Purification and characterization of a plasma membrane ferricyanide-utilizing NADH dehydrogenase from Ehrlich tumour cells

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A ferricyanide-utilizing NADH dehydrogenase (NADH-ferricyanide oxidoreductase) from the plasma membrane of Ehrlich ascites tumour cells has been purified about 1500-fold to apparent homogeneity. The method comprises the isolation of an enriched plasma membrane fraction, solubilization with Triton X-100, ion-exchange chromatography, ammonium sulphate precipitation, Cibacron Blue chromatography and fast-protein liquid chromatography with a Superose-6 gel filtration column. The specific activity of the final pool was more than 61 units/mg protein. The pure enzyme examined by SDS/PAGE displayed only one type of subunit with an apparent molecular mass of

32.0 kDa. The molecular mass of the native protein (117.0 kDa) was estimated by gel filtration; these results suggest a protein composed of four subunits of identical molecular mass. The enzyme was stable in the pH interval between 6 and 9, with maximum activity at pH values from 7.5 to 8.5. The purified enzyme showed Michaelis–Menten kinetics for the substrates, with apparent K_m values of 4.3×10^{-5} M and 6.7×10^{-5} M for NADH and ferricyanide respectively. The isolated protein was strongly inhibited by Zn^{2+} and the thiol-specific reagents mersalyl and *p*-chloromercuribenzenesulphonic acid.

INTRODUCTION

Electron transport systems have been detected in the plasma membrane of different eukaryotic cells [1,2]. Considerable interest has been aroused by the assignment of key essential functions that could be regulated by these redox activities [3]. One of these functions is the control of cell growth; for example, a stimulation of cell proliferation has been found when impermeant external oxidants such as ferricyanide are added to the culture medium [4]. Furthermore in the presence of ferricyanide an increased expression of several genes related to the mitogenic stimulation of mammalian cells occurred [5]. In transformed cells, plasma membrane redox systems are different from those present in normal untransformed cells; for example, in neuroblastoma cells a redox activity correlates with *N-myc* oncogene expression [6]. However, the mechanisms by which plasma membrane redox systems contribute to cell growth and proliferation are unknown.

Wallach and Kamat [7] first reported the presence of an NADH-ferricyanide oxidoreductase (FR) in Ehrlich ascites cell plasma membrane. This activity was later confirmed by Kilberg and Christensen [8] in native plasma membrane vesicles. We have shown that transmembrane FR activity of intact Ehrlich cells was stimulated by increasing the cytosolic NADH levels and strongly inhibited by cytotoxic drugs at concentrations that inhibit cell growth [9]. A detailed kinetic characterization of this enzymic activity was also made in whole cells [9], in highly pure plasma membrane vesicles [10] isolated from Ehrlich tumour cells by two-phase compartmentation [11] and in a reconstituted proteoliposome system [12]. The enzyme showed Michaelis–Menten kinetics for both NADH and ferricyanide. Two phases, one fast and transient and the other slow, were observed in the oxidation of NADH and in the reduction of ferricyanide by using plasma membrane vesicles; in assays carried out in low ionic strength and osmolarity, both phases were indistinguishable [10].

Even though there are a few recent reports dealing with the isolation of several plasma membrane oxidoreductases from

bacteria [13,14] and plant cells [15,16], the number of enzymes isolated in a highly pure state from animal cells is very small [17–19]. The major aim of our work was the purification of the plasma membrane FR from Ehrlich ascites tumour cells and its characterization. The isolated pure enzyme should also provide a useful tool for further biochemical and biophysical studies of tumour plasma membrane redox systems.

MATERIALS AND METHODS

Materials

β -NADH, Triton X-100, reduced Triton X-100, digitonin and potassium ferricyanide were from Sigma (St. Louis, MO, U.S.A.). Reagents for SDS/PAGE were from Serva (Heidelberg, Germany) and Boehringer (Mannheim, Germany). HPLC ultra-pure water, generated by a Milli-RO 60 coupled to a Milli-Q water purification system (Millipore), was used to prepare the aqueous solutions. The chromatography media and fast-protein liquid chromatography (FPLC) columns were from Pharmacia (Uppsala, Sweden), with the exception of the Cibacron Blue 3GA affinity medium (Sigma).

Purification of FR

All the purification steps were performed in a cold room at 4 °C except those employing FPLC, which were done in a thermostated room at 17 °C.

Isolation of an enriched plasma membrane fraction

A hyperdiploid Lettré strain of Ehrlich ascites tumour cells was maintained in 2-month-old female albino Swiss mice OF1 (SPF Ico), as described elsewhere [20]. The life span of the animals after inoculation with 5×10^6 tumour cells was 16 ± 1 d. Tumour

cells from 30 inoculated mice were harvested on day 10 or 11 after inoculation, as described [20], pooled and adjusted to a cellular density of 2×10^8 cells per ml and treated with digitonin (4 mg per 10^9 cells) under the conditions described by Moreadith and Fiskum [21]; this method was originally used for the isolation of mitochondria. The digitonin permeabilized cells were homogenized by 10 passes in a glass–Teflon Potter–Elvehjem homogenizer and centrifuged at 1000 *g* for 5 min. The mitochondria-enriched supernatant was discarded; the gelatinous pellet was retained and washed twice with 20 mM Tris/HCl, pH 7.6 (buffer A). This constitutes the plasma membrane-enriched fraction; the redox activity was conserved when stored at -80°C .

FR solubilization and DEAE chromatography

The enriched plasma membrane fraction, at 20–30 mg/ml protein concentration in buffer A, was treated with Triton X-100 at a detergent-to-protein ratio of 1:1 (by wt.). After incubation for 30 min at 4°C with gentle stirring, the suspension was centrifuged at 100 000 *g* for 30 min. The supernatant (Triton X-100 extract) was applied to a DEAE-Sephacel column (2.5 cm $\times$ 8 cm) coupled to an FPLC system (Pharmacia) and equilibrated with buffer A plus 0.5% (w/v) reduced Triton X-100. The column was washed with the same medium, then eluted with a linear NaCl gradient (0–0.4 M) in buffer A plus 0.5% (w/v) reduced Triton X-100. The flow rate was 60 ml/h and the volume of the gradient 80 ml. Fractions of 2.5 ml were taken and those showing FR activity were pooled.

Ammonium sulphate precipitation

Solid ammonium sulphate was slowly added to the pool of active fractions from the DEAE-Sephacel column until 20% (w/v) saturation was achieved. The solution was stirred for 30 min and centrifuged at 25 000 *g* for 30 min. The supernatant was brought to 40% saturation in ammonium sulphate and left overnight at 4°C . The solution was finally centrifuged at 25 000 *g* for 30 min. The precipitate was dissolved in a small volume of 20 mM Tris/HCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 0.5% (w/v) reduced Triton X-100, pH 7.0 (buffer B).

Dye-affinity chromatography

A Cibacron Blue 3GA column (bed volume 4 ml) was equilibrated with buffer B. The active FR sample was loaded and recirculated twice at a flow rate of 4 ml/h. After washing with buffer B, the enzyme was eluted with buffer B containing 1 M NaCl. Fractions of 1 ml were taken and concentrated by precipitation with 40% (w/v) ammonium sulphate.

Gel filtration chromatography

A Superose-6 gel filtration column was coupled to the FPLC system and equilibrated with 20 mM Tris/HCl, 50 mM NaCl, 0.5% (w/v) reduced Triton X-100, pH 7.0 (buffer C). The concentrated active sample from the affinity chromatography step was applied to the Superose-6 column. The column was eluted with the same buffer at a flow rate of 0.4 ml/min. Fractions of 0.4 ml were collected.

Determination of FR native molecular mass

Gel filtration was used to estimate the apparent molecular mass of native FR. FPLC was performed with a Superose-6 column at the conditions described above. The column was calibrated with

the following standards (Sigma): rabbit IgG (150 kDa), BSA dimer (132 kDa) and monomer (66 kDa), human transferrin (81 kDa) and horse heart cytochrome *c* (12.4 kDa). Salmon sperm DNA (Sigma) was used as a marker for the column void volume.

Antibody production and purification

Purified FR was used as an antigen and injected intradermally into a New Zealand rabbit. For the initial immunization, 375 μg of FR were emulsified with Freund's complete adjuvant (1:1, v/v). Three additional injections of antigen (about 200 μg of protein each) with incomplete Freund's adjuvant were given every two weeks after the initial injection. One week after the last injection the serum was collected from the rabbit. Decomplemented serum was purified by ammonium sulphate precipitation and protein-A Sepharose chromatography. In addition an IgG fraction was affinity purified against the purified FR protein as described elsewhere [22]. Briefly, the enzyme was resolved by SDS/PAGE. The gel was blotted on to a nitrocellulose filter and two 1 cm strips from the vertical ends were immunostained as described below, to locate the FR. Then a horizontal strip corresponding to the enzyme band was cut from the unstained nitrocellulose and the specific IgGs were eluted with 2 M sodium thiocyanate in PBS (140 mM NaCl, 3 mM KCl, 5 mM Na_2HPO_4 , pH 7.4). The immunopurified antibodies were desalted by FPLC with a Phast-desalting column (Pharmacia) running in PBS.

Western blot analysis

Western blotting was performed essentially as described elsewhere [23]. Samples were subjected to SDS/PAGE and then transferred to nitrocellulose by using a semi-dry blot system (LKB 2117-250 Novablot) and an electrophoresis unit LKB 2117-Multiphor II setting at 0.8 mA/cm² for 2 h. Protein standards were located on nitrocellulose by staining a strip from the filter with amido black or by using prestained molecular mass standards. The blot was processed for immunolabelling by overnight incubation in a blocking solution containing 3% BSA, 0.05% (v/v) Tween-20 in PBS. The filter was then incubated for 1 h with purified FR antibodies. Antibodies were diluted in PBS containing 0.5% BSA and 0.05% Tween-20. After three 10 min washes in PBS/0.05% Tween-20, the immunocomplexes were detected with a biotin–avidin peroxidase kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, U.S.A.) in accordance with the manufacturer's directions. Finally, the nitrocellulose sheet was washed another three times with PBS/0.05% Tween-20 and the peroxidase activity revealed by incubation in 0.04% (v/v) H_2O_2 , 0.05% 3,3'-diaminobenzidine in 20 mM potassium phosphate, pH 6.0. The reaction was stopped by incubation in 5% acetic acid followed by several washes with distilled water.

Enzyme assays

Ferricyanide reductase activity was determined by following the ferricyanide reduction in a Shimadzu UV-160 spectrophotometer at 420 nm and 37°C . The assay medium contained 0.25 mM ferricyanide, 0.2 mM NADH and plasma membrane vesicles (60–80 μg of protein) or 2 μg of pure protein in Chance–Hess buffer [24] (154 mM NaCl, 6.16 mM KCl, 11 mM sodium phosphate, pH 7.4) at 1 ml final volume. The NADH oxidation was monitored at 340 nm. One unit of enzyme activity is defined as the reduction of 1 μmol of ferricyanide per min. To study the effect of pH, different buffered media at 5 mM final concentrations were used: acetate (pH 5), phosphate (pH 6, 6.8 and 7.4), Tris (pH 8, 8.5 and 9), and carbonate (pH 10); both

substrates (NADH and ferricyanide) were dissolved at the indicated concentrations in these media.

To assess the contamination of the enriched plasma membrane fractions, the following marker enzymes were assayed: cytochrome *c* oxidase [25] and succinate dehydrogenase [26] as mitochondria marker enzymes; glucose 6-phosphatase as endoplasmic reticulum marker [27]; and 5'-nucleotidase as a plasma membrane marker [26]. Inorganic phosphate was measured by the Fiske-SubbaRow method as described by Esmann [28].

Analytical procedures

SDS/PAGE was performed as described by Laemmli [29] with 12% isocratic polyacrylamide gels with a 3% polyacrylamide stacking gel. Apparent molecular masses of proteins were determined by using the Low Molecular Weight Calibration Kit from Pharmacia, containing: phosphorylase *b* (94.0 kDa), BSA (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α -lactalbumin (14.4 kDa). Protein concentrations were determined by a method of Markwell et al. [30] recommended for membrane proteins or by the Bradford method [31]. Specific glycoprotein staining of the purified FR was performed after SDS/PAGE and blotting on to nitrocellulose. The filter was revealed with the Glycotrack system (Oxford Glycosystem) in accordance with the manufacturer's instructions.

RESULTS AND DISCUSSION

Enzyme solubilization and purification

A procedure for the purification of the Ehrlich ascites tumour cells FR was established. As a prerequisite, a method for the obtention of an enriched plasma membrane fraction was standardized. The traditional two-phase compartmentation method, widely used to obtain highly pure plasma membrane [11], was

discarded because of its extremely low yields. This becomes even more important when there is a limitation on raw material (tumour cells). The enriched plasma membrane fraction obtained by the digitonin method made the yield requirements compatible with acceptable levels of contamination with other subcellular organelles, mainly mitochondria and endoplasmic reticulum. In Table 1 the efficacy of the digitonin method is compared with the two-phase method with different cell organelle marker enzymes. Although the purity of the plasma membrane fraction was lower than that obtained by two-phase compartmentation, an enrichment of 13.3-fold was achieved, while maintaining a similar mitochondrial contamination. The enrichment shown by the endoplasmic reticulum (1.9), although higher than that achieved with the two-phase method, was considered to be a tolerable degree of contamination; moreover, a similar relative specific activity has been reported by Morré et al. [32] for the endoplasmic reticulum contamination of an enriched plasma membrane preparation of tumour cells. Furthermore the yield referred to a protein basis increased 100-fold; thus the plasma membrane fraction was considered a suitable starting material for FR purification.

FR activity was extracted from the plasma membrane fraction by treatment with the detergent Triton X-100; we were unable to obtain a significant amount of soluble activity by several physical and chemical treatments of the membranes, including sonication, alkaline extraction in the presence of high salt concentrations and freezing-thawing cycles (results not shown). An optimal detergent-to-protein ratio of 1.0 (by wt.) was determined for Ehrlich cells FR extraction with Triton X-100, which agrees with typical values reported for membrane protein solubilization [22,33]. The isolation of FR from its native membrane domain usually takes place with no appreciable deleterious effect of the detergent, allowing a high recovery of activity (83%); 30% of the total protein was not solubilized (Table 2).

The Triton X-100 extract was immediately applied to a DEAE-

Table 1 Marker enzymes

Enzyme activities were determined in two different preparations as described in the Materials and methods section. Values are expressed as m-units per mg of protein. Values in parentheses are the enrichment of specific activity relative to the cell homogenate. The vesicles were obtained by the two-phase compartmentation method.

	Cell homogenate	Enriched membrane fraction	Vesicles
5'-Nucleotidase	0.3	4.0 (13.3)	11.0 (36.7)
Glucose 6-phosphatase	3.6	7.0 (1.9)	2.1 (0.58)
Succinate dehydrogenase	51.6	6.7 (0.13)	3.3 (0.06)
Cytochrome <i>c</i> oxidase	336.0	59.5 (0.18)	41.2 (0.12)

Table 2 Purification of FR from Ehrlich ascites tumour cells

Step	Total activity (units)	Total protein (mg)	Specific activity (units per mg)	Yield (%)	Purification (fold)
Cell	752	18800.0	0.04	100.0	1
Membrane fraction	400	1000.0	0.40	53.2	10
TX-100 extract	330	300.0	1.10	43.9	27
Anion-exchange chromatography	260	10.0	26.00	34.6	650
(NH ₄) ₂ SO ₄ precipitation	140	3.5	40.00	18.6	1000
Affinity chromatography	112	2.2	51.00	14.9	1275
Gel-permeation chromatography	61	1.0	61.00	8.1	1525

Figure 1 Anion-exchange chromatography

The Triton X-100 extract was applied to a DEAE-Sephacel column (2.5 cm $\times$ 8 cm) coupled to a FPLC system. Symbols: $\bullet$, A_{280} ; —, NaCl concentration; ∇ , FR activity. The flow rate was 60 ml/h and fractions of 2.5 ml were collected. Abbreviation: U, units; O.D., absorbance, in this and subsequent figures.

Sephacel column for ion-exchange chromatography; the FR was eluted at approx. 250 mM NaCl (Figure 1). The specific activity increased 24-fold with respect to the Triton X-100 extract with a recovery of about 80%; this step in the protocol was the most efficient at resolving FR. In the mobile phase of the chromatographic process it was necessary to maintain a Triton X-100 concentration never lower than 0.5%; otherwise the activity was completely lost, probably owing to aggregation and irreversible precipitation of the protein. The active fractions from the DEAE-Sephacel column were pooled and treated with ammonium sulphate; this salting-out step was not only effective in purifying FR (Table 2) but also in preparing the sample for dye-affinity chromatography (Figure 2). The use of affinity chromatography after the salting-out step avoided the need for dialysis, which was found to be deleterious to the FR activity. The FR was always retained on the column and the presence of ammonium sulphate decreased the binding of other proteins with lower affinity for the dye. Elution of the protein from the dye column was performed with 1 M NaCl; the fractions showing activity were pooled and concentrated by precipitation with 40% (w/v) ammonium sulphate. The resulting pellet was dissolved in a small volume of buffer C and subjected to gel filtration (Figure 3); even though this step was unfavourable for the enzyme's activity, a result expected because of dilution of the sample, a final preparation was achieved with a specific activity of 61 units/mg, which represents a purification of 1525-fold with regard to the starting cells (Table 2). The yield was satisfactory, allowing 1 mg of pure enzyme to be recovered at the end of the protocol when starting from about 19 g of total protein. The very low yield has been one of the main drawbacks of several of the reported purification procedures for plasma membrane redox proteins of plant cells [16,34].

Molecular characterization

The silver-stained SDS-gel of the purified enzyme is shown in Figure 3 (inset). A clear major band of apparent molecular mass 32.0 kDa was observed for the isolated FR; although minor

Figure 2 Dye-affinity chromatography

The active fractions after anion-exchange chromatography were fractionated with ammonium sulphate. The pellet from 40% ammonium sulphate saturation was dissolved in a small volume of buffer B and applied to a Cibacron Blue 3GA column (bed volume 4 ml). The enzyme was eluted with buffer B containing 1 M NaCl. Symbols: $\square$, A_{280} ; ∇ , FR activity. The flow rate was 4 ml/h and fractions of 1 ml were collected. Abbreviation: U, units.

polypeptide bands can be detected, the 32.0 kDa peptide was the only polypeptide clearly concentrated when purer forms of the enzyme were obtained. From the relative intensity of the bands in the silver-stained gel, we can conclude that FR was more than 90% pure. Furthermore when this purified protein was submitted to N-terminal analysis, no signal was detected after Edman degradation, which suggests that the protein is blocked but also reinforces the notion that the preparation was essentially homogeneous.

The molecular mass of the purified Ehrlich FR was also evaluated. Gel filtration chromatography on the Superose-6 column gave an apparent molecular mass of 117.0 kDa. The estimation by size-exclusion chromatography is consistent with FR's being a tetramer of 32.0 kDa subunits. However, we cannot discard the possibility of the native enzyme's consisting of a trimer of identical subunits, because it is well known that the Stokes radius of solubilized membrane proteins can be overestimated by 10–20% on gel filtration columns [35]. The serum anti-Ehrlich FR was purified as described in the Materials and methods section and used for immunoblot analysis of the isolated Ehrlich FR; the 32.0 kDa band was clearly visible although some background appeared at the top of the blot (results not shown). To remove this interference, the serum was immunopurified against the 32 kDa band; this IgG population was also used for Western blot analysis. The immunological characterization of highly pure plasma membrane vesicles isolated by two-phase compartmentation confirmed the pattern already shown by the purified enzyme: a band of 32.0 kDa was revealed (Figure 4, lane A). Given that these vesicles are essentially devoid of endoplasmic reticulum or mitochondrial contamination, this result confirms the purified protein as being a genuine plasma membrane oxidoreductase. In addition the whole-cell immunostaining *in situ* with antibodies raised against the purified enzyme strongly suggests that the purified enzyme is mainly associated with the cell surface (results not shown).

The Ehrlich FR was then characterized with regards to the presence of oligosaccharides attached to the protein. The purified

Figure 3 Gel-permeation chromatography

The concentrated active sample from the affinity chromatography was applied to a Superose-6 column coupled to an FPLC system. The column was eluted with buffer C at a flow rate of 0.4 ml/min and fractions of 0.4 ml were collected. Symbols: ○, A_{280} ; ●, FR activity. Abbreviation: U, units. Inset, SDS/PAGE of purified FR. About 2 μ g of purified FR was applied and the gel stained with silver. The arrowhead at the right indicates the position of the 32 kDa band. The relative position of the molecular mass standards are indicated by arrowheads at the left of the gel (composition described in the Materials and methods section), from top to bottom 94, 67, 43, 30 and 20.1 kDa.

Figure 4 Molecular characterization of purified FR

Lane A, Western blot: highly pure plasma membrane vesicles (about 40 μ g of protein) from Ehrlich tumour cells were analysed by electrophoresis and blotted as described in the Materials and methods section. The blot was revealed by using immunopurified anti-Ehrlich FR antibodies. Lane B, glycoprotein staining: purified FR (about 0.5 μ g) was analysed by SDS/PAGE and blotting onto nitrocellulose. The filter was revealed with a specific glycoprotein stain (Glycotrack system). The 32 kDa band is indicated with an arrowhead at the right; arrowheads at the left indicate the positions of the molecular mass standards used in both experiments (as in Figure 3).

enzyme is a glycoprotein (Figure 4, lane B) and the glycosidic moiety seems to be essential for function, as judged by the strong susceptibility shown by the activity to treatment with several glycosidases, both in the membrane-bound state [36] and in the purified form (results not shown).

Some effort was also devoted to the identification of cofactors bound to the enzyme. One of the most frequent redox cofactors is flavin nucleotide [37]. The UV and visible spectra of the purified FR at 8.5 μ M concentration, well above the limit of detection of flavin, did not show any significant peak in the visible region. As a more sensitive detection method the emission fluorescence spectrum of FR was recorded, after selection of an excitation wavelength typical for flavin prosthetic groups in proteins. Again, the protein fluorescence emission spectra did not reveal any flavin peak, showing the same pattern as the buffer spectra, which probably fluoresces because of the presence of Triton X-100 (results not shown). However, the enzyme shows a high specificity for NADH as an electron donor: NADPH cannot be substituted for NADH. In the presence of NADPH only 7.5% of the activity with NADH is measured. This result agrees quite well with the behaviour shown by whole Ehrlich cells, where the metabolic NADH produced in the lactate dehydrogenase reaction was the physiological electron donor of this activity [9].

Kinetic properties and inhibition studies

The dependence of NADH oxidation on NADH concentration at a fixed concentration of ferricyanide (0.25 mM) fitted a hyperbola; a K_m of 4.3×10^{-5} M was calculated. The specificity constant, k_{cat}/K_m , for NADH oxidation was fairly efficient, being $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. Analysis of the dependence of the ferricyanide reduction on ferricyanide concentration also showed a hyperbolic kinetic, with an apparent K_m value of 6.7×10^{-5} M when assayed at a saturating concentration of NADH (0.5 mM). The specificity showed by FR towards ferricyanide was remarkable; several

Figure 5 The effect of pH on the activity of the purified FR from Ehrlich ascites tumour cells

Both substrates, NADH and ferricyanide, and about 2 μ g of pure protein were dissolved in different buffered media and the activity assay was done at the indicated pH. Values are means $\pm$ S.E.M. for three different determinations and are corrected for the spontaneous decay of NADH. Abbreviation: U, units.

electron acceptors, including oxygen, duroquinone, cytochrome *c*, dichlorophenol-indophenol and Methyl Viologen, failed to elicit any enzyme-catalysed oxidation of NADH. Nevertheless the physiological acceptor remains unknown.

The dependence of the enzyme's activity on pH is shown in Figure 5. Although the enzyme retained a significant activity between pH 6 and 9, the highest values were found in the pH range 7.5–8.5. This behaviour contrasts with the optimal pH values obtained with plasma membrane vesicles, where maximal activity was found at more acidic pH, namely pH 5–6 [38]; a conformational change after protein extraction of the native membrane could explain this discrepancy. Alternatively, the inactivation or even loss of other redox proteins after detergent solubilization has also been argued to explain changes even more drastic on the optimal pH values [16].

The purified enzyme was also strongly inhibited by Zn^{2+} ions. The deduced IC_{50} was 1.25 μM , a value one-tenth of that obtained with isolated plasma membrane vesicles [38]. The inhibitory effect of Zn^{2+} is a hallmark of this protein; inhibition is shown by the membrane-bound protein as well as by solubilized and partly purified enzymes [37]. Furthermore the inhibition was fully reversed in all cases when a chelating agent (EDTA) was added to the medium. Zn^{2+} ions can induce conformational changes in proteins, altering their catalytic properties; in fact Zn^{2+} ions have been reported to inhibit several plasma membrane proteins [39–41]. In contrast, it is noteworthy that other divalent cations such as Ca^{2+} , Mn^{2+} , Sr^{2+} and Mg^{2+} at micromolar concentrations induced a stimulation of FR activity in plasma membrane vesicles [36], which emphasizes the specificity of the effect of Zn^{2+} . The binding of Zn^{2+} ions might move the acid–base equilibrium of histidyl residues towards the formation of a metalloprotein complex with the loss of the catalytic activity. The existence of essential histidyl residue(s) in Ehrlich ascites cells was proposed on the basis of the total loss of the FR activity after treatment with diethyl pyrocarbonate and Rose Bengal [38].

Finally, the susceptibility of the purified protein to several thiol reagents is shown in Table 3. Of the reagents tested, *p*-chloromercuribenzenesulphonate and mersalyl showed an

Table 3 Effects of thiol reagents and electron flow inhibitors on the purified Ehrlich FR activity

Purified FR (0.2 $\mu\text{g}/\text{ml}$) in buffer C was incubated at room temperature with various compounds at the concentrations indicated in parentheses. After 5 min, the substrates NADH and ferricyanide were added at 0.5 mM final concentration and FR activity was assayed as described in the Materials and methods section. The inhibitor concentration during the activity assay was 0.1 mM in all cases. Values are means $\pm$ S.E.M. for at least three different determinations. Abbreviations: PCMBs, *p*-chloromercuribenzenesulphonate; NEM, *N*-ethylmaleimide.

Inhibitor	Activity (%)
Control	100.0 $\pm$ 7.9
NEM (0.5 mM)	72.1 $\pm$ 7.3
PCMBs (0.5 mM)	9.7 $\pm$ 1.8
Mersalyl (0.5 mM)	2.5 $\pm$ 0.3
KCN (1 mM)	97.7 $\pm$ 1.7

almost complete inhibition of the enzymic activity, whereas *N*-ethylmaleimide was the least efficient; a similar inhibition pattern was obtained in plasma membrane vesicles [10]. The results suggest the involvement of cysteinyl residue(s) on protein functionality. A partial inhibition of plasma membrane redox system activity by thiol reagents has previously been detected in whole animal and plant cells [15,42,43]. FR activity remained unaffected in the presence of KCN (Table 3), which reinforces the view that it is not associated with plasma membrane peroxidase activity [44]. The results presented confirm the existence of a ferricyanide-NADH oxidoreductase in the plasma membrane of tumour cells, which probably belongs to a more complex redox system implicated in amino acid transport [3]. The isolation of a highly purified form will give further insight in the important physiological functions of plasma membrane redox systems.

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