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".

Signature of Student: Production Note: Signature removed prior to publication.

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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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Conference Poster Presentations

1. Moghaddasi. S., Ali. H.M., Khondker. R. H., Al Khamici. H., Valenzuela. S. M. Biochemical Characterization of CLIC members and the Influence of the Six Histidine Tag on Enzymatic Activity. The New Horizon, Sydney, Australia. 2016.

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List of Abbreviations

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

GSH ..... Reduced glutathione

GSSG ..... Oxidised glutathione

GST ..... Glutathione-S-transferase

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

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

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

H-site ..... Hydrophobic region

H_2O_2 ..... Hydrogen peroxide

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

HEDS ..... 2-hydroxyethyl disulfide

HCl ..... Hydrochloric acid

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

His ..... Histidine

IAA ..... Indanyloxyacetic acid

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

IPTG ..... Isopropyl-β-thiogalactopyranoside

K^+ ..... Potassium ion

KCl ..... Potassium cholride

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

K_d ..... equilibrium constant

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

KH_2PO_4 ..... Mono-potassium phosphate

$K2HPO_4$ ..... dipotassium phosphate

M ..... Molar

MW ..... molecular weight

mg ..... Milligram

min ..... Minute

mM ..... Millimolar

NaCl ..... Sodium chloride

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

NaN_3 ..... Sodium Azide

Na_2SeO_3 ..... Sodium selenite

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

NTA ..... nitrilotriacetic acid

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

NTA ..... Nitrilotriacetic acid

N-domain ..... Amino terminal domain

nm ..... Nanometer

Ox ..... oxidized state

OD ..... Optical density

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

PBS ..... Potassium buffered saline

Phe ..... Phenylalanine amino acid

Pro ..... Proline amino acid

P ..... product

pKa ..... measure of acid strength

PDI ..... protein disulfide isomerase

ROS ..... Reactive oxygen species

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

S.E ..... Standard error

SEC ..... Size exclusion chromatography

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

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

Ser ..... Serine amino acid

S ..... substrate

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

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

Triton-X100 ..... Octylphenyl-nonaoxyethylene

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

Trx-1 ..... Thioredoxin reductase

TrxR ..... Thioredoxin reductase

Trxs ..... Thioredoxins

Tyr ..... Tyrosine amino acid

µg ..... Microgram

µM ..... Micromolar

UV ..... Ultraviolet

V_0 ..... reaction rate

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

WT ..... WT

X ..... Any amino acid

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ABSTRACT

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

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

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

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

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

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

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