

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

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CERTIFICATE OF ORIGINAL AUTHORSHIP

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

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DEDICATION

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

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List of Abbreviations

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

GSH.....	Reduced glutathione
GSSG.....	Oxidised glutathione
GST.....	Glutathione-S-transferase
GST- β	Glutathione-S-transferase beta class
GST- Ω	Glutathione-S-transferase omega class
GST- Ω 1.....	Glutathione-S-transferase omega group 1
H-site.....	Hydrophobic region
H ₂ O ₂	Hydrogen peroxide
HcTrx-5.....	Thioredoxin-related protein in Haemonchus contortus
HEDS.....	2-hydroxyethyl disulfide
HCl.....	Hydrochloric acid
HEPES.....	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His.....	Histidine
IAA.....	Indanyloxyacetic acid
IUBMB.....	The International Union of Biochemistry and Molecular Biology
IPTG.....	Isopropyl- β -thiogalactopyranoside
K ⁺	Potassium ion
KCl.....	Potassium chloride
K _m	Dissociation constant of the enzyme-substrate complex
K _d	equilibrium constant
k _{cat}	catalytic rate constant
KH ₂ PO ₄	Mono-potassium phosphate
K ₂ HPO ₄	dipotassium phosphate
M.....	Molar
MW.....	molecular weight
mg.....	Milligram
min.....	Minute
mM.....	Millimolar
NaCl.....	Sodium chloride
NADH.....	Nicotinamide adenine dinucleotide (NAD) + hydrogen(H)
NaN ₃	Sodium Azide
Na ₂ SeO ₃	Sodium selenite
NCC27.....	Nuclear chloride channel protein-27kDa
NTA.....	nitrilotriacetic acid
Ni ²⁺	Nickel ion
NTA.....	Nitrilotriacetic acid

N-domain Amino terminal domain
 nm Nanometer
 Ox oxidized state
 OD Optical density
 P64 Bovine chloride channel protein -64kDa
 PBS Potassium buffered saline
 Phe Phenylalanine amino acid
 Pro Proline amino acid
 P product
 pKa measure of acid strength
 PDI protein disulfide isomerase
 ROS Reactive oxygen species
 rPsGrx Glutaredoxin protein from antarctic sea-ice
 S.E Standard error
 SEC Size exclusion chromatography
 SeO(OH)_2 Selenite
 $\text{SeO}_2(\text{OH})_2$ Selenate
 Ser Serine amino acid
 S substrate
 SDS Sodium dodecyl sulphate polyacrylamide gel electrophoresis
 TCEP Tris (2-carboxyethyl)phosphine
 Triton-X100 Octylphenyl-nonaoxyethylene
 Trx(SH)_2 reduced thioredoxin
 Trx-1 Thioredoxin reductase
 TrxR Thioredoxin reductase
 Trxs Thioredoxins
 Tyr Tyrosine amino acid
 μg Microgram
 μM Micromolar
 UV Ultraviolet
 V_0 reaction rate
 V_{max} maximal reaction rate
 WT WT
 X Any amino acid

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ABSTRACT

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

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

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

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

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

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

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