Chapter 7

Preliminary Attempt to Purify WST-1 and Ferricyanide PMOR Activities from RBC Membranes

7.1 Introduction

Although much is known about the composition and mechanism of redox systems in organelle membranes (oxidative phosphorylation in mitochondrial membranes and the photosystems I and II in the thylakoid membranes of the chloroplast) relatively few studies have been performed on the elucidation of the protein composition and reaction mechanism of redox systems in plasma membranes.

One of the earliest reported purifications of a PMOR was from RBCs (Wang and Alaupovic, 1978). The 40 kDa glycoprotein was solubilized by Triton X-100 and purified by affinity chromatography, ammonium sulphate precipitation and isoelectric focussing. The role of the NADH:(acceptor) oxidoreductase (EC 1.6.99.3) was not known at the time, but its specificity for NADH suggested to the authors a role in metHb reduction.

Following on from this work, Kitajima *et al.* (1981) purified an NADH-cyt b_5 reductase from RBC plasma membranes. The enzyme was solubilized with Triton X-100 then purified using DEAE-cellulose anion exchange, hydroxyapatite adsorption and 5'-ADP-hexane-agarose affinity chromatography. The purified enzyme had an apparent mass of 36 kDa as indicated by SDS-PAGE, and a mass of 144 kDa according to gel filtration data, suggesting it might have a tetrameric native form. The protein, containing one equivalent of FAD as a prosthetic group, reduced a large range of oxidised substrates. At the same time another group purified an NADH-cyt b_5 reductase from RBC plasma membranes, its higher apparent molecular weight, 45 kDa (Choury, *et al.*, 1981), perhaps reflecting insufficient removal of

detergent molecules, and/or a degree of glycosylation within the sample. Interestingly, both groups found that digestion with cathepsin D, gave smaller fragments, 22 and 29 kDa from the 36 and 45 kDa membrane proteins, respectively. The shortened proteins had striking similarity with the soluble NADH-cyt b_5 reductase found in the cytoplasm of human RBCs, suggesting that the soluble form may well be derived from proteolytic cleavage of the membrane-bound form. Confirmation that the two proteins were identical was established via their cross-reacting to antibodies raised against liver microsomal NADH-cyt b_5 reductase (Kitajima, *et al.*, 1981).

NADH-cyt b_5 reductase is found on the cytoplasmic face of the plasma membrane. Hence, although this protein may be a possible component of a PMOR complex, it is unlikely that it provides the transmembrane activity. More recently a few candidates for the transmembrane component of the PMOR have been suggested.

A CoQ reductase has been purified from pig liver plasma membranes (Villalba, *et al.*, 1995). This reductase was purified after removal of peripheral proteins and membrane solubilization with CHAPS, using gel filtration, anion exchange chromatography and affinity chromatography. The CoQ reductase activity also co-eluted with ferricyanide reductase activity. The 34 kDa flavoprotein, specific for NADH, was sensitive to quinone analogues such as capsaicin. Another protein of similar size, a 32 kDa glycoprotein, was isolated from Ehrlich tumour cells (del Castillo-Olivares, *et al.*, 1996). This NADH:ferricyanide reductase, isolated from an enriched plasma membrane fraction by solubilization with triton X-100, ammonium sulphate precipitation, and a variety of chromatographic techniques, also showed high specificity for NADH as an electron donor. The native protein had an estimated mass of 117 kDa, suggestive of a tetrameric form.

An NADH:DCIP oxidoreductase activity, purified from bovine and rat brain synaptic plasma membranes and vesicles, and from the neuroblastoma cell line NB41A3 (Bulliard, *et al.*, 1997), was found to be a complex of at least 5 tightly associated proteins. These proteins; GAPDH, hsc70, TOAD64, aldolase C, and enolase-γ, remained in tight association through solubilization, ammonium sulphate

precipitation, gel filtration, affinity chromatography and native-PAGE before separation by SDS-PAGE. The authors suggested that GAPDH was the most likely NADH oxidoreductase component of the complex. However, this protein is only known to be associated with the cytoplasmic surface of the plasma membrane and, therefore, is unlikely to be the transmembrane component of the PMOR.

Of final note, a 35 kDa plasma membrane NADH:ferricyanide oxidoreductase was recently purified from Namalwa cells (Baker, *et al.*, 2004b) and identified as porin isoform 1 or voltage-dependent anion-selective channel 1 (VDAC1). VDAC1 is a transmembrane protein predominately expressed in the outer mitochondrial membrane, but has also been found in the plasma membrane (Baker, *et al.*, 2004b). It has not previously been associated with redox activities.

7.2 Motivation

As mature circulating RBCs have no intracellular organelles, pure plasma membranes are obtained relatively easily. RBCs have been shown to have high levels of ferricyanide reductase activity in the plasma membrane (Himmelreich and Kuchel, 1997), and can also reduce WST-1 at the plasma membrane (Chapter 6). This Chapter presents preliminary efforts to purify the NADH:ferricyanide and NADH:WST-1 oxidoreductase activities from RBC membranes, and specifically to (a), identify the protein(s) responsible and (b), determine whether these oxidants are reduced by the same or different systems.

7.3 Results

7.3.1 Solubilizing RBC membrane ghosts

A variety of ionic, non-ionic, and denaturing detergents were tested for their ability to solubilize ferricyanide and WST-1 reductase activity from RBC ghosts. Activity was completely lost on solubilization with SDS, probably due to a loss of protein secondary structure (Van Renswoude and Kempf, 1984). In contrast, addition of Lubrol, Triton X-100, Tween-80 or CHAPS successfully solubilized both WST-1 and ferricyanide reductase activities (Figure 7.1). The extent of solubilization of

activity increased with detergent concentration for Lubrol and Tween-80. Lubrol appeared to be the most effective in preserving specific activity. Hence, this detergent was chosen for the subsequent solubilization of RBC ghosts, and in the purification protocols described below. Membranes isolated from 3 packs of blood-bank blood (~1.2 L) contained 21.88 and 3377 nkatal (nmol of substrate reduced per s) of WST-1 and ferricyanide reductase activity, respectively. The specific activity of ferricyanide reduction observed in whole ghosts, at 386 nmol min^{-1} mg $protein^{-1}$, was similar to values previously reported (Zamudio and Canessa, 1966; Zamudio, *et al.*, 1969; Crane, *et al.*, 1982; Miner, *et al.*, 1983). Likewise, the specific activity of WST-1 reductase observed in whole ghosts (2.5 nmol min^{-1} mg $protein^{-1}$) was similar to that of the RBC membrane NADH oxidase activity as described by Vijaya and colleagues (1984). On solubilization with 2% Lubrol both of the enzyme activities were purified 1.5 fold (Table 7.1).

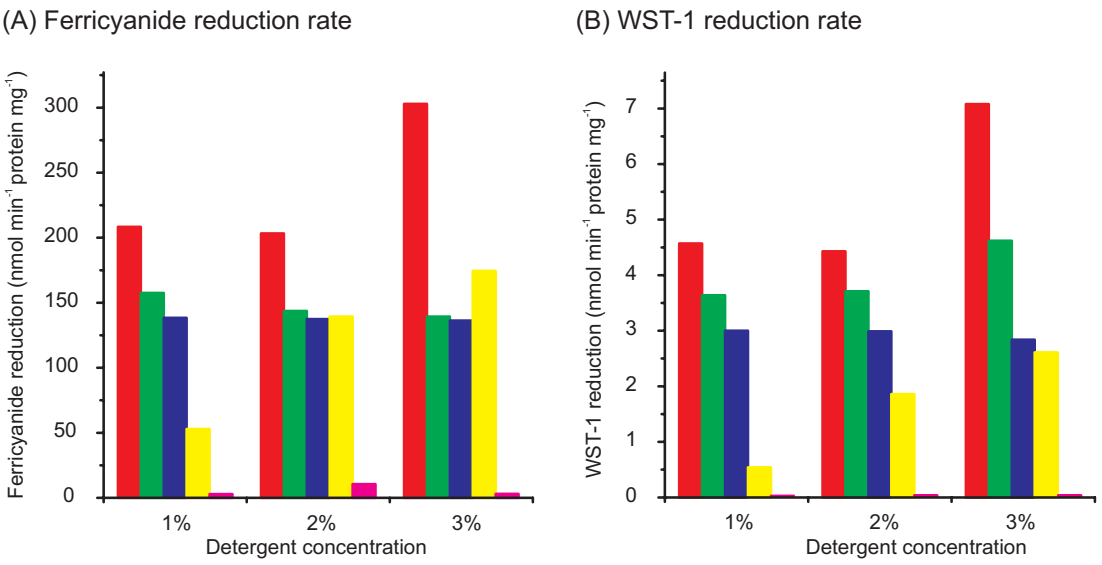

Figure 7.1: Optimization of detergent concentrations used to solubilize redox activity. RBC ghosts (3 mg/mL protein) were incubated overnight at 4 °C in 50 mM Tris-HCl, pH 8.0 containing 50 mM NaCl, with the detergents, Lubrol (■), CHAPS (■), Triton X-100 (■), Tween 80 (■) and SDS (■) at 1, 2 and 3%, then centrifuged at 13.2 rpm for 1 h at 4 °C. The protein concentration of the supernatant was measured (§2.7.6) then assayed for (A) ferricyanide (§2.7.7.1) and (B) WST-1 (§2.7.7.2) reductase activity.

The specificities of the WST-1 and ferricyanide reductase assays were tested on a variety of different control protein preparations (data not shown). At protein

concentrations above 5 mg/mL, commercial samples of alcohol dehydrogenase, BSA, Hb, LDH, and lysozyme, all reduced ferricyanide and WST-1 to various extents. Below this concentration negligible activity was observed. The rates of reduction from these protein samples at 5 mg/mL were ~50 fold lower than observed in isolated membranes of comparable protein concentration.

7.3.2 Purification of ferricyanide and WST-1 redox activity

7.3.2.1 DEAE-Sepharose anion-exchange chromatography on Lubrol solubilized membranes

Lubrol solubilized plasma membranes (175 mL, ~3 mg/mL) were loaded onto a DEAE-Sepharose column (10 mL) and washed with 5 column volumes prior to elution with a linear gradient of 0.05–2 M NaCl in buffer A. The flow-through, wash, and collected fractions were assayed for protein concentration, and ferricyanide, and WST-1 reductase activities. WST-1 and ferricyanide reductase activities co-purified, eluting from the column with 300–400 mM NaCl (Figure 7.2). Some WST-1 and ferricyanide reduction was also recovered in the wash-fraction, the further purification of which is discussed in §7.3.2.4. Ferricyanide reductase specific activity was purified 2.1 fold in the active fractions, and 85% of total activity was recovered. WST-1 reductase specific activity did not appear to be purified in the active fractions, but 87% of the total activity was recovered (Table 7.1).

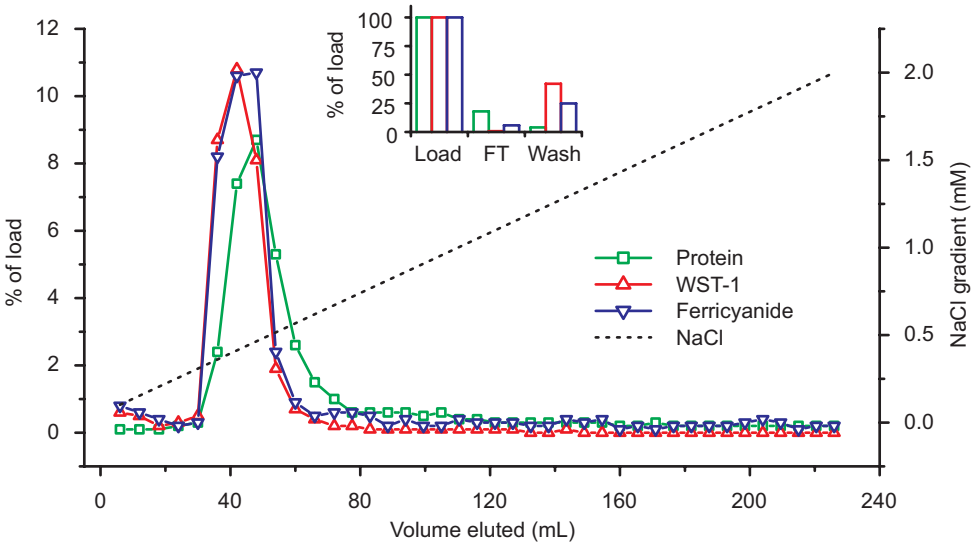

Figure 7.2: Fractionation of redox activity by DEAE-Sepharose chromatography. Plasma membrane proteins, solubilized in 2% Lubrol, were loaded onto a DEAE-Sepharose column and eluted with a linear NaCl (⋯) gradient (elution details in §2.7.3). Six mL fractions were collected and measured for protein concentration (□), and ferricyanide (▽) and WST-1 (△) reductase activity (§2.7.6, §2.7.7.1 and §2.7.7.2, respectively). The inset shows the recovery of protein and activity in the flow-through (FT) and wash fractions compared to the load.

7.3.2.2 Hydroxyapatite adsorption of active fractions from anion-exchange chromatography

The active fractions from the DEAE-Sepharose column were pooled and diluted to ~50 mM salt prior to loading on a 10 mL Hydroxyapatite Bio-Gel® HTP gel column. The column was washed with 40 mL of buffer A prior to elution with a linear gradient of 0–0.5 M NH_4SO_4. The flow-through, wash, and collected fractions were again tested for protein concentration, and ferricyanide, and WST-1 reductase activities. Essentially no activity was recovered in the wash-fraction. The fractions containing WST-1 and ferricyanide reductase activities co-eluted between 180–220 mM NH_4SO_4 and corresponded with the main protein peak (Figure 7.3). The ferricyanide and WST-1 reductase activities were purified 4.3 and 16 fold, respectively (Table 7.1).

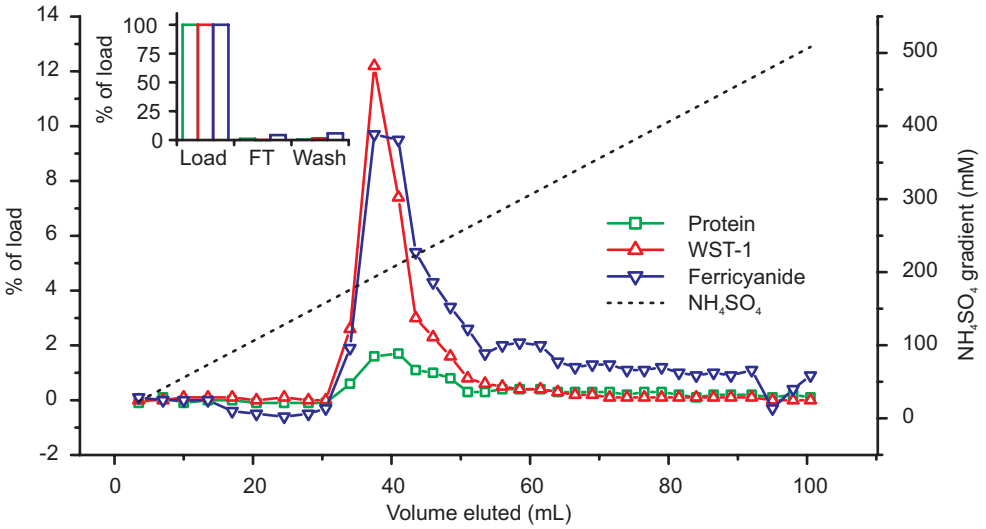

Figure 7.3: Fractionation of redox activity from combined active fractions from DEAE-Sepharose column by hydroxyapatite adsorption chromatography. The active fractions were loaded onto a hydroxyapatite column, eluted with a linear NH_4SO_4 (⋯⋯) gradient (elution details in §2.7.4), and fractions assayed for protein concentration (□), and ferricyanide (▽) and WST-1 (△) reductase activity (§2.7.6, §2.7.7.1 and §2.7.7.2, respectively). The inset shows the recovery of protein and activity in the flow-through (FT) and wash fractions compared to the load.

7.3.2.3 Size exclusion chromatography of active fractions from hydroxyapatite adsorption

The active fractions from hydroxyapatite fractionation were pooled (8.5 mL) and concentrated to 0.5 mL in a stirred cell concentrator using a YM10 diaflo ultrafiltration membrane (Amicon Inc., Beverly, MA, USA). All solutions and samples were passed through a 0.22 µm filter prior to application to a 25 mL Sephadex-200 HR 10/30 column (Amersham Pharmacia) coupled to a BioRad Laboratories FPLC system. Samples were eluted at a constant flow rate of 0.5 mL/min and assayed for protein concentration and redox activity. WST-1 and ferricyanide reductase activities eluted between 7 and 11 mL with the highest activity for each occurring at 8.5 mL (Figure 7.4), this suggested a molecular weight of approximately 500 kDa.

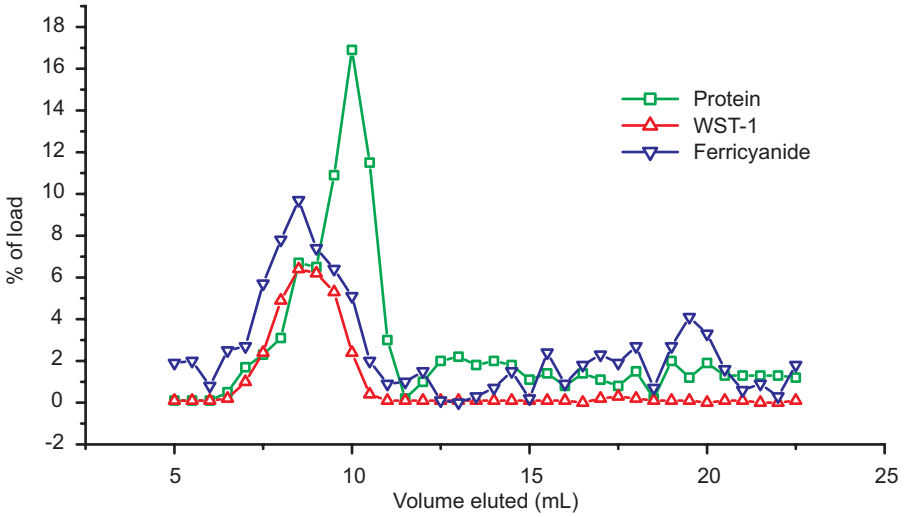

Figure 7.4: Elution of redox activity from gel filtration S-200 column. The active pool from hydroxyapatite fractionation was loaded onto Sephadex-200 HR30/10 column (elution details in §2.7.5) and 0.5 mL fractions were assayed for protein concentration (□), and ferricyanide (▽) and WST-1 (△) reductase activity (§2.7.6, §2.7.7.1 and §2.7.7.2, respectively).

The ferricyanide and WST-1 reductase activities were concentrated 6.7 and 15.6 fold, respectively, by the combination of anion-exchange, hydroxyapatite and gel filtration chromatography (Table 7.1).

Table 7.1: Purification of membrane redox activity from RBC membranes

	Total protein (mg)	Total volume (mL)	Specific activity (nmol min^{-1} mg^{-1})		Purification factor		Total activity (nkatal)	
			FeCN	WST-1	FeCN	WST-1	FeCN	WST-1
Ghosts	525	200	386	2.5			3378	21.88
Solubilization	310	175	559	3.7	1.4	1.5	2888	19.12
DEAE-Sepharose	23.5	15	796	1.2	2.1	0.5	312	0.47
Hydroxyapatite	0.41	8	1679	40	4.3	16.0	11.3	0.27
Gel filtration	0.03	0.5	2593	39	6.7	15.6	1.2	0.02

The redox activities co-eluted with the main protein peak in both the DEAE-Sepharose and HA chromatography steps, whilst in the gel filtration step they appeared to co-elute with a shoulder component of the main peak. The large estimated size of the protein(s) responsible for the redox activity from the gel filtration experiment and the limited extent of purification achieved, suggested either

incomplete separation of proteins due to aggregation or, that the redox activity was associated with a complex of proteins. The effectiveness of the purification techniques was examined by SDS-PAGE (Figure 7.5).

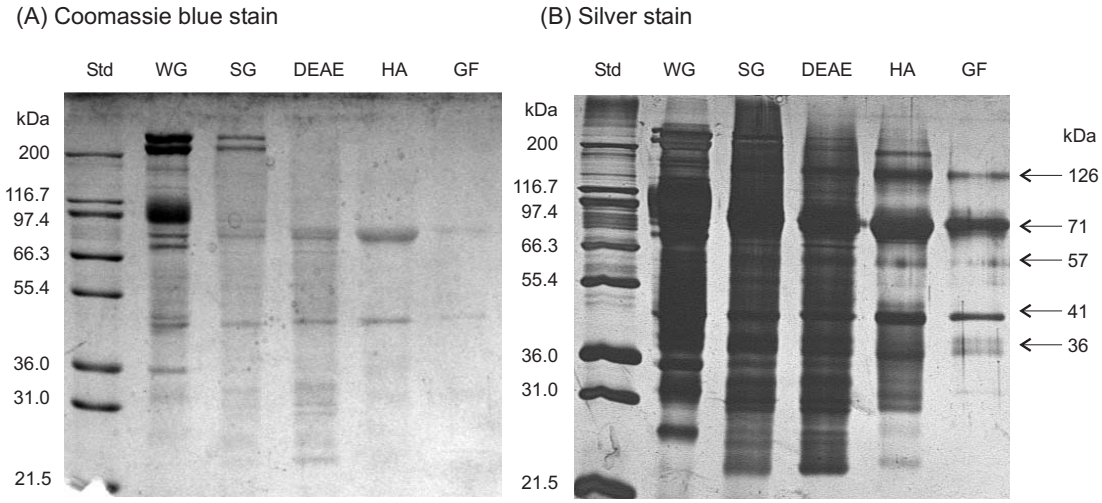

Figure 7.5: Purification of plasma membrane ferricyanide and WST-1 reductase activities as analysed by SDS-PAGE. Mark 12® molecular weight standards (Std) were loaded in lane 1. The active sample from whole RBC ghosts (WG), solubilized ghosts (SG), DEAE-Sepharose chromatography (DEAE), hydroxyapatite chromatography (HA), and gel filtration column (GF) were mixed with loading buffer and run on a 10% SDS-PAGE gel (see §2.7.8 for details). Proteins were stained with (A) Coomassie blue stain and (B) silver stain as described in §2.7.9 and §2.7.10, respectively. The molecular weights of the standards are indicated on the left edge of the gels and the approximate sizes of the bands in the active fraction after gel filtration are indicated to the right of the silver stained gel.

As the low protein concentrations in the purified fractions limited the effectiveness of the visualization by staining with Coomassie blue (Figure 7.5A), the more sensitive silver staining technique was also used (Figure 7.5B). Overall, the extent of purification of one or more protein(s) was unclear. The protein profile observed in the gel filtration (GF) lanes is approximately analogous to that observed in the solubilized ghosts (SG) lanes (Figure 7.5A). The shoulder to the main peak observed in the gel filtration step (which contained the ferricyanide and WST-1 reductase activities) also contained an essentially identical SDS-PAGE profile to the main peak (data not shown). The final active fraction from gel filtration contained 6 prominent protein bands with approximate sizes of 126, 71, 57, 41, and 36 kDa (the latter being two bands but treated as one species). These bands were excised from a Coomassie

blue stained gel and sent to BMSF (University of New South Wales, Sydney, Australia) for in-gel trypsin digest, zip-tip clean up, and liquid chromatography-mass spectrometry (LC-MS) analysis (Table 7.2). It should be noted that the protein content of each band was low, making positive identifications difficult. The proteins identified from the LC-MS data had, in most cases different molecular weights to those estimated from the SDS-PAGE gel. The bands sized as approximately 41 kDa and 126 kDa, are consistent with the molecular weight of the actin monomer and (presumably covalently-linked) trimer (Stryer, 1995). In contrast, keratin from hair and skin particles is known as a common contaminant in MS spectra. Hence, it is considered unlikely that the keratin signals identified correlate with the true identity of the 57 kDa band. Glycophorins AM and C, as the names suggest, are transmembrane glycoproteins which possess peptidic and glycopeptidic epitopes (Lisowska, 2001). The function of glycophorin AM is not clearly established, while Glycophorin C is involved in anchoring the red cell skeleton to the membrane (Tanner, 1993).

Table 7.2: MASCOT search results on peptide fragments identified by LC-MS.

Estimated size from SDS-PAGE	MASCOT result	Location	Molecular weight
126 kDa	actin	Component of the cytoskeleton	42 kDa monomer
71 kDa	glycophorin AM	Integral membrane glycoprotein	14.8 kDa monomer
57 kDa	cytokeratin 1	In human hair	65.8 kDa monomer
41 kDa	actin	See above	42 kDa monomer
36 kDa	glycophorin C	Integral membrane glycoprotein	13.8 kDa monomer

Thus, it is unlikely that any of the proteins listed in Table 7.2 contribute to the redox activity being investigated. The active component(s) of the fraction may in fact be present at only very low abundance and may not have been identifiable on the SDS-PAGE gel, or by LC-MS.

7.3.2.4 Hydroxyapatite adsorption of wash fractions from anion-exchange chromatography

Due to the low levels of activity noted in the DEAE-Sepharose active fractions, the wash-fraction from the DEAE-Sepharose column was applied to a hydroxyapatite column for further purification (Figure 7.6). Approximately 40% of the loaded protein eluted in the wash-fraction, but the majority of the ferricyanide and WST-1 reductase activities were bound to the column and eluted with between 120–150 mM NH_4SO_4. As with the fractionations reported above, the ferricyanide and WST-1 activities corresponded with the main protein peak. The ferricyanide and WST-1 reductase activities were purified 158 and 117.2 fold, respectively (Table 7.3). Four protein bands were identified with SDS-PAGE from the active fractions from the hydroxyapatite column (Figure 7.7). The sizes of these bands (100, 65, 57, and 36 kDa) were similar to those identified from the DEAE-Sepharose active fractions (Figure 7.5). Unfortunately, the low protein recovery from the hydroxyapatite column prevented further purification and identification.

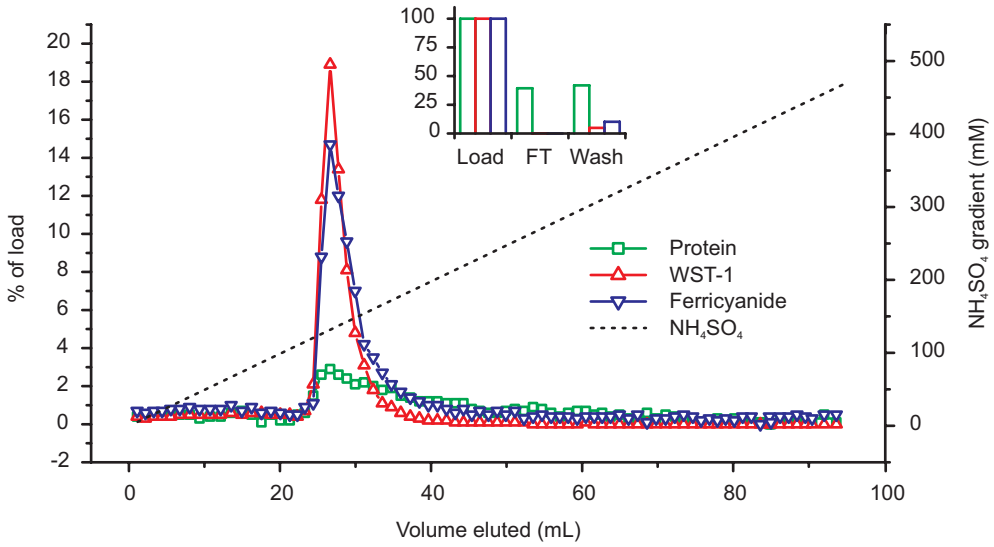

Figure 7.6: Fractionation of redox activity from the DEAE-Sepharose column-wash by hydroxyapatite adsorption chromatography. The active fractions were loaded onto a hydroxyapatite column; eluted with a linear NH_4SO_4 (⋯) gradient (elution details in §2.7.4) and resultant fractions were assayed for protein concentration (□), ferricyanide (▽), and WST-1 (△) reductase activity (§2.7.6, §2.7.7.1 and §2.7.7.2, respectively). The inset shows the recovery of protein and activity in the flow-through (FT) and wash fractions compared to the load.

Table 7.3: Purification of membrane redox activity from the DEAE-Sepharose wash-fraction.

	Total protein (mg)	Total volume (mL)	Specific activity (nmol min^{-1} mg^{-1})		Purification factor		Total activity (nkatal)	
			FeCN	WST-1	FeCN	WST-1	FeCN	WST-1
Ghosts	525	200	386	2.5			3378	21.88
Solubilization	310	175	559	3.7	1.4	1.5	2888	19.12
DEAE-Sepharose	2.82	20	5756	16.7	14.9	6.7	271	0.79
Hydroxyapatite	0.07	1.2	60986	293.0	158.0	117.2	72	0.35

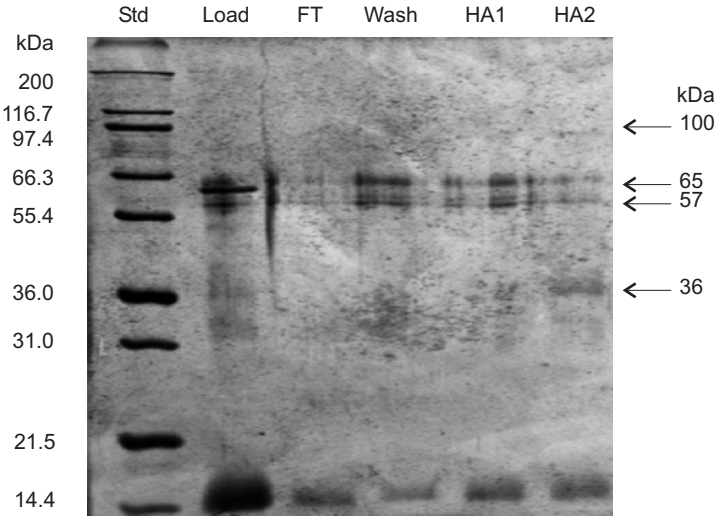

Figure 7.7: SDS-PAGE gel of active fractions from hydroxyapatite fractionation of DEAE-Sepharose wash-fraction. Mark 12® molecular weight standards (Std) were loaded in lane 1, wash-fraction from DEAE-Sepharose (load), column flow-through (FT), wash, and two active fractions from the hydroxyapatite fractionation (HA1, HA2) were diluted with loading buffer before running on 10% polyacrylamide gel (see §2.7.8 for details). Proteins were stained with silver stain as described in §2.7.10. The molecular weights of the standards are indicated to the left of the gel and the approximate sizes of the bands in the active fraction are indicated on the right side of the gel.

7.4 Discussion

The presence of redox activity in the plasma membrane is indisputable. However, the process and proteins involved in the reduction of extracellular oxidants are still under investigation. Until recently, those proteins identified as being involved in PMOR activity have not been transmembranous. These include cyt b_5 reductase (Choury, *et al.*, 1981; Kitajima, *et al.*, 1981; Kim, *et al.*, 1995) and GAPDH (Bulliard, *et al.*, 1997), which are both associated with the cytosolic side of the

plasma membrane. Transmembrane candidate proteins include CoQ reductase, an integral membrane protein, which has been shown to be involved in CoQ mediated transmembrane redox activity (Villalba*, et al.*, 1995), and plasma membrane VDAC1 which has ferricyanide oxidoreductase activity (Baker*, et al.*, 2004b).

The preliminary attempts reported in this Chapter to purify ferricyanide and WST-1 reductase activities from the RBC plasma membrane were inconclusive. High levels of reductase activity for both oxidants were found in the RBC plasma membrane, 3378 and 21.88 nkatal for ferricyanide and WST-1, respectively, and activity was retained throughout the purification steps (Tables 7.1 and 7.3). However, active fractions collected from most of the columns co-eluted with the main protein peak. Exceptions to this were: the high activity and low protein content of the wash-fraction collected from the DEAE-Sepharose column, the high protein and low redox activity content of the wash-fraction collected from the hydroxyapatite column of the DEAE-Sepharose wash-fraction (insets in Figures 7.2 and 7.6, respectively) and the redox active shoulder peak in the gel filtration profile (Figure 7.4). The high activity in the wash-fraction of the DEAE-Sepharose column suggested either, that the protein(s) had low binding affinity for the column or, that there are in fact two activities/protein(s) which have both WST-1 and ferricyanide reductase capacity.

The DEAE-Sepharose wash-fraction was purified to a greater extent than the active fractions eluted off the column with high salt, with over a hundred fold increase in purity achieved for both ferricyanide and WST-1 reductase activity after hydroxyapatite adsorption (Table 7.3). Thus, this might represent a starting point for future purification attempts. Interestingly, a similar number and size of protein bands were present after SDS-PAGE separation of the DEAE-Sepharose wash-fraction (Figure 7.7) as were found after the purification of the DEAE-Sepharose active fractions (Figure 7.5). This suggests that the ferricyanide and WST-1 reductase activities of RBC membranes could be attributable to a complex of proteins which themselves are heterogeneous in nature, some able to bind to DEAE-Sepharose, whilst others were unable to do so. Such complexes may be comprised of numerous "carrier" proteins (such as actin) which themselves are unlikely to play any specialised role in the activity of the purified material. The proteins identified by LC-MS, apart from actin, did not correspond in molecular weight to the estimated

sizes from the SDS-PAGE gel (Figure 7.5). Glycophorin C forms a bridge with protein 4.1 and spectrin/actin between the plasma membrane and the cytoskeleton of RBCs (Tanner, 1993; Chang and Low, 2001). Glycophorin A is the main glycoprotein in the human RBC membrane (Cochet, *et al.*, 2001) and may play a role in facilitating the movement of Band 3 to the cell surface during its biosynthesis (Tanner, 1993). Membrane glycoproteins have previously been suggested to play a role in PMOR activity, as glycosidases (del Castillo-Olivares, *et al.*, 1994) and lectins (Navas, *et al.*, 1988) can inhibit PMORs. However, although the proteins identified were either transmembrane or membrane associated, the lack of consistency with the estimated molecular weights raises suspicion as to whether they are truly components of a PMOR. The very identification of such proteins suggests that the solubilization step in itself was not sufficient to separate different proteins into different detergent micelles, which would have greatly facilitated higher levels of purification.

The preliminary studies performed with detergents indicated that Lubrol was the most effective detergent for purifying ferricyanide and WST-1 reductase activities from the RBC plasma membrane. The successful identification of transmembrane proteins in the resultant fractions suggests that the concentration of detergent (2%) was indeed sufficient to solubilize integral membrane proteins. However, different concentrations might enable more successful separation of the membrane components. The identification of the cytoskeletal protein actin suggests that in future protocols, membranes should be washed with 0.1 M EDTA to remove peripheral proteins prior to further purification steps (Van Renswoude and Kempf, 1984).

The co-elution of ferricyanide and WST-1 reductase activities with the main protein peak in most of the fractionations suggests the conditions used for each column were sub-optimal. The ferricyanide and WST-1 reductase activities were found to be resistant to high salt and detergent concentration. Hence, the purification protocol might be improved by optimising the concentrations of these components. The purification could also be improved by scaling up the amount of starting material by at least an order of magnitude (perhaps using animal, rather than human blood) and

by introducing different fractionation techniques into the protocol. Blue Sepharose-affinity chromatography, for instance, has been used successfully to purify NADH utilising dehydrogenases including GAPDH (Liu and Stellwagen, 1986) and, more recently, the putative PMOR activity VDAC1 (Baker, *et al.*, 2004b), and may be appropriate for the purification of RBC plasma membrane ferricyanide and WST-1 reductase activities. An additional technique which might be useful in the elucidation of whether a single protein or a protein complex gives rise to the PMOR activity is Blue Native-PAGE (Nijtmans, *et al.*, 2002). This technique was first developed to study the components of the mitochondrial respiratory chain (Schagger and von Jagow, 1991) and in combination with SDS-PAGE can be used to identify protein complexes and the components of the complex (Nijtmans, *et al.*, 2002). Alternatively a proteomics based approach could be used where the total components of solubilized ghosts are separated by two-dimensional electrophoresis and individual bands identified by mass spectrometry.

From this preliminary attempt to purify NADH:ferricyanide and NADH:WST-1 oxidoreductase activities from RBC membranes, it was, unfortunately, not possible to determine with any certainty whether the two activities were performed by the same or different systems, or what the component(s) of these systems were. However, it is possible that further development of the methods employed might allow for these activities to be successfully purified and identified.