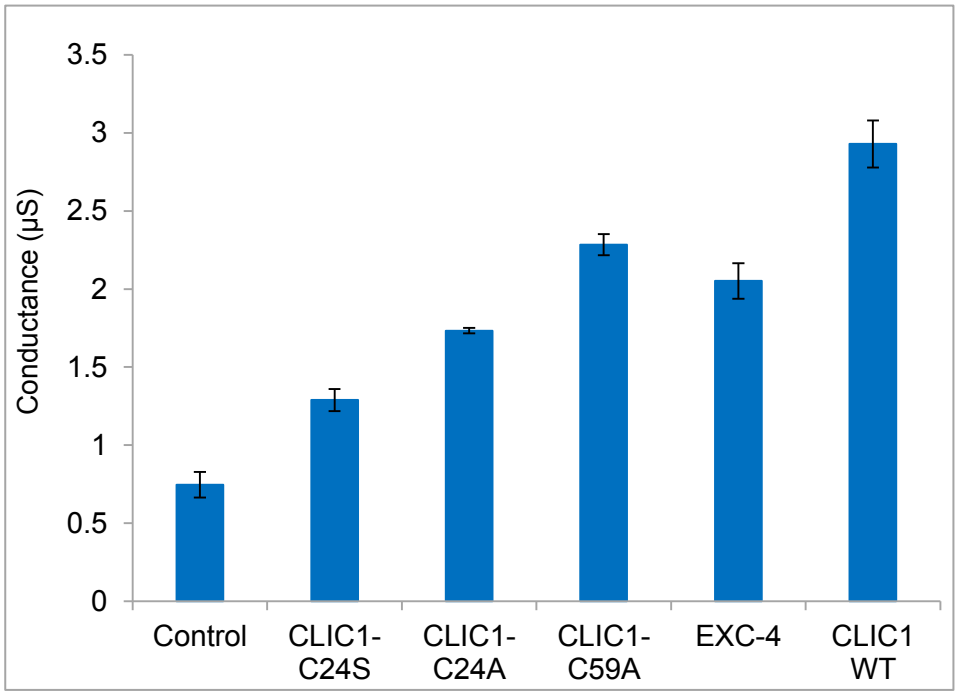

Figure 3.11: Conductance of CLIC1 mutants and EXC-4 in membranes containing 25mol% cholesterol. 20µg of CLIC1-C24A; CLIC1-C24S; CLIC1-C59A; EXC-4 and CLIC1 (WT) proteins in 100µL HEPES/KCl buffer (pH 6.5) were reconstituted in tethered bilayer membranes containing 25mol% cholesterol and the conductance was measured and analysis was performed using excel 2010 and Graph pad prism 6. Control sample is buffer only containing 0.5mM TCEP with no protein added to membrane containing 25mol% cholesterol. The error bars represent the standard error of three independent repeats of conductance measurements. (n=3)

Proteins	Difference in activity
CLIC1 (WT) vs CLIC1-C24A	Significant
CLIC1 (WT) vs CLIC1-C24S	Significant
CLIC1 (WT) vs CLIC1-C59A	Significant
CLIC1-C59A vs C24A	Significant
CLIC1-C59A vs C24S	Significant
EXC-4 vs CLIC1-C59A	Significant
CLIC1 (WT) vs EXC-4	Non-significant
CLIC1-C24A vs CLIC1-C24S	Non-Significant

Table 3.1: Tukey's test results comparing the activity of CLIC1 (WT), EXC-4 and CLIC1 mutants in tBLMs containing 25mol% cholesterol. p values obtained by performing post hoc Tukey's test for multiple sample comparisons and the significance of results was established when the p value is less than 0.05.

Chapter 3 Discussion

3.13 Conductance of CLIC1 is Dependent on Cholesterol in tBLMs

Our tBLMs and impedance spectroscopy results (Figure 3.1) shows that CLIC1 demonstrates high conductance in membranes containing varying concentrations of cholesterol (0mol%-50mol%), Also the initiation of activity or conductance of CLIC1 in tBLMs containing cholesterol is faster and higher when compared to CLIC1 in tBLMs without cholesterol. This cholesterol-dependent response of CLIC1 conductance holds similarities to cholesterol concentration effect on CDCs conduction in membranes (as is the case for listeriolysin-O in Figure 2.16).

CLIC1 reduced monomer and CLIC1 oxidised dimer were able to conduct in tBLMs with 25mol% cholesterol as shown by the representative impedance spectroscopy recording of CLIC1 in Figure 3.2. However this same result was observed in multiple repetitions of the experiment, confirming that the initiation of conductance by CLIC1 reduced monomer in membranes containing cholesterol is faster in comparison to CLIC1 oxidised dimer. These findings are similar to the results obtained by Goodchild et al, 2009 (87). The faster conductance rates of CLIC1 reduced monomer was speculated to be due to the oxidation of CLIC1 monomer in the presence of a membrane that cause the formation of membrane insertion intermediate structure of the protein which can be stabilised by the formation of disulphide bond between Cys24-Cys59 of CLIC1.

Despite using a reducing agent and covering the membrane in order to minimise protein oxidation, it was still not possible to provide a fully reducing environment for the protein as it was exposed to air while adding it into membranes, therefore it is there was a possibility for oxidising the protein over time. So the observed activity of CLIC1 reduced monomeric protein in membrane can be due to the slow oxidation of protein

due to air exposure which has resulted in an increase in protein insertion and therefore an increase in protein conductance in membranes. Due to the presence of up to 8mM GSH in cellular cytosol (222) that makes the cellular environment to be relatively reducing, it is more likely that the soluble CLIC1 protein exists as a reduced monomer in solution rather than being oxidised. Therefore it was proposed that oxidation cannot be the only factor for CLIC1 insertion and chloride ion channel function in membranes.

CLIC1 oxidised dimer is protein prepared in the presence of 2mM H_2O_2, pre-incubated in buffer containing 2mM H_2O_2 where this same buffer was used for the formation of tethered bilayer lipid membranes. We speculate that the slow conduction rate of CLIC1 oxidised dimer is due to the locked structure of CLIC1 by the strong pre-formed disulphide bond between Cys24 and Cys59. Therefore CLIC1 oxidised dimer may not be able to associate with the membranes; however over time some of the oxidised dimeric CLIC1 protein showed slower initiation of conduction in membranes which indicates that CLIC1 protein may insert slowly into membranes as some of CLIC1 dimers may convert back to be monomers as suggested by Goodchild et al, 2009 (88).

Interestingly, adding varied concentrations of CLIC1 into membranes containing 25mol% cholesterol showed a linear relationship with protein conductance (Figure 3.3). These results indicate that CLIC1 dimer and CLIC1 monomer in solution already exist in the multimerisation or oligomerisation state required for the formation of functional ion channels in membranes. However at higher concentrations of CLIC1 monomer there is an apparent saturation in the membranes that may not be a real saturation of the relationship between CLIC1 monomeric protein concentration and the ion channel conductance. As the protein concentration increases in the same area or size of membrane, there will be rapid multiple insertion events occurring causing the membrane to saturate in a quicker time, as was described previously by Tulk et al, 2002

(8). Also CLIC1 protein does not seem to cause major changes or alterations to membrane capacitance (as in Figure 3.4) which indicates that CLIC1 (reduced monomer, oxidised dimer or oxidised monomeric) proteins do not just associate with the membranes containing cholesterol but instead they insert to form ion channels that do not significantly affect the thickness of membranes.

It is clearly shown from all the above results that cholesterol plays regulatory roles on the ability of CLIC1 monomeric or dimeric protein to bind, insert and assemble to form stable chloride ion channels in membranes. There are many mechanisms that would explain why proteins tend to assemble in membranes with high cholesterol concentrations and one of which involves the interaction of specific segments or motifs of the proteins at the membrane interface with certain lipid components such as cholesterol in membranes (223,224). It has been suggested that there are specific segments in the integral membrane proteins that was thought to facilitate the interactions of proteins with membranes, e.g. the interaction of scaffolding protein flotillin and caveolins with cholesterol rich domains in membranes (223,224). Such segments called the CRAC (cholesterol recognition amino acid consensus) motif which is located near the transmembrane helix of some proteins and is represented by **L/V**XXXXX**R/K** or **Y**XXXXX**R/K**, where the X represents any amino acid (225,226). Also another CRAC segment that has a main role in sequestering proteins into cholesterol rich domains is the rich in tryptophan residue motif found in the fusgenic protein of HIV glycophorin-41 or gp41, represented by **LWYIK** (227,228). When the leucine was substituted with isoleucine the interaction of protein with cholesterol was found to be fully supressed, where the replacement of leucine with alanine or valine both protein mutants were found to be weakly active in sequestering cholesterol when compared to the wild type protein that contains the **L**WIYK motif (228). Interestingly,

human CLIC proteins (except for CLIC3) contain the conserved motif of **L35**WLKG adjacent to their PTMD domain (Figure 3.12). Further investigations are required in order to study the contribution of this motif to the high conductance of CLIC1 in membranes containing cholesterol.

Further investigations of CLIC1 amino acid sequence alignments, it has been shown that it also contains the motif (**G**XXX**G** as in the sequence **G18**AKI**G22**) near the PTMD structure that is also highly conserved amongst all the human CLIC proteins as shown in Figure 3.12. This motif (GXXXG or GXXXG-like motif where the Glycine residues are substituted by alanine or serine) was detected mediate the interactions of transmembrane helices and therefore it is important for the homo and hetero-oligomerisation of membrane proteins such as Glycophorin A (229), ErbB (230) family members of growth factor receptor tyrosine kinases; the multispan membrane protein APH-1 (231) and also F_0F_1-ATP synthase that contains a single **G**XXX**G** motif and was detected to loose ability to oligomerise following glycine mutation to leucine amino acid (232). Also recently the **G**XXX**G** motif was speculated to be the cholesterol binding site in the Amyloid precursor protein (233). Interestingly some of the CDCs also contain this motif such as Listeriolysin 529aa that contains one copy of the motif (**G91**YKD**G95**); Perfringolysin-O 499aa which contains 2 copies (**G68**KKA**G72** and **G151**KVS**G155**); Intermedilysin 532aa contains (**G163**LKN**G167**) and also α-Hemolysin protein seems to also contain the motif (**G84**AST**G88**) in its structure however it does not contain the amino acid lysine **(K)** which seems to be present in the motif of all human CLIC proteins and the mentioned CDCs.

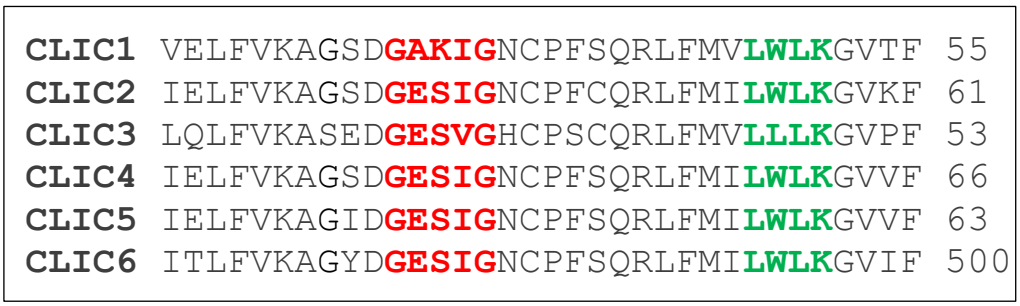

Figure 3.12: Amino Acid Sequence Alignment of Human CLIC proteins showing the CRAC motif. Highlighted in red is the GXXXG motif and in Green highlighted the LWLK motif in human CLICs. CLIC1 (accession number: CAG46868.1), CLIC2 (accession number: CAA03948.1), CLIC3 (accession number: NP_004660.2), CLIC4 (accession number: CAG38532.1), CLIC5 (accession number: AAF66928.1), CLIC6 (accession number: NP_444507.1). The alignment was produced using Clustalw.

Using the previous findings of CLIC1 conductance in membranes with little or no cholesterol, in conjunction with our cholesterol-dependent response of CLIC1 conductance in tethered bilayer lipid membranes, we speculate that cholesterol may facilitate the quaternary assembly of CLIC1 ion channels within the membrane. Further investigations are required in order to determine how cholesterol may affect the oligomerisation/assembly of CLIC1 in order to form functional ion channels in membranes.

3.14 CLIC1 Possesses Higher Conductance in tBLMs Containing Ergosterol

As shown in Figure 3.6, incorporating CLIC1 into tBLMs containing 25mol% ergosterol resulted in a dramatic increase in membrane conductance compared to membranes containing cholesterol of equal molar fraction, which is a behaviour highly similar to the ergosterol favouritism by the polyene antifungal drugs (Amphotericin B and Nystatin A) as was mentioned in the previous chapter.

The linear curve and the high conductance of CLIC1 monomeric protein in comparison to the horizontal parabola-like curve of CLIC1 dimer (Figure 3.7) indicate that the conductance increase for CLIC1 dimer saturates faster than CLIC1 monomer. This can be due as explained in the previous section that the dimeric protein is structurally locked by the Cys24-Cys59 intramolecular disulphide bond resulting in initially less protein assembly or insertion into membranes as an additional step could be needed in order achieve the continuous formation protein multimers required for the final channel assembly to create conductive ion channels in membranes.

To further confirm these results we have incorporated CLIC1 monomeric protein into membranes containing yeast lipid extracts that contain ergosterol as the main sterol component, *E-coli* lipid extracts and zwitterionic lipids were used as controls as they do not contain any sterols. As shown in Figure 3.8, CLIC1 monomeric protein has demonstrated higher conductance in yeast lipid extracts than membranes containing *E-coli* or zwitterionic lipids only.

According to previous studies, it was shown that at temperatures higher than 15ºC the transition of POPC membranes into the liquid ordered phase occurs at lower concentrations of ergosterol than cholesterol. This suggests that ergosterol is a more effective promoter in POPC membranes, where it may promote the formation of raft-

like domains more strongly than cholesterol (234,235). This finding raises the question whether the formation of sterol-raft domains in membranes aid the structural unfolding of CLIC1, and increase the aggregation or oligomerisation of protein in order to insert more efficiently.

The stability of channels in membranes is also an important factor in maintaining the rate of ion transport across membranes (127,143); this further suggests that the high conductance of CLIC1 in membranes with ergosterol is due to the ability of ergosterol to increase the stability of channels formed by CLIC1 via increasing the rigidity of protein aggregate structures. This may also suggest that CLIC1 monomeric protein aggregates experience a higher rigidity in the presence of ergosterol than in cholesterol membranes and therefore CLIC1 showed higher conductance with faster initiation rates in membranes with ergosterol than membranes containing cholesterol.

As was previously hypothesised, sterols may affect the function of membrane proteins by exerting changes to the physical properties of the lipid bilayer membrane or by the establishment of specific protein-sterols interactions (236,237) such as those that might arise via the GXXXG motif in some proteins, and their interaction directly with cholesterol. Further investigations are required in order to understand or characterise the role of ergosterol and its influence on CLIC1 protein- associations with membranes.

3.15 Inhibition of Ion Channel Activity of CLIC1 by Free Sterols

Cholesterol was speculated to be the binding site or "receptor" in membranes for the cholesterol-dependent cytolysins (CDCs). This was based on experimental observations following pre-incubation of the CDCs with exogenous cholesterol, resulting in suppression of their cytolytic activity (238-249). It was speculated that the pre-

incubation of CDCs with free cholesterol causes the receptor or the binding site of CDCs to be saturated with cholesterol preventing the toxin from binding to the membranes or the cholesterol in the membranes (4,146-148,150,250). It was also observed that listeriolysin-O (LLO), intermedilysin (ILY), streptolysin-O (SLO) and perfringolysin-O (PFO) were able to insert into membranes with ~90% cholesterol depletion but their lytic activity was dramatically decreased (more than 11,000 fold decrease for PFO toxin) (251).

Further studies suggested that a specific concentration of cholesterol is required in order to induce the structural changes of toxins in order to form the pre-membrane complex (251). Herein, we report that pre-incubation of CLIC1 monomeric protein with free sterols (cholesterol or ergosterol) prior to its addition to membranes containing 50mol% sterols resulted in a full abrogation of the ion channel activity of CLIC1 in tBLMs as shown in Figure 3.19. This strongly suggests that CLIC1 binds membrane sterols, and they act as receptors, binding or docking sites for the protein for initial insertion followed by oligomerisation and then the subsequent final quaternary structural assembly forming functional ion channels in membranes.

Membrane capacitance (Figure 3.10) following addition of pre-incubated CLIC1 was found to be slightly lower which indicates that membranes become slightly thicker with time, which most likely reflects the insertion and arrangement of the protein within the membrane.

3.16 Role of Critical Cysteine Residues in CLIC1 Function

The importance and contribution of the two critical cysteine residues to the activity and structural conformation of proteins have been extensively investigated over the past decade. Cholesterol-dependent cytolysins (CDCs) share structural homology near their

C terminal domain which is highly conserved across family members and called the undecapeptide (**E<u>C</u>TGLAWEWWR**) motif. The cysteine residue in the conserved motif was thought to be essential for the haemolytic activity of the cytolysin (252-255). According to experimental studies of Listeriolysin-O mutants where the cysteine residue was substituted with serine or alanine amino acids, it was reported that there was no detectable activity of Listeriolysin-O mutant at pH 7.4. Therefore it was suggested that the cysteine amino acid residue in the motif has a main role in maintaining the structure of the conserved undecapeptide motif rather than being directly involved in CDC activity (256).

Similar studies have been performed with CLIC1, where the importance of Cys24-Cys59 and the dimer formation of the protein were investigated. This is also significant, given that Cys59 is unique to CLIC1, unlike Cys24 which is conserved across all human CLICs (88). In a former study by Littler et al, 2004 (84), it was suggested that the formation of the disulphide bond in CLIC1 (Cys24-Cys59) is essential for its ion channel activity. Chloride efflux and tip-dip experiments of CLIC1 mutants were performed where the hydrophobic Cys24 and Cys59 residues were substituted with the hydrophilic amino acid serine. The result was that none of the mutants demonstrated ion channel activity. It was predicted that upon insertion of CLIC1 into membranes, the Cys24 residue would be located on the luminal (*trans*) side of the membrane whereas the other cysteine, Cys59 in CLIC1 will be at the cytosolic (*cis*) side. Following experiments with NEM (cysteine reactive compound) it was found to block the channel activity of CLIC1 from the *trans* side only, it was therefore proposed that the activity of CLIC1 is trans-redox regulated at which Cys24 plays an important role in regulating the ion channel activity of CLIC1 (47).

In order to further investigate the role of Cys24, Singh et al, 2006 (47) generated a mutant of CLIC1 in which Cys24 was replaced by alanine which has similar properties to the cysteine, both are hydrophobic and neutral amino acid residues. CLIC1-C24A has demonstrated the ability to insert into planar bilayer membranes to form functional chloride ion channels that show the same ions selectivity and characteristics to CLIC1. Therefore it was concluded that Cys24 was not essential for protein insertion into membranes but that it may be involved in the regulation of CLIC1 ion channel selectivity or gating activity.

Here in, we report that under reducing conditions all CLIC1 mutants (C24A, C24S and C59A that according to size exclusion chromatography profiles they were all found to have the similar structural fold to CLIC1 (WT) protein (84)) were conductive in tethered bilayer lipid membranes containing cholesterol as shown in Figure 3.11. In comparison to wild-type CLIC1 and EXC-4 which is CLIC-like protein from *Caenorhabditis elegans* that contains an aspartic acid instead of the active cysteine residue, where leucine amino acid residue located next to the predicted transmembrane domain was thought to contribute to the ion channel activity of EXC-4 in the luminal membranes of many cell types (35,38). It can be shown that the conductance level is different amongst all CLIC1 mutants studied here, as CLIC1-C24A and CLIC1-C24S were found to show similar ion channel activity in membranes as there was no significant difference indicated by statistical test analysis of three experimental repeats, whereas CLIC1-C59A showed higher conductance than the Cys24 mutants. These results suggest that we oxidation and CLIC1 dimer formation are not essential for the insertion and ion channel function of CLIC1 in membranes. However the decrease in the conductance of the mutants CLIC1-Cys24 and Cys59 reveals that both cysteine residues are important for the optimal conductance of CLIC1 in membranes, and the

further decrease in the activity of CLIC1-C24A or C24S suggests that this cysteine residue likely plays an important role in the insertion or assembly of the protein in membranes via interacting with other residues in the protein structure that enhances the assembly, oligomerisation and insertion of protein into membranes or it assists in the stabilisation, maintenance and regulation of ion channel activity of CLIC1 in membranes. There are still other likely candidate regions and other structural components in CLIC1 that may contribute to the protein function as an ion channel.

Stomatin family members of peripheral membrane proteins (including stomatin like proteins in mammals: STOML 1-3 and the kidney specific podocin) were originally thought to regulate the ion channel activity of other proteins. Stomatin proteins associate with membranes enriched with lipid rafts, cholesterol and sphingolipids via palmitoylation, where the major and minor cysteine residues in the protein structure (Cys30 and Cys87) is covalently attached to fatty acids such as palmitic acid in membranes. The structure of stomatin consists of an N-terminal domain, which is the membrane inserting domain containing a conserved proline amino acid residue. The N and C-domains were predicted to face the cytoplasm of cells suggesting a unique hairpin loop topological structure for stomatin where the proline residue was found to maintain the anchoring domain of stomatin in membranes (257). CLIC proteins also contain a conserved proline residue near the protein active site, Cys24 in the PTMD that require further testing in order to investigate the potential role of these proline residues in CLIC1 and other CLIC members.

Conclusion

Using tBLMs and impedance spectroscopy as a feasible system to study and characterise the function of membrane inserting proteins and peptides and according to the results discussed in this chapter, membrane sterols appear to play an essential role in the spontaneous membrane insertion of CLIC1 protein. The high cholesterol-dependent conductance of CLIC1 in tBLMs suggests that membrane sterols appear to act as receptors or binding candidates for CLIC1 that may aid in protein's unfolding, oligomerisation or aggregation, insertion and functional formation of ion channels in membranes.

Chapter 4

CLIC Proteins Demonstrate Glutaredoxin-Like Enzymatic Activity

4.1 Introduction

Aerobic respiration is one of the processes in the body that cause the generation of oxidant molecular products and reactive oxygen species (ROS) that include: hydrogen peroxide, superoxide radical and hydroxyl radical (258-260). ROS may interact with proteins, lipids, nucleic acids and modify them to cause different levels and degrees of toxicity that are found to be associated with numerous diseases such as cancer, neurodegenerative and cardiovascular diseases (261-264). Due to the redox properties and high sensitivity of the cysteine amino acid in proteins to ROS, it is considered to be the key amino acid for protein folding, metal coordination and enzyme catalysis (265-268). ROS can cause modifications to the cysteine thiols in proteins, resulting in the generation of more oxidised forms of the cysteine residues, as either sulfenic (-SOH) or sulfinic ($-SO_2H$) acids, which are considered to be reversible oxidation derivatives of the protein thiol. In addition, ROS modifications can also result in the disulfide bonds or sulfonic acid ($-SO_3H$), which are more stable or irreversible oxidation products (269-271). The disulfide bonds between protein cysteine residues can be intra or intermolecular bonds that contribute to the tertiary or quaternary structure of proteins (269). Therefore the redox state of cysteine residues within a protein can regulate the protein's structure and as a consequence, alter protein's cellular functions. Furthermore, mixed disulfide bonds may form between the cysteine thiol of a protein and different molecules such as the reduced tripeptide glutathione (GSH) in a reaction called glutathionylation, which is another reversible protective mechanism of protein thiols that can occur under oxidative stress (272-275).

There are several protective antioxidant systems in cells that help to maintain a reduced intracellular environment in order to achieve homeostasis and normal cellular

functioning. One of these systems is a family of proteins known as thioredoxins (Trxs). These proteins were discovered in 1964 as electron donors participating with the ribonucleotide reductase (RNR) which is an enzyme providing deoxyribonucleotides for DNA synthesis and proliferation (276,277). Trxs are low molecular weight thiol-disulfide oxidoreductases (12kDa) (278). They are highly abundant in cells with a great number of isoforms existing in different species. Their structure contains the thioredoxin fold that consists of four to five stranded anti-parallel β-sheets surrounded by 3 to 4 α-helices. Within the loop connecting β-sheet 1 and α-helix 1, exists a conserved G-site with the motif [**Cys**-Gly-Pro-**Cys]** (279,280)**.** The main function of these proteins is to maintain the redox state of proteins by a cysteine-thiol-disulfide exchange process. Their own regeneration occurs via a thioredoxin reductase (TrxR) enzyme and electrons derived from NADPH (281,282) as illustrated in Figure 4.1.

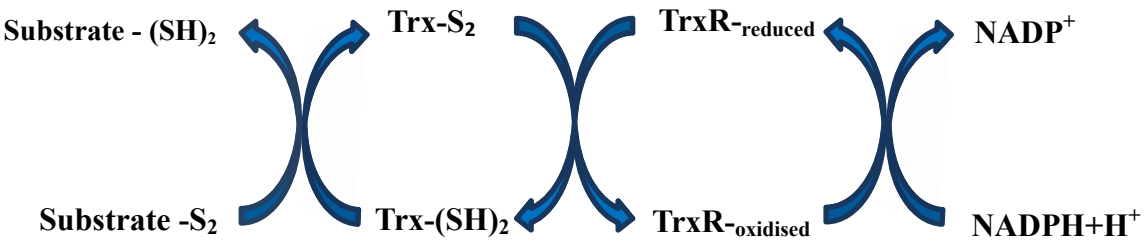

Figure 4.1: The thioredoxin system. Target substrates (which can be proteins) are reduced by thioredoxin enzymes causing the Trxs themselves to become oxidised. TrxR in turn transfers electrons from NADPH to the oxidised Trx, hence regenerating the Trx, which is then ready for another round of substrate reduction. Modified figure was taken from (282).

In 1976, a second group of proteins, the glutaredoxins (Grxs), were discovered as a dithiol hydrogen donor system for ribonucleotide reductase in *E-coli* cells lacking thioredoxin-1 (Trx-1) (283). Grxs (molecular weight ~10-12 kDa) are conserved in both prokaryotes and eukaryotes and are found to function as general thiol-disulfide oxidoreductases to reduce protein disulfides/or mixed disulfides formed between GSH-

and proteins (284). Their structure is highly similar to the Trxs in that they also contain a G-site motif that is highly conserved across all the Grxs (285-287). Grxs can be monothiol, containing a single cysteine residue at their G-site motif located at the N-terminal domain [**Cys**-Gly-Phe-Ser] or a dithiol motif [**Cys**-Pro-Tyr-**Cys**] (280,288).

Two monothiol Grx systems have been identified in mammals, the mitochondrial Grx-5 (also called PICOT-protein kinase C-interacting cousin of thioredoxin) and Grx-3 (289-291). Monothiol Grxs were found to be involved in many functions such as in biogenesis of iron-sulfur clusters, iron homeostasis, cell growth and proliferation (269).

Protein disulfide reduction occurs via the single cysteine residue at the active site motif of monothiol Grxs that interact with the GSH of the GSH-mixed disulfide moiety of the target protein (292). Due to the high affinity of Grxs to GSH (293,294), a covalent Grx-SG complex is formed causing the release of reduced protein or the non-GSH moiety (295,296). The mixed intermediate, Grx-SG will be reduced by another GSH molecule causing the generation of oxidised glutathione or glutathione disulfide (GSSG) that then will be regenerated and reduced back to GSH by the enzyme glutathione reductase (GR) by accepting electrons from NADPH as shown in Figure 4.2 (282,295,296).

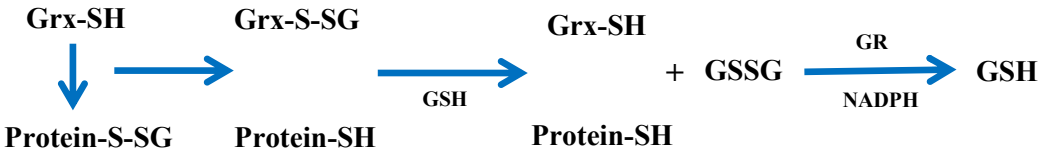

Figure 4.2: The monothiol mechanism of glutaredoxins. Grxs reduce the mixed disulfide bonds of proteins bound to GSH by interacting with the GSH moity of the proteins resulting in the release of reduced proteins. Grxs then will be reduced again by GSH that gets regenerated by GR and NADPH. Modified Figure was taken from (282).

On the other hand, in the dithiol glutaredoxin system, the reduction of the disulfide bonds of proteins can be achieved by the use of the two cysteine residues contained within their active site motif (286). The first cysteine residue initiates a nucleophilic attack on the sulphur atoms of the disulfide bonds causing the formation of mixed disulfides between Grx and the target protein (288). The second cysteine residue becomes deprotonated and attacks the mixed disulfides causing the target protein to be reduced and as a consequence the Grxs become oxidised (Figure 4.3) (282,288). The oxidised Grx is then reduced by GSH. As a result GSH is oxidised (GSSG) and is regenerated by GR via accepting electrons from NADPH. In humans there are also two dithiol Grxs, Grx-1 (297) and Grx-2 (298,299) that were found to participate in multiple activities including ribonucleotide, dehydroascorbate reduction and also in the glutathionylation reactions (297,298).

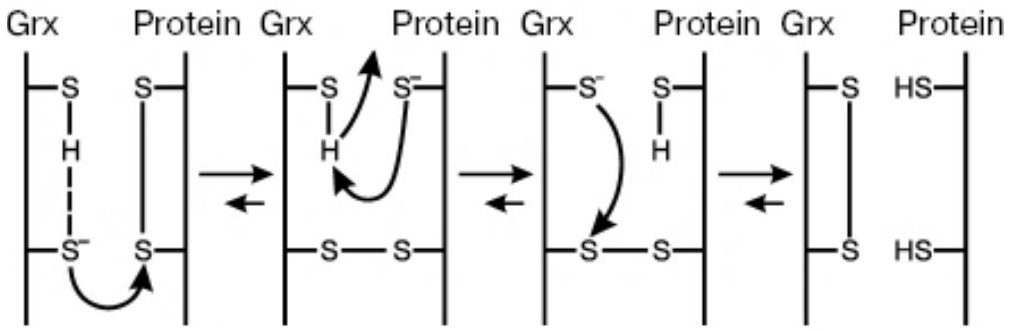

Figure 4.3: Dithiol mechanism of glutaredoxins. Grxs initiate a nucleophilic attack on one of the sulfur atoms of the oxidised protein via the first cysteine residue in the active site motif of Grxs. This process results in the formation of mixed disulfides between the Grx and the oxidised protein. The second cysteine residue attacks the mixed disulfide bond causing the release of reduced protein and oxidised Grxs. Figure was taken from (282).

The Glutathione S-Transferase (GST) superfamily of enzymes is another important detoxification system in living cells that participates in this process by removing the by-products generated as consequences of oxidative stress in cells (300). As mentioned in Chapter 1 section 1.3, that GSTs are involved in many critical cellular functions, one of which is detoxification of drugs and xenobiotics. Their enzymatic detoxification function involves the conversion of the lipophilic xenobiotics or non-soluble toxic chemicals, by conjugating them to water soluble molecules such as reduced glutathione or GSH in order for the metabolites to become less toxic or more water-soluble, which allows them to be more easily eliminated out of the cellular environment (300,301).

The GST-Ω class was found to be distinct to other GSTs in that it exhibits glutathione-dependent thiol transferase activity and GST-Ω has been shown to catalyse the glutathionylation reactions and glutathione-dependent reduction of dehydroascorbate (DHA) (72,302). The enzymatic activity of GST-Ω resembles that of the glutaredoxins (74,303).

As mentioned previously, X-ray crystallography of CLIC1 revealed that the soluble form of CLIC1 adopts a fold that is similar to the GST superfamily, and in particular the GST-Ω class (70). The structure of CLIC1 consists of an all α-helical C-terminal domain and an N-terminal domain with a thioredoxin fold comprised of four β-strands sandwiched between three α-helices that contains the glutaredoxin-like monothiol motif [**Cys**-Pro-Phe-Ser]. The active cysteine residue, Cys24 in CLIC1, was found to covalently bind GSH in a manner similar to GST-Ω class of proteins that possesses a monothiol G-site [**Cys**-Pro-Phe-Ala] (72). Most human CLIC proteins (CLICs1, 4, 5 and 6) contain the monothiol active site motif [**Cys**-X-X-Ser], while CLICs 2 and 3 contain the dithiol motif [**Cys**-X-X-**Cys**] (70).

Due to the high level of structural homology between CLIC proteins, GST-Ω, Grxs and Trxs which are well-known enzymes, it has been speculated that the soluble form of the CLICs would also function as oxidoreductase enzymes (70,78). This chapter describes the investigations undertaken to ascertain any putative enzymatic activity of the soluble form of the CLIC proteins using a set of assays typically designed to measure the activity of glutathione-dependent enzymes.

Chapter 4

Materials and Methods

4.2 Chemicals and Reagents

The following reagents were all purchased from *Sigma Aldrich*: Recombinant thioredoxin-1 (Trx-1) and glutaredoxin-1 (Grx-1) Thioredoxin reductase (TrxR) from rat liver and glutathione reductase (GR) from yeast, reduced glutathione (GSH), sodium selenite (Na_2SeO_3), reduced nicotinamide adenine dinucleotide phosphate (NADPH), dehydroascorbic acid (DHA), 2-hydroxyethyl disulphide (HEDS), cholesterol (99% purity), dithiothreitol (DTT), indanyloxyacetic acid (IAA-94), anthracene-9-carboxylic acid (A9C), and 4,4`-diisothiocyano-2,2`stilbene-disulfonic acid (DIDS), bovine plasma thrombin and insulin.

Saxitoxin was supplied by National Research Council (NRC) of Canada Institute of Marine Biosciences (*Halifax, NS, Canada*).

4.3 Expression and Purification of Recombinant Wild-type CLIC1, CLIC2, CLIC4, Dimeric CLIC1, HcTrx-5 and CLIC1 Mutant Proteins

CLIC1 (WT) protein was expressed in *E. coli* BL21 (DE3) using the His-tag pET28a vector and purified as previously described in Chapter 2, sections 2.6.1-2.10. CLIC2, CLIC4 and CLIC1 dimeric proteins were kindly provided by Dr Louise Brown and group at Macquarie University, Sydney, Australia. They were expressed and purified as outlined previously in (169) for CLIC2, CLIC4 as in (71) and CLIC1 dimer as previously described (89).

Mutant versions of CLIC1: CLIC1-C24A, CLIC1-C24S and CLIC1-C59A were expressed and purified as previously described in Chapter 3, sections 3.2 and 3.3.

4.4 Preparation of Recombinant HcTrx-5 Protein

The cDNA clone encoding the protein HcTrx-5 isolated from *Haemonchus contortus* (304) was kindly provided by Associate Professor Mary Davey, from the University of Technology, Sydney, Australia. Purified recombinant protein HcTrx-5, was prepared as described below

4.4.1 Small Scale Cultures

Glycerol stocks of *E-coli* bacteria containing pTrcHisB vector (Invitrogen, Australia) to express HcTrx-5 WT protein were provided by Professor Mary Davey and group at University of Technology, Sydney, Australia. The glycerol stock containing was then inoculated in 100µL of 2xYT media (1g Yeast extract, 1.6g Tryptone and 0.5g NaCl; pH 7.0) containing 100µg/mL ampicillin antibiotic (Sigma Aldrich) and left to grow overnight at 37°C, 200rpm.

4.4.2 Large Scale Culture and Induction of HcTrx-5 Protein Expression

A stab from the overnight small scale cultures were inoculated to 1L of 2xYT medium containing 100µg/mL ampicillin antibiotic (Sigma Aldrich) and left to grow with shaking at 37°C, 200rpm for about 2 hours until an OD of 600 is reached. The expression of protein was then enhanced by inducing the bacterial cultures with 1mM IPTG. Bacterial cultures incubated with agitation and left to grow overnight at 20°C, 200rpm.

4.4.3 Harvesting and lysing HcTrx-5-transformed *E-coli* Cells

The IPTG induced cells were centrifuged at 12000 rpm for 15 minutes at 6°C and the resultant pellets were scraped resuspended in the Native IMAC lysis buffer containing 1mg/mL lysozymes and 10% N-lauryl sarcosine from (Bio-Rad). Resuspended *E-coli* cells were incubated on ice for 1 hour before sonication on ice, with 10 seconds pulses at 60% output. Cell lysate was then centrifuged at 12000g for 20 minutes at 4°C. The supernatant was kept on ice and prepared to the purification of protein.

4.4.4 Purification and Quantification of HcTrx-5 (WT) Protein

His-tagged HcTrx-5 protein was purified using the Native IMAC purification Kit and the Profinia purification system (Bio-Rad), following manufacturer's instructions. Then the purified HcTrx-5 protein was quantified as described previously in Chapter 2, section 2.9.1. Also for further confirmation of protein purity, it was run on SDS-PAGE as outlines in Chapter 2, section 2.10.

4.5 Condition for Enzyme Assays

All assays were performed in flat 96-well plates, with a final volume of 200µL and absorbance read using a BioTek PowerWave^{TM} Microplate Spectrophotometer.

4.6 HEDS Enzyme Assay

HEDS enzyme assays were carried out following the method described in (303). 5µM final concentration of CLIC proteins: wild type CLLIC1 reduced monomeric, CLIC1 oxidised dimeric, CLIC2, CLIC4, and CLIC1 mutants: CLIC1-C24A, CLIC1-C24S, CLIC1-C59A, 5µM of HcTrx-5 or Grx-1 (control proteins) were added into a mixture of 5mM potassium phosphate buffer (pH 7), 1mM EDTA, 250µM NADPH, 50nM GR and 1mM HEDS. The mixture containing the proteins was incubated for 5 minutes at 37ºC; with the reaction being initiated by addition of 1mM GSH and the consumption of NADPH was monitored at A_{340nm}.

4.7 HEDS Enzyme Assay for CLIC Proteins in the Presence of Thioredoxin Reductase

5µM final concentration of either CLIC1, CLIC2, CLIC4 or Trx-1 (control protein) were added to 0.1M Tris-HCl buffer (pH 7.5) containing 1mM EDTA, 200µM NADPH and 50nM TrxR (from rat liver). The mixture was incubated for 5 minutes at 37ºC, with the reaction initiated by addition of 750µM HEDS. Consumption of NADPH was monitored at A_{340nm}.

4.8 Insulin Disulfide Reductase Assay

Insulin disulfide reductase assay was used to measure the reduction of insulin disulfides by dithiothreitol (DTT) in the presence of 5µM of Trx-1 or CLIC1 following the method described in (305,306). A stock solution of 20mM concentration insulin was obtained by dissolving lyophilized insulin in diluted HCl (pH 3). Insulin was further diluted to 0.13mM final concentration in the insulin disulfide reductase assay. The reaction was performed in the presence of 50mM Tris, 2mM EDTA buffer (pH 7.5)

containing 0.33mM DTT, 5μM CLIC1 or Trx-1 (control protein). The change in solution turbidity due to insulin reduction was measured by monitoring absorbance at λ_{650nm} for 30 minutes.

4.9 Glutaredoxin-like Activity of CLIC1 using Sodium Selenite

The assay was performed following the method in (307). 5μM final concentration of CLIC1 or Grx-1 (control protein) was added to a mixture of 0.1mM Tris-HCl buffer (pH 7.5) containing 1mM EDTA, 200μM NADPH, 50nM GR, 0.1mg/mL bovine serum albumin and 15μM sodium selenite (Na_2SeO_3). The mixture was incubated for 5 minutes at 37ºC before initiating the reaction by adding 50μM of GSH. Consumption of NADPH was monitored at A_{340nm}.

4.10 Assays for Dehydroascorbic Acid Reductase (DHAR) Activity of CLIC1

The assay was performed as described in (308). 5μM final concentration of CLIC1, CLIC4 or HcTrx-5 (control protein) was added to 137mM sodium phosphate buffer (pH 7.5), containing 0.35mM NADPH, 50nM GR and 2mM GSH. The mixture was incubated for 1 minute at 30ºC prior to initiation of reaction with 1mM DHA. Consumption of NADPH was monitored at A_{340nm}.

4.11 Pre-incubating CLIC1 with Ion Channel Blocker Drug and Cholesterol

Stock solutions of 560μM of IAA-94, A9C and DIDS were freshly prepared by dissolving 0.2mg/mL of each drug in deionised water with vortexing until a homogeneous solution of each drug in water was achieved. IAA-94, A9C and DIDS

were further diluted to 10μM in 5mM potassium phosphate buffer (pH 7.5). 5μM final concentration of CLIC1 was incubated with 10μM IAA-94, A9C, DIDs or saxitoxin for 1 hour prior to performing the HEDS enzyme assay. Similarly, 5μM of CLIC1 in 156μL of 5mM potassium phosphate buffer (pH 7.5) was incubated with 0.4, 0.8 and 1.6mM of cholesterol (a concentration 34mM cholesterol in ethanol) for 1 hour on ice (as previously described in (174)) prior to use of the protein sample in the HEDS assay as outlined in section 4.5 above.

Chapter 4 Results

4.12 Investigating the Enzymatic Activity of CLIC Proteins in the HEDS Assay

The activity of the Grxs have typically been investigated using an *in vitro* enzyme assay containing β-hydroxyethyl disulfide, HEDS, a low molecular weight compound found to act as a specific and sensitive substrate for the Grxs (309,310). The HEDS enzyme assay was therefore employed to investigate the putative enzymatic activity of members of the CLIC family.

As seen in Figure 4.4, that Reaction mixtures containing the positive control proteins HcTrx-5 or Grx-1, which are well-known enzymes found to have glutathione-dependent activity, are characterized by an increase in NADPH consumption as indicated by a decrease in the absorbance (A) as measured at 340nm over time. Similar consumption of NADPH is observed when CLIC1, CLIC2 and CLIC4, were substituted for the control proteins in the HEDS assay. This indicates that all the three CLIC proteins tested were able to reduce the disulfide bond of the HEDS substrate when coupled with reduced glutathione (GSH) and glutathione reductase (GR) in the presence of NADPH. CLIC1 and CLIC4 seem to demonstrate similar kinetic and enzymatic activity; however CLIC2 showed an apparent lower activity in the HEDS enzyme assay.

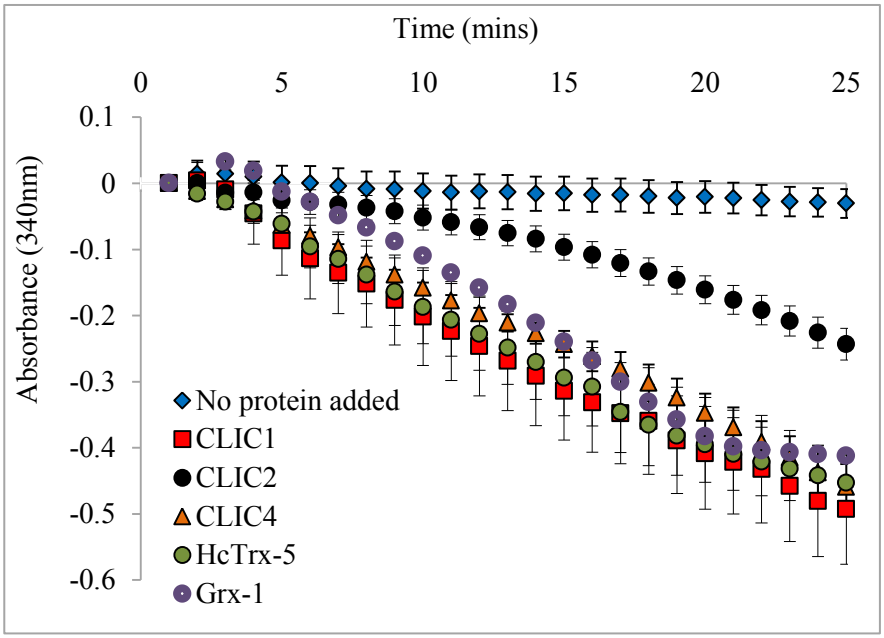

Figure 4.4: Activity of the CLIC proteins in the HEDS enzyme assay. The HEDS enzyme assay was carried out using 5mM potassium phosphate with 1mM EDTA, pH 7 containing 5µM of CLIC proteins (CLIC1, CLIC2or CLIC4) or HcTrx-5 or Grx-1 (control proteins), 250µM NADPH, 1mM HEDS and 50nM GR. The mixture was incubated at 37ºC and then the reaction was initiated by the addition of 1mM GSH and the absorbance of NADPH was monitored at A_{340nm}. Error bars represent the standard error of at least three independent measurements.

4.13 CLIC Proteins Demonstrate Glutathione-Dependent Enzymatic Activity

As outlined in the previous sections, that Trxs are maintained in a reduced state in cells by accepting protons from NADPH via the enzyme thioredoxin reductase (TrxR) (311,312). In order to further investigate the enzymatic activity of CLIC proteins; it was essential to determine whether they resemble the Trxs whereby enzymatic activity is coupled to TrxR and GSH is not required.

CLIC proteins (CLIC1, CLIC2 and CLIC4) were added into an assay system containing TrxR instead of GR with no GSH present. As seen in Figure 4.5, in the presence Trx-1, the absorbance of NADPH decreased over time which indicates that the consumption of

NADPH increased. As expected that Trx-1 was able to reduce the disulfide bonds to the HEDS and gets regenerated by TrxR and NADPH.

However none of the three tested CLIC proteins were able to reduce the HEDS substrate in the presence of TrxR, demonstrating the CLIC proteins do not function in a manner similar to the thioredoxins.

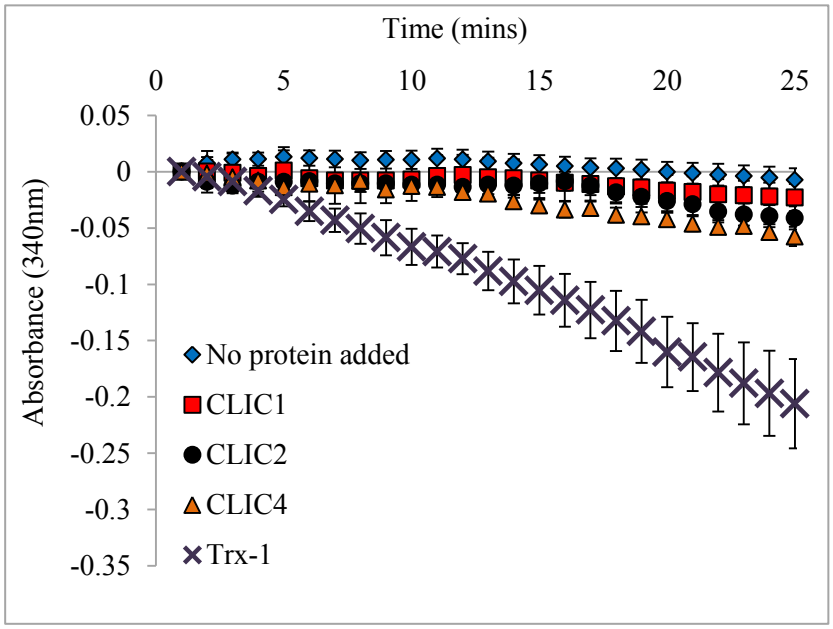

Figure 4.5: Thioredoxin activity of CLIC proteins. The reaction mixture contained 0.1M Tris-HCl (pH 7.5) with 2mM EDTA, containing 5µM reduced CLIC1, CLIC2 or CLIC4 (WT) protein, 200Mm NADPH, 750µM HEDS, 50µM Trx-1 (included as a positive control). The absorbance of NADPH was monitored at A_{340nm}. The control in this assay represents a reaction vessel containing buffer and all the reaction agents except CLICs or control proteins. Error bars represent the standard error of at least three independent measurements.

Another common assay used to assess oxidoreductase activity by the thioredoxins is the insulin disulfide reductase assay as described by Holmgren (305,306). In this assay the reduction of insulin disulfides by DTT is catalysed by Trx-1, resulting in increased solution turbidity via precipitation of the free insulin B chain (305,306). CLIC1 was found to have no catalytic activity in this system when compared to the positive control Trx-1 (Figure 4.6).

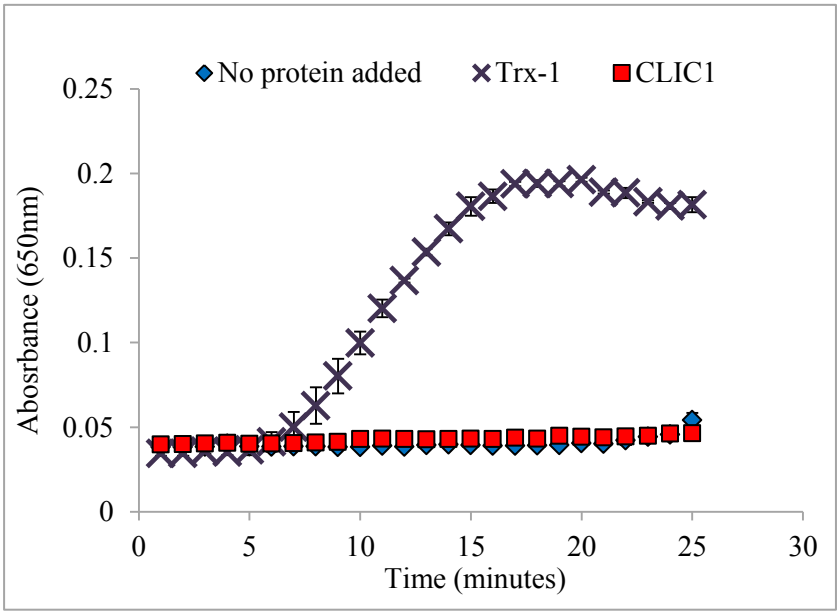

Figure 4.6: Insulin reductase activity of CLIC1. Insulin disulfide reductase assay was performed by having a reaction mixture containing 2mM EDTA in 50mM Tris-HCl (pH 7.5), 0.17mM insulin, 0.33M DTT, 5µM reduced CLIC1 or 5µM Trx-1 (included as a positive control). The reaction was monitored at A_{650nm} over 30 minutes. Error bars represent the standard error of at least three independent measurements.

4.14 Cysteine-24 Residue is Essential for the Enzymatic Activity of CLIC1

In order to determine critical structural residues within CLIC1, which are involved in its oxidoreductase enzymatic activity, a number of CLIC1 mutants were produced. The Cys_{24} residue located within the monothiol active site in CLIC1 was replaced by either

alanine (CLIC1-C24A) or serine (CLIC1-C24S). The enzymatic function of these mutants was then measured in the HEDS enzyme assay. In addition, Cys59, which is unique to CLIC1 and in involved in forming an intramolecular disulphide bond with Cys24 that results in CLIC1 dimer formation, was also mutated to alanine (CLIC1-C59A). All CLIC1 mutants were tested in the HEDS assay. As seen in Figure 4.7, both Cys24 mutants, CLIC1-C24A and CLIC1-C24S demonstrated no enzymatic activity. However, the mutant CLIC1-C59A was capable of reducing the HEDS substrate in the presence of GR and GSH, with a Km of 1.25±0.65µM, which is equivalent to that of the wild type CLIC1 monomer (Km of 1.28±0.65µM) (Figure 4.8).

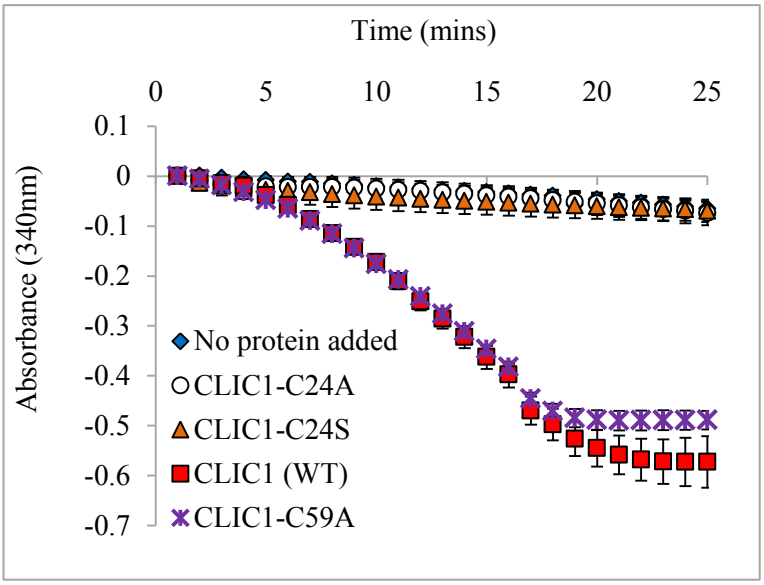

Figure 4.7: Comparison of the oxidoreductase activity of CLIC1 (WT) monomer and CLIC1-Cys mutants. A reaction mixture of 5mM potassium phosphate (pH 7) with 1mM EDTA buffer containing 250µM NADPH, 50nM GR, 1mM HEDS and 5µM CLIC1 (WT) monomer, CLIC1-C24A, CLIC1-C24S or CLIC1-C59A that was incubated for 5 mins at 37ºC. The reaction was initiated with the addition of 1mM GSH and the absorbance of NADPH was monitored at A_{340nm}. Error bars represent the standard error of at least three experimental repeats.

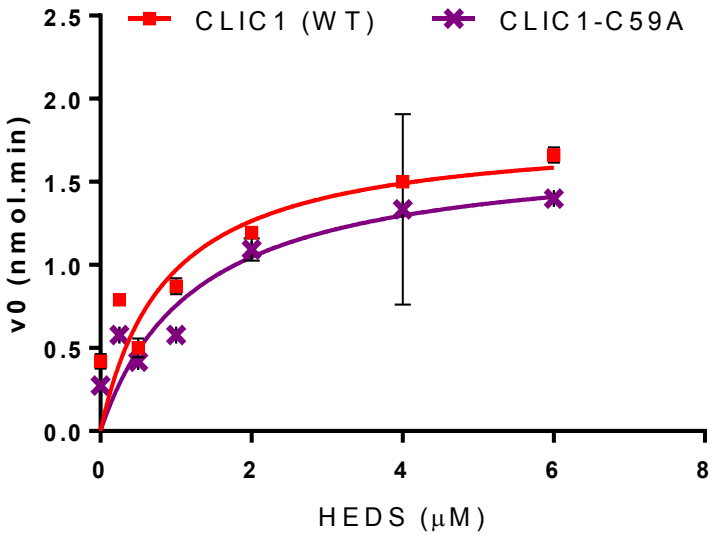

Figure 4.8: Michaelis menten plot of CLIC1 and CLIC1-C59A mutant in the presence of HEDS. A mixture of 5mM potassium phosphate (pH 7) with 1mM EDTA containing 250µM NADPH, HEDS (0, 0.25, 0.5, 1, 2, 4 or 6mM), 50nM GR and 5µM of CLIC1 (WT) monomer or CLIC1-C59A protein was incubated at 37ºC for 5 minutes, prior addition of 1mM GSH. The absorbance of NADPH was monitored at A_{340nm}. (n=3)

As was explained previously that upon oxidation, soluble CLIC1 forms a non-covalent dimer, where the N-terminal thioredoxin fold domain structure is completely altered, disrupting the glutaredoxin-like active site (84). The dimer is stabilized via an intramolecular disulfide bond between Cys24 and Cys59. The dimer form of CLIC1 was therefore tested for oxidoreductase enzymatic activity in the HEDS assay system in order to investigate the involvement of Cys59 residue in the enzymatic function of CLIC1. As seen in Figure 4.9, that CLIC1 dimer was also found to function as an enzyme in the HEDS enzyme assay as it was able to reduce the disulphide bonds of the HEDS substrate. It is also demonstrated that the rate of oxidised NADPH production of by CLIC1 dimer is 0.02µM/min, which is similar to the monomeric CLIC1, with a rate of 0.03µM/min.

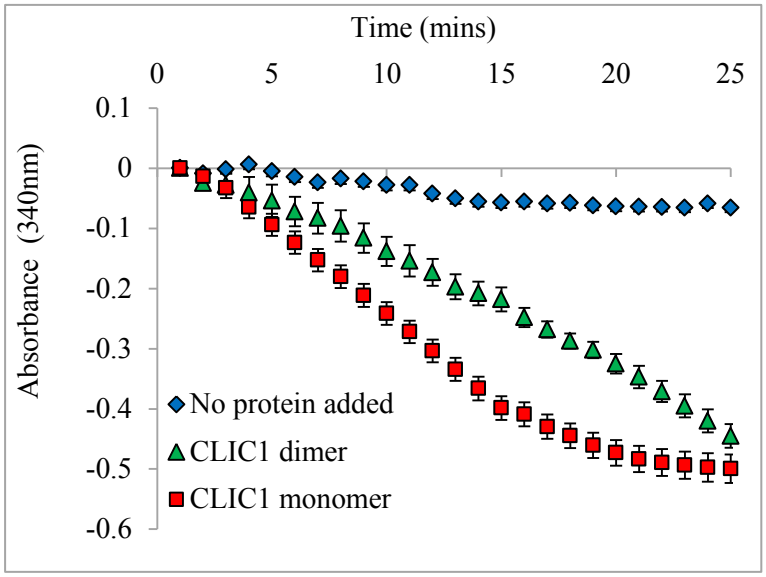

Figure 4.9: HEDS enzyme assay for CLIC1 monomer and dimer proteins. 5mM potassium phosphate with 1mM EDTA, pH 7 buffer containing 250µM NADPH, 1mM HEDS and 50nM GR and 5µM of CLIC monomer or CLIC1 dimer proteins were all incubated at 37ºC. The reaction was then initiated by the addition of 1mM GSH and the absorbance of NADPH was monitored at A_{340nm}. The control in this assay represents a reaction vessel containing buffer and all the reaction agents except CLICs or control proteins. Error bars represent the standard error of at least three independent measurements.

The rates were calculated in Microsoft Excel using the linear equation ($y = mx + b$), where m is the slope of the linear curve of the graph (in order to determine the change in the absorbance of NADPH at 340nm wavelength (Δ340nm)), and b is the y-intercept. The determined slope was then multiplied by the extinction coefficient of NADPH (6.22 x $10^{-3}\mu M^{-1}cm^{-1}$.

4.15 Sodium Selenite and Dehydroascorbic Acid are Substrates for CLIC1

Glutaredoxins are known to be involved in the reduction of dehydroascorbic acid (DHA) to ascorbic acid (AA) (308,312), which is a vital process for normal cellular function (303). Also Grxs were found to reduce the selenite anion (307). Therefore the

HEDS substrate was substituted with sodium selenite and DHA in the enzyme assay in order to investigate the ability of CLIC1 to reduce the DHA and selenite.

A glutaredoxin-like activity assay was carried out in the presence of CLIC1 (WT) or hGrx-1 (as a control protein) and sodium selenite (Na_2SeO_3) as the substrate, with the reaction initiated by the addition of GSH. Figure 4.10 shows, that in the presence of CLIC1 or Grx-1, the consumption of NADPH is stoichiometric to the selenite anion which suggests that CLIC1 was also able to reduce the sodium selenite, in a manner similar to Grx-1.

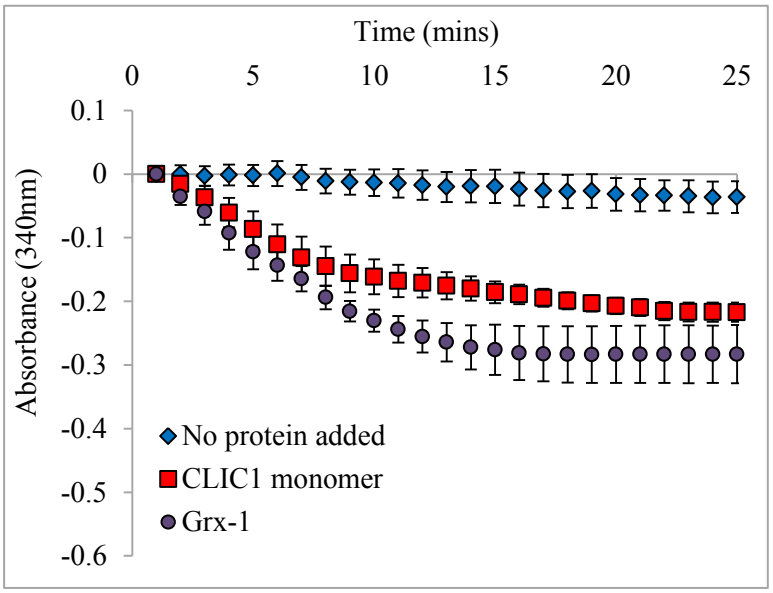

Figure 4.10: Enzyme assay of CLIC1 in the presence of sodium selenite. A reaction mixture of 0.1mM Tris-HCl (pH 7.5) with 1mM EDTA containing 200mM NADPH, 50nM GR,15µM sodium selenite, 0.1mg/mL BSA and 5µM CLIC1(WT) monomer or 5µM Grx-1 as a control was incubated for was initiated by the addition of 50µM GSH at 20ºC. The consumption of NADPH was measured at A_{340nm}. Error bars represent the standard error of at least three experimental repeats.

A titration of the sodium selenite substrate (0-16µM) demonstrated that CLIC1 has a relatively high Km (4.81±3.00µM) (Figure 4.11), relative to the normally lower

concentration of selenium found in most cells (<1µM) (313). This result suggests that the binding affinity of CLIC1 for sodium selenite is low and as a result product formation is dependent on the availability of sodium selenite.

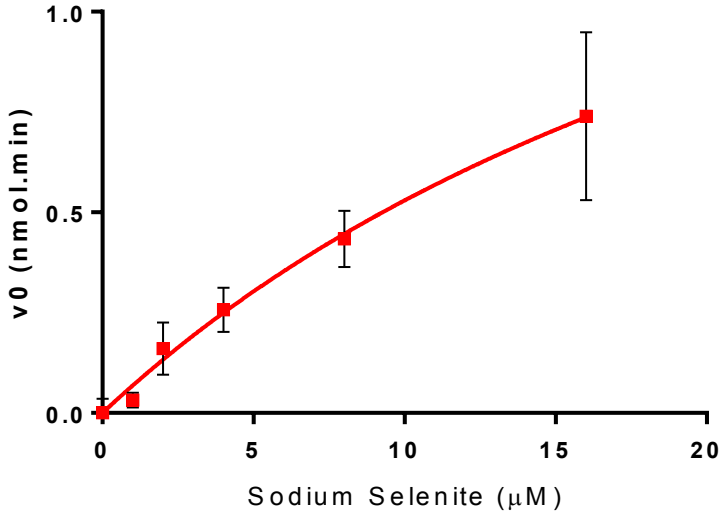

Figure 4.11: Michaelis Menten plot of CLIC1 and sodium selenite. 0.1mM Tris-HCl (pH 7.5) with 1mM EDTA containing 0.2mM NADPH, 50nM GR, 5µM CLIC1 (WT) monomer and sodium selenite (0, 1, 2, 4, 8 or 16µM). The initiation of the reaction was achieved by adding 50µM GSH at 20ºC where the consumption of NADPH was measured at A_{340nm}. (n=3)

In Figure 4.12, it can be seen that NADPH consumption increased in the presence of CLIC1 or CLIC4 and they demonstrate similar activity to *HcTrx-5,* a known dehydroascorbate reductase (DHAR) from the parasitic worm *Haemonchus contortus* (304).

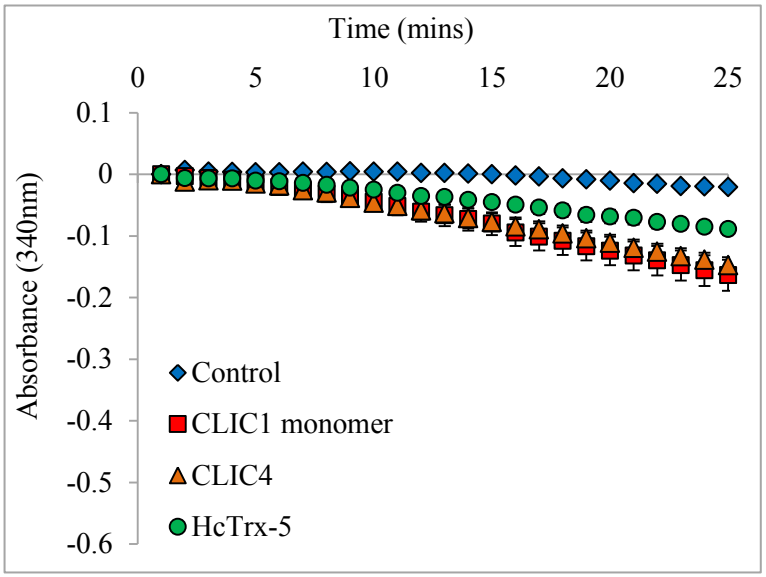

Figure 4.12: Dehydroascorbic acid as a substrate for CLIC1. The oxidoreductase enzymatic reaction using DHA as a substrate was performed in 137mM sodium phosphate buffer (pH 7.5) containing 2mM EDTA, 0.35mM NADPH, 50nM GR, 2mM GSH and 1mM DHA. The reaction was initiated after addition of 5µM reduced CLIC1, CLIC4 or HcTrx-5 (as control). The consumption of NADPH was measured at A_{340nm}. Error bars represent the standard error of at least three experimental repeats.

Kinetic studies using 5µM CLIC1 protein and different concentrations of DHA (0-6mM) indicate a linear relationship (Figure 4.13). This indicates CLIC1 has a strong binding affinity for DHA, suggesting soluble CLIC1 would likely be saturated with by DHA under normal intracellular conditions.

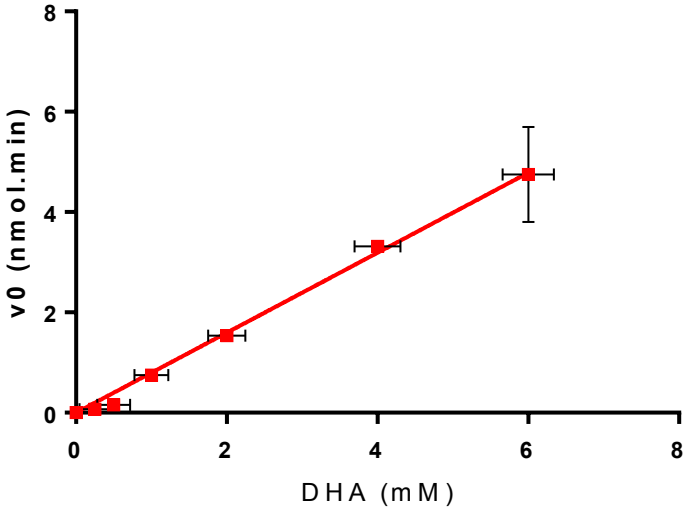

Figure 4.13: Michaelis Menten plot of CLIC1 and dehydroascorbic acid (DHA). A mixture of 137mM sodium phosphate buffer (pH 7.5) with 2mM EDTA, 0.35mM NADPH, 50nM GR, 2mM GSH and DHA (0, 0.25, 0.5,1, 2, 4 or 6µM). The reaction was initiated after the addition of 5µM CLIC1 (WT) protein and the NADPH consumption was monitored at A_{340nm}. (n=3)

4.16 Inhibition of CLIC1 Enzymatic Activity by Chloride Ion Channel Blocker Drugs But Not Cholesterol

According to previous electrophysiological studies it was shown that the drugs IAA-94 and A9C block CLIC1 ion channel activity in cells and artificial membranes, while DIDS has no significant effect on the ion channel function of CLIC1 (44). *In vitro* studies also confirm that IAA-94 inhibits CLIC channels produced by adding recombinant soluble CLIC1 into artificial bilayers (8,49,70).

In order to investigate the effect of IAA-94, A9C and DIDs exert any effect on the enzymatic activity of CLIC1; the protein was pre-incubated with the each of the drugs for approximately 1 hour.

As seen in Figure 4.14, both IAA-94 and A9C were able to completely block the enzymatic activity of CLIC1, however DIDS had no effect. In addition, a known sodium ion channel blocker, saxitoxin, which was used a control was found to have no effect on the enzymatic activity of CLIC1 in the HEDS enzyme assay

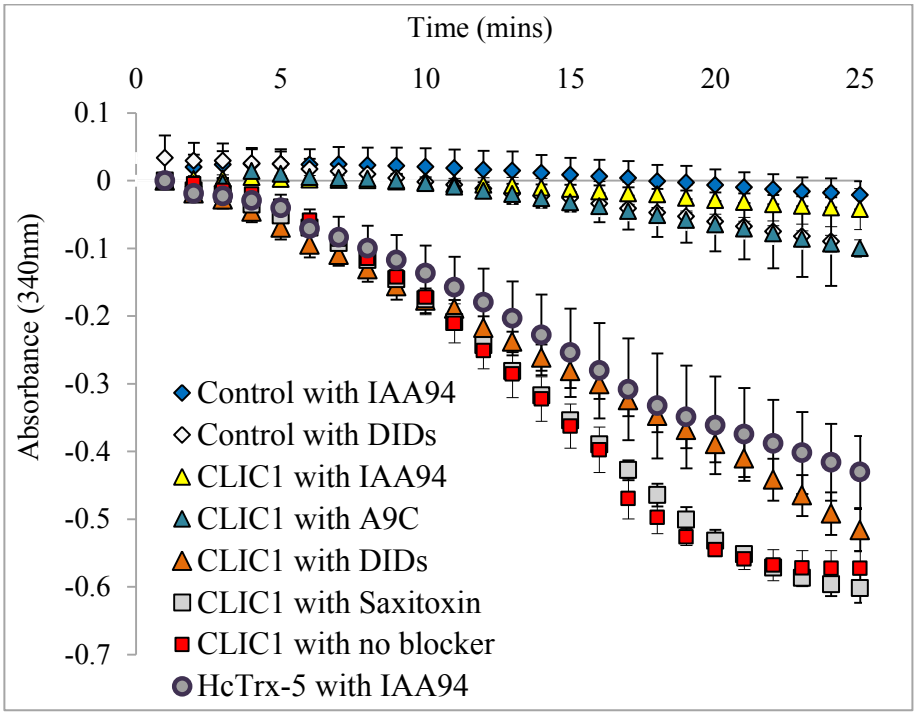

Figure 4.14: Effect of chloride ion channel inhibitor drugs on the enzymatic activity of CLIC1 in the HEDS enzyme assay. 5mM potassium phosphate buffer (pH 7) with 1mM EDTA containing 250µM NADPH, 1mM HEDS, 50nM GR and5µM of CLIC1 reduced (WT) or HcTrx-5 protein pre- incubated with 10µM IAA-94, A9C, DIDS or Saxitoxin for ~1 hour prior use of the protein in the assay. The reaction mixture was incubated for 5 mins at 37ºC and then was initiated by the addition of 1mM GSH. The consumption of NADPH was monitored at A_{340nm} post addition of 1mM GSH. Error bars shown represent the standard error of at least three experimental measurements.

As described in the earlier chapters of this thesis, cholesterol is critical for the insertion and conductance of CLIC1 in artificial membrane systems and that pre-incubation of CLIC1 with 0.4, 0.8 or 1.6 mM cholesterol prior to addition of the protein to membranes inhibited CLIC1 membrane insertion and ion conductance (174). We

therefore assessed whether cholesterol could also regulate CLIC1 enzymatic activity. As seen in Figure 4.15, pre-incubation of the protein with cholesterol resulted in no obvious change in CLIC1 enzymatic activity in the HEDS enzyme assay.

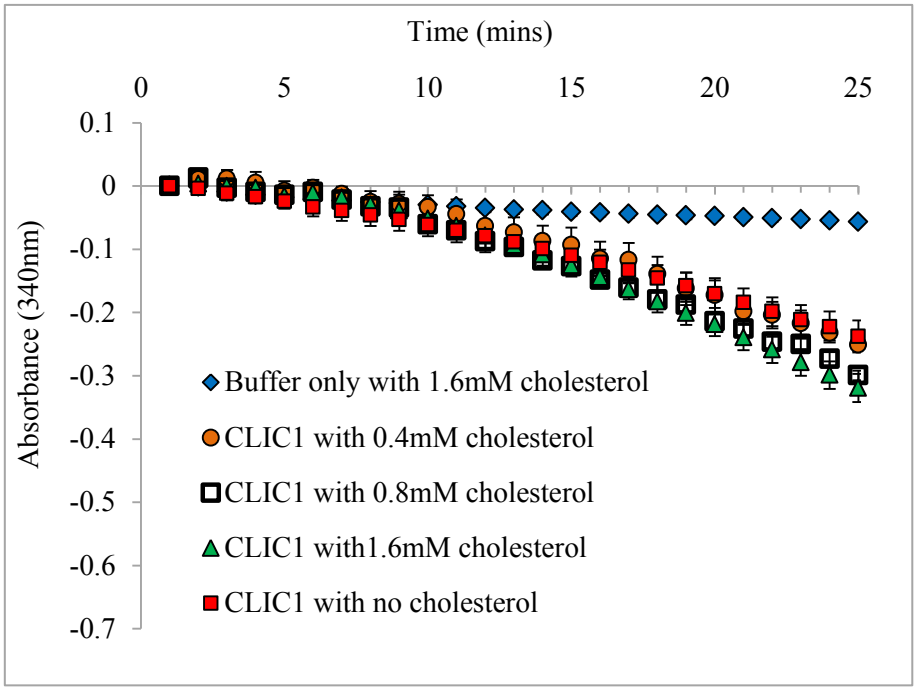

Figure 4.15: HEDS enzyme assay for pre-incubated CLIC1 with cholesterol. 5µM of CLIC1 monomer (WT) protein was incubated with 0.4, 0.8 and 1.6mM cholesterol for ~1 hour prior to its addition to a reaction mixture of 250µM NADPH, 1mM HEDS, 50nM GR in 5mM potassium phosphate buffer with 1mM EDTA, pH 7, at 37ºC. The consumption of NADPH was monitored at A_{340nm} post addition of 1mM GSH. Control included all the reaction components including 1.6mM cholesterol, except with no CLIC1 protein. Error bars shown represent the standard error of at least three experimental measurements.

Chapter 4 Discussion

4.17 CLIC Proteins Demonstrate Oxidoreductase Enzymatic Function

Members of the CLIC family exist mainly as soluble proteins, and under specific conditions they spontaneously insert into lipid membranes to form chloride selective ion channels (78,314,315). The ion channel activity of CLIC proteins has been extensively studied and characterized, however no distinct function has previously been assigned to their soluble form. Based on the high structural homology of CLIC proteins to well-known enzymes such as GST-Ω proteins, it was speculated that the soluble CLIC proteins may function as enzymes (37). However to date, no experimental evidence had been provided to support such assumptions. Here in, we investigate the function of the soluble CLIC proteins by assaying their activity using well-known systems used to measure the glutathione dependent enzymatic activity of proteins.

Protein glutathionylation is an important regulatory mechanism in biochemical processes where reversible modifications of protein thiols occur. It involves the addition of GSH to protein thiols and it aids in the activation and inhibition of some proteins (168,316). As an example, α-ketoglutarate dehydrogenase (317) was found to be inhibited by reversible glutathionylation and the retinal pigment epithelium cells were shown to be activated by the glutathionylation process (318). Glutathionylation of proteins contributes to the regulation of many vital cellular activities such as energy metabolism, ion channel activity, redox signalling as well as protein folding (316,319-321).

Glutaredoxins and GST-Ω class were found to catalyse protein deglutathionylation in order to maintain cellular sulfhydryl homeostasis (168,322). Recent studies suggest that the change in glutaredoxin levels affect protein glutathionylation status and,

subsequently, downstream signalling events (323). The ability of the Grxs to deglutathionylate the mixed disulfide bond forms between the GSH and proteins was measured using an *in vitro* enzyme assay, where Grxs reduce the disulfide bonds form between GSH and the mercaptoethanol region of the HEDS substrate. In this assay the Grxs are maintained in the reduced state by GSH which gets regenerated by accepting electrons from NADPH via GR enzyme (303) (as shown in Figure 4.16)

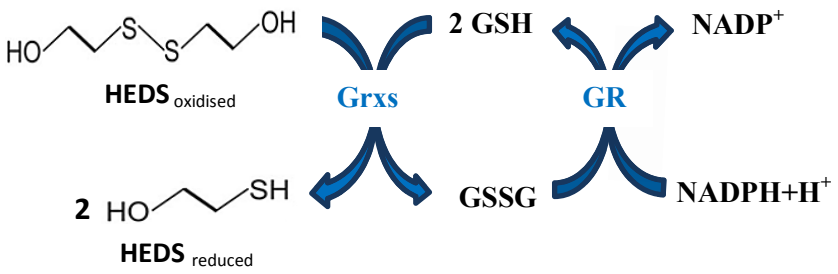

Figure 4.16: Glutaredoxins activity in the HEDS enzyme assay. Grxs reduce the disulfide bonds of the HEDS causing the Grxs to be oxidised. The oxidised Grxs will be reduced again by GSH which will be left oxidised (GSSG) which will be maintained in the reduced form again by accepting electrons from NADPH via glutathione reductase (GR). Modified Figure was taken from (324).

By performing the HEDS enzyme assay substituting the CLIC proteins in place of Grx-1 and HcTrx-5 and as shown in Figure 4.4, CLIC1, CLIC2 and CLIC4 were able to reduce the disulfide bonds of the HEDS substrate in the HEDS enzyme assay, thus demonstrating that they can also function as oxidoreductases. However in our results it is apparent that CLIC2 has the lowest enzymatic activity in the HEDS assay when compared to either CLIC1 or CLIC4. Also according to a study by Board et al, 2004 (160), they found that CLIC2 demonstrated no significant enzymatic activity in the HEDS enzyme assay (160). This difference in the enzymatic activity of CLIC2 compared to CLIC1 and CLIC4 can be due to the dithiol active site motif in CLIC2 which contains two cysteine residues (CPFC) rather than the monothiol active site motif

(CPFS) as exists in CLIC1 and CLIC4 proteins. Further studies are needed in order to establish the differences between CLIC2 and the other CLICs, as enzyme with a distinct dithiol catalytic mechanism, compared to the monothiol members CLIC1 and CLIC4.

Under oxidative conditions, the CLIC1 monomer forms a dimer where the N-terminal domain of the dimeric CLIC1, no longer resembles the glutaredoxin fold and the reactive cysteine, Cys24, forms a disulphide bond with Cys59 in CLIC1 protein. From our results, it appears that CLIC1 dimer which has a structure that is radically different from CLIC1 monomer (84), is also able to function as an oxidoreductase enzyme in the HEDS assay which is an unexpected result (Figure 4.9). The solution to this conundrum comes by examining the HEDS assay conditions, which include 1mM of reduced glutathione or GSH. These reducing conditions will rapidly convert the CLIC1 oxidised dimer back into its reduced monomeric form as the structural transition is fully reversible (84). This dimeric to monomeric protein conversion process would explain the slightly slower rate of NADPH consumption by the CLIC1 dimer sample compared to the monomer. On the other hand, it was speculated by Cromer et al, 2002 (37), that the formation of the CLIC1 dimer could be essential for GSH binding. That as an acidic residue of the neighbouring monomer may form a salt bridge with the N- terminal amino group of the γ-glutamyl moiety of the GSH in a similar fashion to the GSTs, such as GST-Ω which contains an acidic residue (glutamic amino acid, E85) whereas, CLIC1 contains an aspartic acid residue (D109), which is equivalent to the glutamic amino acid found in the GST-Ω group as shown in Figure 4.17.

Also aspartic acid residue in CLIC1 appears to be conserved in all the CLIC proteins except for CLIC2 which contains the polar amino acid residue, asparagine (N115). Furthermore, one could speculate that the lower activity of CLIC2 compared to CLIC1

and CLIC4 may be also related to the variation in the GSH attachment sites in the CLIC2 structure (76).

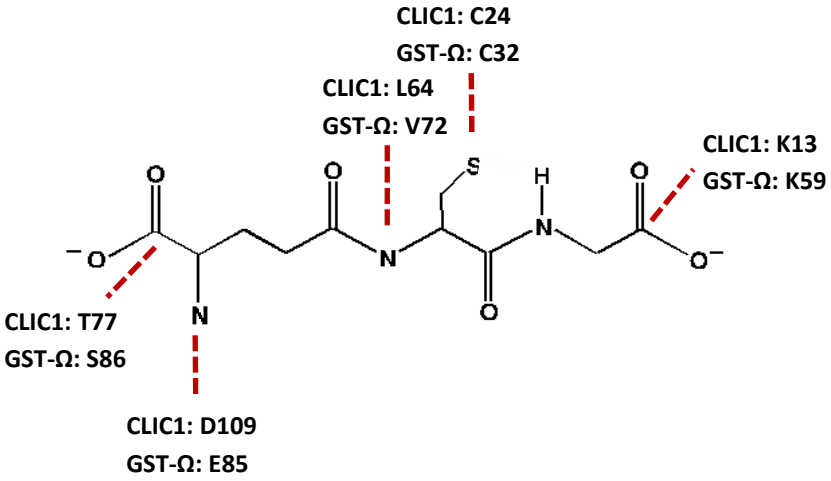

Figure 4.17: A schematic diagram showing some of the residues in CLIC1 and GST-Ω that interact with the GSH. Modified Figure was taken from (37).

We could also expect that members of the CLIC family are capable of carrying out target protein de/glutathionylation activity. This is supported by the X-ray crystallographic studies that reveal an open slot adjacent to the GSH binding site in CLIC1 that is large enough to accommodate a protein substrate (70). Therefore, de/glutathionylation may well be a mechanism by which CLIC proteins control ion channel activity and other cellular processes (78,157).

4.17.1 CLIC Proteins Oxidoreductase Activity is Specific to Glutathione and Glutathione Reductase

It was demonstrated previously that Grxs reduce the highly specific disulfides coupled to GSH and studies using human Grx-1 and Grx-2 demonstrate that mixed disulfides containing compounds other than GSH are not substrates for this family of proteins (294,325). However, several dithiol glutaredoxins such as human Grx-2 have been characterised to have the uncommon active site motif of (Cys-Ser-Tyr-Cys) instead of (Cys-Pro-Tyr-Cys) known for other dithiol Grxs. Grx-2 was found to perform diverse activities, including being reduced by thioredoxin reductase (TrxR) as well as GSH and glutathione reductase (GrxR) (326). According to our results (Figure 4.5), CLIC1, CLIC2 and CLIC4 were not reduced by the selenoenzyme, thioredoxin reductase (TrxR). Of these the most surprising was CLIC2, which even though it contains the dithiol active site motif, and would be expected to show similar activity to these dithiol Grxs was also negative. Given the lack of activity by all three CLIC proteins, one could speculate that they have GSH-dependent enzymatic activity that is distinct to the dithiol glutaredoxins.

Also as a classic and general assay to detect the disulfide reductase activity of enzymes, the insulin reductase assay was also performed. Trx and some of the Grxs were found to have insulin reductase activity (306,327). In Figure 4.6, it can be seen that Trx-1 was able to catalyse the reduction of the disulfide bond formed between the A and B chains of insulin in the presence of a reducing agent like DTT. As a consequence the turbidity of the assay will increase from the aggregation or precipitation of the free insulin B chain. Using the two free thiols at the active site motif of Trx-1 (CGPC), the disulfide bridge in insulin was reduced in the presence of DTT in the assay solution (327,328). In order to test the non-specific disulfide reductase ability of CLIC1, the insulin

reductase assay was carried and it can be shown that CLIC1 was not able to reduce the disulfide bridge of insulin, which could be due to the single thiol in the active site motif and also the glutathione-dependent oxidoreductase activity of CLIC1. Further investigations and comparative studies are needed in order to ascertain a distinct CLIC1 enzymatic profile.

4.18 Cysteine-24 Residue is Essential for the Enzymatic Activity of CLIC1

Enzymes exhibiting a glutaredoxin-like activity were speculated to require an active cysteine residue (265,267). It was determined previously that the cysteine residues in proteins play important roles in mediating cellular responses to redox status in cells through the ability to detect changes in the redox state and transduce changes in protein structure and functions (265-267).

In the glutaredoxin system and as it was outlined in the previous sections that the first cysteine residue in the G-site motif is reported to attack the sulfur atom in disulfide bridges - as occurs within glutathione mixed disulfide bonds - and therefore promotes thiol transfer (329). Similarly in the GSTs groups, GST-Ω (contains Cys32) and GST-β (containing Cys10) demonstrate glutaredoxin-like activity in the HEDS enzyme assay with the first cysteine residue in their active site motif also found to be essential for their enzymatic activity (37,322).

Our HEDS enzyme assay of CLIC1 mutants, CLIC1-C24A or CLIC1-C24S (as in Figure 4.7) shows that Cys24 is the essential catalytic cysteine in the structure of CLIC1 protein (70). Although Cys59 is essential for the transition of CLIC1 from the reduced monomer to the oxidized dimer state (84), mutation of Cys59 to alanine was found to

have no effect on the enzymatic activity of CLIC1. This confirms that Cys24 in CLIC1 is the central redox catalytic residue, essential for the enzymatic function of CLIC1, with no apparent involvement of Cys59.

Also our finding is reminiscent of the results obtained by Shi et al, 1999 (287), where the first cysteine residue in the active site of *E-coli* dithiol Grxs (1, 2 and 3) was found to be essential for catalysing the reduction of arsenate reductase intermediate. That was determined as a result of abrogating the catalytic activity of the Grx-mutant that does not contain the first cysteine residue located at the N-terminal domain, however the removal of the cysteine residue at the C-terminal domain did not affect the enzymatic activity of the Grxs (287).

Interestingly, there are other proteins that were found to demonstrate glutaredoxin-like enzymatic activity; however their structure does not contain a single cysteine residue such as Ure2 protein from yeast *Saccharomyces cerevisiae* (330). The main structural features of Ure2 that were predicted to be important for the redox activity of the protein are asparagine (N124) but it was not known how this amino acid residue may facilitate the thiol transfer reactions of Ure2 (330). The conservation of the proline residue in Grxs, GSTs and even in Ure2 was thought to contribute to the enzymatic activity of those proteins. It was demonstrated that introducing a free thiol group before the proline residue in Ure2 protein has caused an increase in some oxidoreduction and dehydroascorbic acid reductase (DHAR) activity by the protein (330,331). Given, that the structure of CLIC1 contains both a conserved asparagine (N23) and proline (P25) residues that were also found to be conserved across all human CLIC members. Therefore, further studies and investigations are required to investigate the contribution of these conserved residues in CLIC1 and other CLICs enzymatic activity.

4.19 CLIC1 Aids Selenite Metabolism

Selenium is a metalloid that belongs to the same group as oxygen and sulfur in the period table. Selenium may exist in inorganic forms such as metallic (Se^0), oxyanion (selenite $SeO(OH)_2$) and selenate ($SeO_2(OH)_2$) or it can be present in organic forms such as the seleno amino acids (SeCys) and selenomethionine (SeMet) where food (animal and plant products) is the main source of this type of selenium (332,333).

Selenium is an important trace element in the body (334-336), it was found to play a crucial role in health and medicine (337) such as the control of bacterial infections (338) and inflammatory reactions (339), it also participates in the control of different cancers (340,341). Selenium in the form of SeCys forms the active centre for the seleno-enzymes such as thioredoxin reductase, glutathione peroxidase (GPx) and other enzymes that participate in the redox reactions (332,337,342-344). Selenium is toxic when found at high concentrations; therefore it is biologically regulated within a narrow range (0.1-1.0µg/g diet) (333).

In cells, selenium compounds are recognised as selenium species that are transformed into the reduced form of selenide (HSe^-, RSe^-) by the help of GSH and Trxs (345,346). In the thioredoxin system, the reduction of selenium in the form of selenite occurs via the formation of a covalent adduct with the GSH, giving rise to selenodiglutathione (GS-Se-SG) which then will be metabolised to into selenide (347,348). Selenide undergoes redox cycling with oxygen and thiol causing a significant production of reactive oxygen species (ROS) (349,350). In turn, increased generation of ROS and superoxides leads to cellular damage and can induce apoptosis. Wallenberg et al,2010 (307) has demonstrated human Grx enzymes including Grx-1 which acts as a redox

active protein catalysing the reduction of disulfides, were also able to also metabolise selenium compounds.

Given that our results of the HEDS enzyme assay show that CLIC proteins also demonstrate monothiol glutaredoxins-like activity, we have investigated CLIC1's ability to reduce selenite in the form of sodium selenite (Na_2SeO_3) (333), which is the most soluble selenium compound and was found to be readily reduced by GSH to selenide.

According to our findings (Figure 4.10), CLIC1 was able to metabolise sodium selenite in a manner similar to Grx-1. This finding supports the hypothesis that soluble CLIC1 may catalyse the reactions of GSH with selenium compounds in order to reduce them to selenite and probably induce their incorporation into the selenoproteins.

However the binding affinity of CLIC1 to sodium selenite is slow (as indicated by the Km value (4.81±3.00μM) obtained from Figure 4.11 that was found to be higher than the physiological value of selenium in cells), one can then speculate that selenite may not be the physiological substrate for the CLIC proteins.

4.20 DHA Acts as a Substrate for CLIC1

Ascorbic acid (AA or vitamin C) is normally found in high concentrations at some body tissues (in millimolar concentrations in aqueous humor and lenses of animals and humans) (351,352). It plays important roles as an active antioxidative agent and scavenger against hydrogen peroxide, superoxide, hydroxyl radical and oxidative stresses that are generated as by-products from cellular metabolism (307). The physiological functions of AA are associated with the univalent oxidation of the acid

leading to the formation of monodehydroascorbic acid that undergoes a spontaneous disproportionation or further oxidation in order to be converted to dehydroascorbic acid (DHA) as a divalent oxidation by-product (353,354). The metal-catalyzed oxidation products of ascorbate which are highly toxic to cells and have been linked to many diseases including senile cataracts in ocular lenses (355). Also since AA cannot be synthesised or produced by animals and human cells, dietary intake and supplements are the only way to obtain this vitamin (354). Therefore reducing the oxidised forms of AA and regenerating it, is considered to be an essential process in order to maintain the normal level of AA in cells (354).

The monoascorbate form of AA was detected to be reduced by NADPH dependent enzymatic reactions occurring at subcellular membranes of the mitochondria and microsomes (356-358). Whereas, the conversion of DHA back to AA is achieved non-enzymatically by GSH (353). Wells et al, 1995 (359), have demonstrated that the glutaredoxins are able to catalyse the reductions of DHA via the use of GSH (as shown in Figure 4.18).

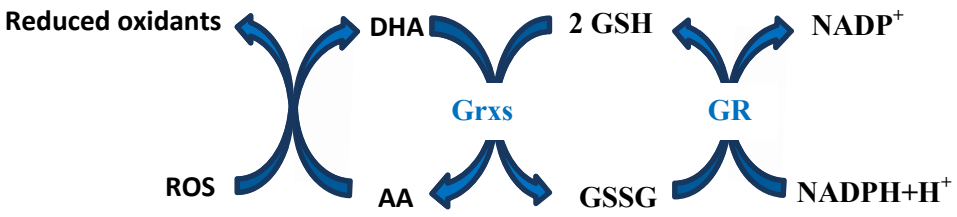

Figure 4.18: Reduction of DHA to AA via GSH involving the Grxs. Grxs catalyses the reduction of DHA to AA by GSH. The resulted oxidised Grxs will be reduced again by GSH causing the formation of GSSG that will be regenerated again to be GSH by accepting electrons from NADPH via glutathione reductase (GR). Modified Figure was taken from (359).

In plants, dehydroascorbic acid reductase (DHAR) enzymes were found to aid the reduction of DHA to AA (360). Phylogenetic studies have shown that the plant DHARs are the closest relatives of the CLIC protein family (78). Thus, these plant DHAR proteins are predicted to adopt a three-dimensional structure similar to the soluble form of CLIC1 (323,361). A recent study has demonstrated the oxidoreductase activity of the DHAR from *Populus tomentosa* (323). Upon alanine substitution of the Cys20 residue which is located in the predicted GSH binding site in the protein PtoDHAR2, its reductase activity was fully abolished (323). These findings correlate closely with our results for the two CLIC1 mutants (C24A and C24S) in the HEDS enzyme assay (Figure 4.7).

From these results and the obtained data as in Figure 4.12, one could speculate that members of the CLIC family serve a protective function in cells by metabolizing substrates such as DHA and thus maintaining the intracellular levels of ascorbate. The DHA would be the more likely physiological substrate for the CLIC proteins as the binding affinity of DHA to CLIC1 appears to be higher as indicated by the linear curve of michaelis menten plot (Figure. 4.13). DHAR activity of the CLIC proteins is therefore consistent with their close structural evolutionary relationship with the plant DHAR proteins (39,78,323). If this putative activity by the CLICs is considered within the context of the ocular lens, reduction of DHA by CLIC proteins could aid in preventing many diseases such as selenite cataract formation.

4.23 Chloride Ion Channel Blockers Inhibit the Enzymatic Activity of CLIC1

Ethacrynic Acid ($C_{13}H_{12}Cl_2O_4$) is a diuretic drug whose mechanisms of action are characterised by inhibiting the sodium-potassium-chloride cotransports. Also ethacrynic acid was found to inhibit enzymatic activity of the GSTs.

Ethacrynic acid interacts with the GSTs as a substrate or as non-substrate ligand that then is conjugated spontaneously or via GST-catalysed reactions with the GSH in order to form an ethacrynic-GSH (EA-SG) conjugate (362-365). The EA-SG adducts also acts as inhibitors for the GST enzymes (364). According to studies of the GST-π class, it was determined that this group of enzymes consists of two domains (N-terminal and C-terminal domains) that are linked by large hydrophobic region (H-site) which is located adjacent to the GSH (or G-binding site) (365). Therefore the GSH of the EA-SG complex will bind into the G-site whereas the EA component will bind into the H-site of the GST protein (366-368).

Furthermore, when the structural complex of GST-π and EA was superimposed onto the CLIC1 structure, it was shown that CLIC1 shares the same structural homology to the existence of an empty space at the H-site that was speculated to be a binding site for a blocker or an inhibitor (37,368). Experiments have demonstrated that CLIC1 channel activity was blocked or inhibited by IAA-94 ($C_{17}H_{18}Cl_2O_4$), which is a diuretic drug and a homologue to ethacrynic acid (34). Also A9C ($C_{15}H_{10}O_2$) as another chloride ion channel blocker demonstrate the same IAA-94 effect on CLIC1 channel activity, however the chloride channel blocker DIDS ($C_{16}H_8Na_2O_6S_4$) did not cause any significant difference to the protein's activity in membranes (44). Also when CHOK1

cells were grown in the presence of IAA-94 and A9C, the cell cycle was arrested at the G2M phase, but this was not the case for DIDS (44).

More recently, an intriguing finding of our work was the inhibitory effect on CLIC1's enzymatic activity in the HEDS enzyme assay by the chloride ion channel blockers IAA-94, A9C but not DIDS (Figure 4.14). These findings are consistent with the structural and evolutionary relationship between the GST and CLIC families as IAA-94 (369). The more likely explanation is that the inhibitors, IAA-94 and A9C, act by binding near the active site of the soluble form of CLIC1 thus inhibiting its enzymatic activity and similarly its channel activity. Another point was also noted with respect to the determination of the structure of CLIC1 (70). Given that these drugs are membrane permeable, their cellular inhibitory effects and arrest of the cell cycle progression, could be due to inhibition of CLIC1 enzymatic activity rather than by directly blocking the integral membrane form of the CLIC1 channel.

Furthermore, pre-incubation of CLIC1 protein with free cholesterol was found to inhibit the chloride ion channel activity of the protein in tethered bilayer lipid membranes (174), however our HEDS enzyme assay of the pre-incubated CLIC1 monomer with different concentrations of cholesterol show that cholesterol did not affect the oxidoreductase activity of CLIC1 (Figure 2.15). This suggests that the interaction site of cholesterol with CLIC1, which could occur via the GXXXG site (233), present in the CLIC proteins, is not involved in the enzymatic activity for CLIC1. Further studies and investigations are required about how cholesterol interacts with CLIC1 protein.

Conclusion

Members of the CLIC protein family, which are known to function as ion channels when integrated into membranes, also demonstrate monothiol glutaredoxin-like enzymatic activity when in their soluble form. This supports an additional role for these proteins in the cellular processes of detoxification and oxidoreduction. Finally, the fact that the same CLIC1 channel blockers inhibit CLIC1 enzymatic function suggests that the enzymatic properties of CLIC1 may also control the function of the channel form.

Chapter 5

Discussion and Future Directions

5.1 Sterols are Essential for the Ion Channel Activity of CLIC1

Cell membranes are composed of phospholipids, sterols, sphingolipids and trans-membrane proteins (370). Different concentrations of these constituents can cause heterogeneous aggregation of the sterols, sphingolipids and some types of phospholipids, and rigid patchs of membranes may arise, resulting in the formation of segregated domains or cholesterol-rich domains known as "lipid rafts" (370,371), such as caveolae that form cave-like morphology (372) at some plasma membrane surfaces. Lipid rafts rich in cholesterol and sphingolipids are associated with glycosyl phosphotidylinositol anchored proteins, caveolins (cave-1) (371) such as palmitoylated proteins and the src-family, as lipid modified proteins. Caveolae structures highly rich in cholesterol and were found to be involved in the processes of endocytosis (373), lipid and cholesterol homeostasis (374-377), signal transduction and are as known to be ideal docking sites for protein kinases (371,378).

Earlier studies reported that CLIC4 is localised within specific cholesterol-rich domains of membranes similar to caveolae (379). Here in, we report that the functional formation of chloride ion channels by CLIC1 is highly dependent on sterol (cholesterol or ergosterol) concentrations in tBLMs which is similar to the CDC proteins that demonstrate cholesterol-dependent activity in membranes. The precise process of CLIC1 binding and its specific interaction with membrane cholesterol requires further investigation.

It has been demonstrated that the CDC proteins stereptolysin (SLO) and intermedilysin (ILY), are able to bind to membranes lacking cholesterol, however, the cytolytic activity of these toxins was fully abrogated in such membranes (251). These findings suggest

that cholesterol is not required for the initial binding or as a receptor for the toxins, however it appears to be essential subsequent processes of the insertion of the transmembrane β-barrel structure and the structural transition of toxins from the prepore to the final pore forming complex. Furthermore, cholesterol depletion of membranes was shown to affect the oligomerisation of the monomeric subunits of the SLO and ILY in order to form the prepore structure required for their effective insertion and final oligomerisation within the membrane (251). In a similar manner, cholesterol-depletion of membranes resulted in decreased binding of the CDC toxin perfringolysin (PFO) into membranes, and cytolytic activity was retained, although it was decreased (251). Therefore, one can speculate that the increased conductance levels observed for CLIC1 in membranes containing sterols, suggests that membrane sterols or sterol-rich domains, facilitate all or some of the following processes including its structural rearrangement, membrane docking, membrane insertion, oligomerisation and ultimately the gating of its ion channel activity. The fact that preincubation of CLIC1 with cholesterol resulted in a complete abrogation of its ion channel activity strongly suggests involvement of sterols in the initial binding of the protein to the membrane, and hence holds further implications for CLICs localised to cholesterol-rich domains.

The observation that all the human CLIC proteins contain the motif GXXXG has led to the proposal that it could act as the binding site for cholesterol, as occurs in other proteins such as amyloid β-protein (AβP) (233,380). On the other hand the GXXXG motif has also been shown to stabilise the oligomerisation of the proteins glycophorin A and human carbonic anhydrase in membrane (381,382). Therefore, the precise role of the GXXXG motif warrants investigation. Studies by our group at UTS are currently underway using site-directed mutagenesis in order to replace either one or both of the

glycine (G) residues of the motif with an alanine amino acid, which should preserve the secondary structure of the protein and allow for determination of the role of the GXXXG motif in the process of CLIC1 insertion into membranes and its interactions with cholesterol.

CLIC1 also showed higher conductance in membranes containing the yeast or fungal sterol, ergosterol. A number of naturally occuring peptides have been purified from insects, cecropins A, B, and D that were found to have antimicrobial and antifungal activity (383-385). However their activity was limited due to their fast degredation by proteases in the intercellular fluids, therefore it was essential to produce synthetic analogs that are more stable and resistant to such degradation (386). D4E1 (17 amino acids in length) is a completely synthetic peptide, composed of β-sheets that was found to share a similar pattern of interaction with membranes as the naturally occuring peptides (387). D4E1 was able to bind to ergosterol and cholesterol in membranes (386). Therefore the lethality of D4E1 was proposed to be due to interaction with the ergosterol in fungal cell membranes (386). Interestingly CLIC1 was shown to be more active in membranes containing ergosterol than membranes with cholesterol, which may indicate that CLIC1 possesses anti-fungicidal activity. This new finding opens a new research area about the function of CLICs in different organisms and their participation in disease processes.

Therefore, investigating the conductances of CLIC1 and other CLIC family members in membranes containing different sterols is highly important, in order for a detailed understanding of the mechanims and,the role of the sterols in CLIC protein insertion and ultimately their functional formation of chloride ion channels in membranes.

5.2 The Residue Cysteine-24 is Not Essential for the Ion Channel Activity of CLIC1 in Membranes Containing Cholesterol

The discovery of the ion channel function of the CLIC proteins can be directly linked to the original work by the Al-Awqati group who isolated the bovine protein p64, while seeking to isolate the cystic fibrosis chloride channel (26). Since then, the CLIC proteins have been demonstrated to form ion channels in the membranes of intracellular compartments as well as the outer plasma membrane of cells. Due to the presence and conservation of an active cysteine residue in the structure of all human CLIC proteins, its been suggested that the activity of the CLICs is highly redox sensitive (84). According to the results obtained by experiments aimed to investigate the binding of CLIC proteins to the lipid bilayers, it was shown that the ion channel activity of CLIC proteins in membranes is highly dependent upon oxidation and reduction processes (84,88). Mutation of Cys24 to alanine resulted in reduced single ion channel conductance of CLIC1 when compared to the wild type protein (47), however when Cys24 residue was replaced by serine, the ion channel activity of the protein was fully eliminated (84).

This thesis reports the investigation of the activity of two CLIC1 Cys24 muntants C24A and C24S. Interestingly, both mutants were found to conduct equally well in tBLMs containing cholesterol. However their conductance was lower compared to CLIC1-C59A mutant and the wild type CLIC1 protein. According to our tBLM and impedance spectroscopy results, we have demonstrated that both Cys24 mutants were able to form fully functional conducting channels in membranes containing 25mol% cholesterol. suggesting that the formation of the disulphide bond between Cys24 and Cys59 may not be as critical for the ion channel activity of CLIC1. These findings on the other hand,

lend support to the importance of cholesterol in the CLIC1 membrane interactions via either its initial membrane binding, oligomerisation or channel gating.

The decreased conductance of both Cys24 mutants in the tBLM membranes is intriguing given the Cys59 mutant demonstrated no such decrease in its conductance. Instead, this result supports a role for the Cys24 residue in either the ion channel gating or pore / selectivity filter of the channel, given Cys24 is the first residue in the putative transmembrane spanning domain of CLIC1 (putative CLIC1 TM domain ranges from residues 24-46).

Of further relevance is the fact that CLIC1 is the only CLIC family member with a Cysteine residue at this position (Cys24). Other CLIC-like proteins such as EXC-4 from *C.elegance,* which contains an aspartic acid residue at this equivalent position, was found to insert and form ion channels in lipid bilayer lipid membranes. On the other hand, replacement of the proline residue in EXC-4 with a leucine, in the middle of the predicted transmembrane domain (TMD), caused EXC-4 to loose the ability to insert into membranes (35). This finding raises the question regarding the importance of the conserved proline residue in the active site motif of all human CLIC proteins (P25 in CLIC1), which may contribute to the ability of the protein to auto-insert into membranes and form functional chloride ion channels. These findings point to the need for further investigation of redox control of CLIC proteins, along with the need to elucidate the role of other critical residues lining the pore and the transmembrane domain of the channel.

5.3 Glutaredoxin-like Enzymatic Activity of the CLIC Proteins

The structural model of the CLIC proteins does not fit the structural characteristics and pattern of the classical ion channel proteins as they lack a well-defined transmembrane domain. It has been determined that the CLIC proteins share high structural homology with the GST superfamily of enzymes and in particular the GST-Ω proteins (55). It is not well known if the CLIC proteins are simply a more developed splice variant of the GSTs. As elaborated by Jacob et al, 1977 (388) and Gancedo et al, 2008 (389). They argue that as there is no end goal in evolution and new novel functions of proteins may develop by adopting existing proteins. Unlike CLIC proteins that were found to function as ion channels in membranes, the GSTs are well-known promiscuous enzymes that have no membrane association properties or any ion channel activity (37,55,56). In this thesis, we have shown for the first time using *in vitro* assays that the soluble CLIC proteins (CLIC1, CLIC2 and CLIC4) are able to function as enzymes and as such, they resemble the activity of the GST-Ω and the monothiol glutaredoxin proteins.

Cys24 residue seems to play a critical role in the enzymatic activity of CLIC1. We note that the mutation of either Cys24 to serine or alanine caused the full elimination for the enzymatic activity of CLIC1 in the HEDS enzyme assay. However, our tBLM results show that CLIC1-C24A or C24S mutants were able to function as ion channels in membranes containing 25mol% cholesterol. These findings lead us to categorise or classify the CLIC proteins as “moonlighting proteins”.

Moonlighting proteins were first described in 1988 (390). They are multitasking proteins that are able to perform more than one biological function (391,392). The moonlighting functions were speculated to reside in more conserved proteins or in ones

that are highly expressed (392,393). They have been identified in animals, prokaryotes, plants and yeast (389,391,394-396). Examples of moonlighting proteins, are the crystallin structural proteins of the vertebrate eye lens, the duck ε-crystallin which was also found to have catalytic function of a lactate dehydrogenase enzyme (397), the turtle τ-cyrstallin turned out to be the glycolytic enzyme α-enolase (398). The *E-coli* thioredoxin enzyme, was later determined to also be the T7 DNA polymerase subunit (278) and the cystic fibrosis transmembrane regulator (CFTR) protein is also able to function as an ion channel besides its regulatory functions (399,400).

The critical features of moonlighting proteins is their ability to carry out multiple, yet unrelated functions, and these are not due to RNA splice variants, gene fusions or promiscuous enzymatic activity (391,401-403). The multiple functions within one polypeptide chain must be independent of each other, indicating that the inactivation of one function by a mutation does not affect the second function of a moonlighting protein (394,403). Changes in the redox state of the cell, modifications to the oligomeric state of a protein, binding to molecules, substrates, ligands or to cellular membranes are all factors that may contribute to the second function of a protein (394). In addition, "neomorphic moonlighting" characteristics may arise from such changes, which means that the new and different function of a protein can be caused as a result of alterations or disruptions to protein structure or state (403).

By taking all these factors into account, one can consider the appropriateness of classifying members of the CLIC protein family, as moonlighting proteins. Members of the CLIC family adopt more than one structural conformation, and perform multiple independent functions. The protein CLIC1 has been identified as "metamorphic": that

is, it can adopt more than one native structure (9,89). Furthermore, members of the CLIC family have the ability to transition between soluble proteins into membrane associated proteins that function as ion channels (3). Furthermore, the recently identified enzymatic activity of the soluble CLIC1, appears to be distinct to its ion channel activity, as demonstrated by cholesterol's regulation of the latter activity but not the former. Also the fact that Cys24 is essential for the *in vitro* enzymatic activity of CLIC1 but not essential for its ion channel function in tBLM membranes. All these factors combine to support the classification of CLIC1 and likely other members of the CLIC family, as moonlighting proteins (391).

5.4 Future Directions

With the exciting new finding that members of the CLIC family also function as enzymes, it is now critical to review and reassess previous findings and studies. For example, the fact that the same CLIC1 channel blockers also inhibit CLIC1 enzymatic function, one must reconsider several of the cellular studies where these blockers were used to assess CLIC function. Furthermore, one can now consider how the enzymatic activity of CLIC1 may also control the function of the channel form. Early experiments have shown that CLIC1 (and other CLIC proteins) can spontaneously form electrophysiologically active anion channels in artificial bilayers where the electrophysiological properties resemble those of the CLIC currents observed in cells (8,49,70). Thus, *in vitro*, the CLIC protein must auto-insert into the bilayer to form the channel. It is possible that the CLIC1 enzymatic activity can either control this membrane insertion process or that once some CLIC1 has inserted and formed a channel, then the remaining soluble CLIC1 controls the channel via its enzymatic

activity. In cells, it is also possible that the soluble CLIC enzyme controls other channels as has been shown for CLIC2 and the ryanodyne receptor Ca^{2+} release channel (160). The scene is now set for a completely new approach in the study of this intriguing and unusual family of proteins. Other suggested studies outlined below include:

- To fully understand the interaction of CLIC1 with sterols in membranes will most likely require further complementary techniques such as small-angle X-ray scattering (SAXS), and neutron reflectometry. These techniques should provide hard data on which to model the structural interaction of CLIC proteins with membranes containing sterols.

  These structural techniques would be greatly enhanced if used in conjunction with site directed mutagenesis to investigate the contribution of the other cysteine residues and the conserved amino residues in the CLIC structure such as lucine-40 (L40) proline-25 (P25) and asparagine 23 (N23) as for CLIC1. Correlations between the enzymatic, ion channel activity and protein structure will provide powerful new insights.

- According to the work presented in this thesis, it can be shown that all the enzymatic activity experiments that have been performed to investigate the function of the soluble CLIC proteins are limited to in *vitro* assays. To fill the gaps in understanding of the dual functionality of these metamorphic and putative "moonlighting" proteins requires elucidation of the cellular mechanisms controlling switching between their distinct functions and their role in health and

disease. It is important to study the role of CLICs using *in vivo* and cellular models.

- From this work, it seems that the soluble CLIC proteins function as general antioxidants and oxidoreductases resembling the glutaredoxins enzymatic activity. Therefore an important investigative theme i the enzymatic activity of different CLIC proteins in the presence of different substrates and macromolecules. Such studies will likely provide important insights into the physiological roles of CLIC proteins and to investigate their potential participation in disease processes.

5.4.1 Investigating the Role of CLIC Proteins in Disease States

The conservation of CLIC proteins among vertebrates and invertebrates strongly favours the hypothesis that the CLICs perform very important biological functions. Localisation, coupled with increased activity of CLIC proteins in sterol-rich membrane domains or artificial membranes has important implications. CLIC proteins have been reported to possess significant roles in major issues of public health. These include a study by Novarino et al, 2004 (46) who has demonstrated that CLIC1 over-expression causes amyloid β-protein (AβP) to increase microglial cell activation, which is a process that can lead to Alzheimer's disease (AD). It was further speculated that high cholesterol levels increase the AβP levels in AD brains and cholesterol lowering drugs (such as statins) were found to decrease the level of AβP (404). Given that CLIC1 ion channel conductance in membranes increases as cholesterol concentration increases, one may ask whether CLIC1 protein plays a role in tissues containing high levels of

cholesterol. An adjunct study would be to investigate the effect of cholesterol lowering drugs on the function of CLIC1 as an ion channel or as a soluble protein. Such a study would have relevance to understanding the causes of Alzheimer's disease and possibly provide a biomarker for the early detection of the disease.

Also CLIC1 was found to be more active in membranes containing ergosterol than membranes with cholesterol; this raises the question regarding the role of CLIC1 in fungal infections in the body and also whether it is possible to use the CLICs as naturally produced proteins to function as anti-fungal agents, similar to nystatin A and amphotericin B.

A further potential public health impact of understanding CLIC1 function is in the treatment of selenite cataracts in ocular lenses. In cells, selenide causes selenite cataract in ocular lenses when found in high concentrations, while vitamin C (ascorbic acid or AA) was found to protect the lens from oxidative stress and cataract formation (355). However over time, ascorbic acid gets oxidised to form dehydroascorbic acid (DHA) which is toxic to cells when found at high concentrations. According to our recent findings, CLIC1 was shown to have oxidoreductive capabilities similar to the glutaredoxins in reducing the DHA to AA and also uses sodium selenite as a substrate. Combining both results, it can be postulated that CLIC1 is involved in preventing selenite cataract by metabolizing sodium selenite and maintaining the AA level in the ocular lens by reducing DHA. In order to confirm the involvement of CLIC1 in cataract formation and its potential role in reducing cataract progression, the use of ocular lenses (cell lines or ocular tissues from animal models) as well as immunohistochemistry methods are required.

Also it was determined that high concentrations of selenium may induce apoptosis in tumour cells, where selenite compound demonstrated 80% mesothelioma malignant cells death with no significant effect to normal cells (405). Also the same results were seen when selenite was administered to malignant prostate cells (406). This means that there is high selective uptake of selenite in malignant cells than normal cells (407). According to a recent study by Tain et al, 2014 (408), it was determined that CLIC1 is involved in the regulation and proliferation of prostate cancer through mitogen-activated protein kinase / Extracellular Signal-Regulated Kinases (MAPK/ERK) pathway. Therefore it is essential to investigate the other pathways and the role of CLIC1 in selenite metabolism or uptake and to study the possibility of using CLIC1 as an effective target for the treatment and therapy of prostate cancer (and probably other cancer types).

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