Transmembrane Electron Transport Systems in Erythrocyte Plasma Membranes

A thesis submitted for the degree of

Doctor of Philosophy

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Declaration

The work performed for this thesis was carried out in the School of Molecular and Microbial Biosciences (formerly the Department of Biochemistry) at the University of Sydney between April 2001 and October 2004. The methaemoglobin reduction assays described in Chapter 5 were done in collaboration with Prof Eri Ogawa and Associate Prof Nihal Agar. Prof Ogawa performed the experiments whose results are depicted in Figures 5.3 and 5.4. All other experimental results were obtained by me alone and to the best of my knowledge have not been published previously by any other person.

I the candidate, Eleanor Clare Kennett, hereby declare that none of the work presented in this thesis has been submitted to any other university or institution for a higher degree.

Eleanor Clare Kennett

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Abstract

Electron transport systems exist in the plasma membranes of all cells. Although not well characterised they play roles in cell growth and proliferation, hormone responses and other cell signalling events, but perhaps their most important role, especially in erythrocytes, is enabling the cell to respond to changes in both intra- and extracellular redox environments. Human erythrocytes possess a transmembrane electron transport capability that mediates the transfer of reducing equivalents from reduced intracellular species to oxidised extracellular species and is concomitant with proton extrusion.

In the work for this thesis I showed that erythrocyte membranes contain a transmembrane WST-1 (water soluble tetrazolium-1) reductase activity that uses reducing equivalents from intracellular NADH to reduce extracellular WST-1. The rate of WST-1 reduction was increased by the presence of phenazine methosulfate and, although of low activity, it showed similar properties to a previously reported transmembrane NADH-oxidase activity. ^{1}H NMR experiments showed that WST-1 was reversibly bound to the membrane and/or proteins in the membrane within the timeframe of the NMR experiment, confirming the location of the WST-1 reduction.

Preliminary attempts to purify NADH:WST-1 reductase and NADH:ferricyanide reductase activities from the erythrocyte plasma membrane were inconclusive. The protein(s) responsible for the reduction of these oxidants appear to be of low abundance in the plasma membrane and may be part of a larger protein complex. Further work on the isolation of these redox activities is required before the protein(s) involved can be identified with any confidence.

The ability of cells to export electrons suggests that an electron import mechanism might also exist to re-establish the cell's redox-buffering equilibrium under

conditions of oxidative stress. This hypothesis was tested in glucose-deprived erythrocytes using reduced glutathione and NADH as extracellular electron donors. It was shown that neither reduced glutathione nor NADH donated reducing equivalents through a transmembrane redox system. Extracellular NADH was, however, able to produce profound changes in starvation metabolism and methaemoglobin reduction rates. The addition of extracellular NADH caused a six-fold increase in the rate of lactate production above that observed in glucose-starved controls, together with a concomitant decrease in pyruvate production. In erythrocytes containing high levels of methaemoglobin, extracellular NADH increased the rate of methaemoglobin reduction in both the presence and absence of glucose. These results were explained by the leakage of lactate dehydrogenase from erythrocytes due to an admittedly low level of haemolysis. This caused the displacement of the intracellular pseudo-equilibrium of the lactate dehydrogenase reaction via transmembrane exchange of lactate, allowing the conversion of extracellular pyruvate to lactate and resulted in an increase in intracellular NADH concentrations. The latter increased the rate of methaemoglobin reduction.

In conclusion, the work described in this thesis showed that erythrocyte membranes do not contain mechanisms for importing electrons or reducing equivalents from extracellular reduced glutathione or NADH. Erythrocytes do, however, contain an electron export system which can reduce extracellular oxidants such as WST-1 and the activity of this system depends on an intricate balance between intracellular antioxidants and enzyme activities. There is much still to be learnt about plasma membrane redox systems, little is known, for example, about the protein composition, mechanism of action, and the *in vivo* conditions under which these systems are most active.

Abbreviations

2,3BPG	2,3-*bis*phosphoglycerate
αCHC	α-cyano-4-hydroxycinnamate
AFR	ascorbate free radical
BCA	bicinchoninic acid
BSA	bovine serum albumin
cNOX	constitutive NADH oxidase
CoQ_{10}	oxidised Coenzyme Q_{10}, ubiquinone
$CoQ_{10}H_2$	reduced Coenzyme Q_{10}, ubiquinol
COSY	correlation spectroscopy
CPMG	Carr-Purcell-Meiboom-Gill pulse sequence
cyt b_5	cytochrome b_5
cyt b_{561}	cytochrome b_{561}
DABS	diazobenzene sulfonate
DCIP	2,6-dichlorophenolindophenol, Tillmans reagent
DHA	dehydroascorbate
DIDS	4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid
DTNB	2,2′-dinitro-5,5′-dithiodibenzoic acid, Ellman's reagent
EDTA	ethylenediaminetetraacetic acid
EPR	electron paramagnetic resonance spectroscopy
ER	endoplasmic reticulum
FID	free induction decay
FPLC	fast performance liquid chromatography
G6PDH	glucose-6-phosphate dehydrogenase
GADPH	glyceraldehyde-3-phosphate dehydrogenase
ghosts	isolated RBC membranes
GR	glutathione reductase
GSH	reduced glutathione

GSHPx	glutathione peroxidase
GSSG	oxidised glutathione
GSSR	glutathione-protein mixed disulfide
GST	glutathione S-transferase
Hc	haematocrit
Hb	haemoglobin
HMBC	heteronuclear multiple bond correlation (spectroscopy)
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum correlation (spectroscopy)
LC-MS	liquid chromatography-mass spectrometry
LDH	lactate dehydrogenase
MeP	methylphosphonate
metHb	methaemoglobin
NADD	[*nicotinamide*-4*R*-^{2}H]adenine dinucleotide
NADH-MR	NADH methaemoglobin reductase
NBT	nitroblue tetrazolium
NEM	*N*-ethylmaleimide
NMR	nuclear magnetic resonance (spectroscopy)
oPPP	oxidative pentose phosphate pathway
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCA	perchloric acid
pCMB	*para*-chloromercuribenzoate
pCMBS	*para*-chloromercuribenzene sulfate
P_i	orthophosphate, inorganic phosphate
PMOR	plasma membrane oxidoreductase
PMS	phenazine methosulfate
RBC	erythrocyte, red blood cell
ROS	reactive oxygen species
ROW	reverse osmosis water
SDS	sodium dodecylsulfate
SOD	superoxide dismutase
tBHP	*tert*-butylhydroperoxide
TCA	trichloroacetic acid

TEMED	*N,N,N′N′*-tetramethylethylenediamine
TEP	triethylphosphate
tNOX	tumour NADH oxidase
TSP	(2,2,3,3-d_4) trimethylsilyl-3-propionate
VDAC1	voltage-dependent anion-selective channel 1
WST-1	water soluble tetrazolium-1

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