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Involvement of essential histidine residue(s) in the activity of Ehrlich cell plasma membrane NADH–ferricyanide oxidoreductase

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Abstract

The existence of histidine residue(s) implicated in the active site of NADH–ferricyanide oxidoreductase in plasma membrane vesicles isolated from Ehrlich ascites tumour cells is investigated. The shape of the pH-dependence curve of the enzyme activity suggests that one or more histidine residues are located at (or near) the active site of the enzyme. This hypothesis is supported by the following experimental data: the loss of activity after treatment with diethyl pyrocarbonate (DEPC) or photooxidation by using Rose bengal, and the strong inhibition caused by Zn^{2+} ions at micromolar concentrations. The combined arguments support the statement that histidine plays an essential role in the catalytic activity of NADH–ferricyanide oxidoreductase from Ehrlich ascites tumour cells.

Key words: Histidine; DEPC; Rose bengal; Chemical modification; Ferricyanide reductase; Ehrlich ascites tumor cell

1. Introduction

Plasma membrane redox systems seem to be ubiquitous in both plant and animal cells (Refs.1,2, and references therein). They are related to several vital functions, including the energization of transport (see Ref. 3 for a review) and the control of cell growth [1]. In transformed cells, plasma membrane redox systems are modified as compared with those present in normal untransformed cells; for instance, in neuroblastoma cells this redox activity correlates with N-*myc* oncogene expression [4].

In intact Ehrlich ascites tumour cells a transmembrane ferricyanide reductase activity is described which is dependent on a cytosolic NADH source [5]. This activity is accompanied by alkalinization of cytosol and is strongly inhibited by amiloride and several antitumour drugs, such as mitoxantrone [5,6]. When highly pure plasma membrane vesicles from Ehrlich cells were used, two phases were observed in both NADH oxidation and ferricyanide reduction [7]. The enzyme is very sensitive to trypsin proteolysis, suggesting that hydrophilic domains are important for the activity [7].

However, little is known about the molecular mechanism of this plasma membrane redox activity. The use of amino acid-specific reagents could provide an insight into amino acid reactive groups implicated in the active site and the characteristics of the enzyme. In this context, it has been shown that, in both intact cells and plasma membrane vesicles, sulfhydryl reagents significantly inhibit this activity, suggesting that sulfhydryl groups are essential for ferricyanide reductase activity [5,7]. In the present report, a triple approach is used in order to gain information about the molecular basis of the pH dependence of NADH–ferricyanide oxidoreductase activity of plasma membrane vesicles isolated from Ehrlich cells: (i) a strong inhbition caused by diethyl pyrocarbonate (DEPC) treatment is shown; (ii) loss of activity after photooxidation in the presence of the dye Rose bengal is studied; and (iii) the reversible inhibitory effect of Zn^{2+} is discussed. All these data suggest that one or more histidine residues are located at (or near) the active site of the enzyme.

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2. Materials and methods

2.1. Materials

DEPC and Rose bengal were obtained from Sigma. All other reagents were of the highest available purity and supplied by Sigma, Merck, and Boehringer-Mannheim.

2.2. Preparation of plasma membrane vesicles

Highly pure plasma membrane vesicles were isolated from Ehrlich ascites tumour cells by two-phase compartmentation in poly(ethylene glycol)/dextran, as described elsewhere [8]. The purity of the plasma membranes obtained was greater than 95%, as determined by marker enzyme assays; mitochondrial contamination was less than 0.4%, and endoplasmic reticulum contamination was less than 2%.

2.3. Treatment of plasma membrane vesicles with DEPC

Solutions of DEPC were prepared fresh every day in absolute ethanol. Plasma membrane vesicles (2–4 mg) were suspended in 20 mM potassium phosphate (pH 6.4), in the presence of different concentrations of DEPC (or an equivalent volume of ethanol in the controls), at a final volume of 1 ml. Final ethanol concentration was never higher than 0.5% by volume. After 20 min of incubation at room temperature, plasma membrane vesicles were centrifuged and washed twice with 5 mM Hepes buffer (pH 7.4). Finally, vesicles were suspended in the same wash buffer and used in the activity assay, as described below.

2.4. Treatment of plasma membrane vesicles with Rose bengal

Photooxidation in the presence of the dye Rose bengal was initiated by placing open vials, containing the vesicles in phosphate buffered saline (PBS: 154 mM NaCl, 7 mM KCl, 11 mM sodium phosphate (pH 7.4)) in an ice bath 20 cm underneath a 150 W white bulb lamp. Controls with the same dye concentrations were kept in darkness throughout. After 10 min, vesicles were centrifuged and washed twice with PBS. Finally, vesicles were suspended in PBS and used in the activity assay, as described below.

2.5. Ferricyanide reductase assay

Ferricyanide reduction was kinetically followed in a Shimadzu UV-160 spectrophotometer at 420 nm. The assay medium contained 0.5 mM ferricyanide, 0.5 mM NADH and plasma membrane vesicles (60–80 μg protein) in PBS or 5 mM Hepes (pH 7.4) at a final volume of 1 ml. To study the effect of pH, different buffered media at 5 mM final concentration were used: acetate (pH 5), phosphate (pH 6, 6.8, and 7.4) and Tris (pH 8, 8.5, and 9). In the experiments with $ZnCl_2$, 0.4 mM ferricyanide and 0.1 mM NADH were used.

3. Results and discussion

3.1. Effect of pH

In the global reaction of NADH–ferricyanide oxidoreductase:

$$NADH + H^+ + 2\ ferricyanide^{3-} \rightleftharpoons 2\ ferrocyanide^{4-} + NAD^+ + 2H^+$$

the reduction of ferricyanide produces a net release of protons. For this reason, taking into account the Le Chatelier principle, it should be expected that the reaction was favoured by basic pH values; just the opposite effect was observed (Fig. 1). However, the hypothesis that histidine residues could be implicated in the active site of the enzyme must be supported by other experimental approaches, such as the use of chemical modifiers.

3.2. DEPC treatment

DEPC is the most commonly used reagent for histidine modification [9]. This reagent shows good specificity in the pH range from 5.5 to 8 [10]. However,

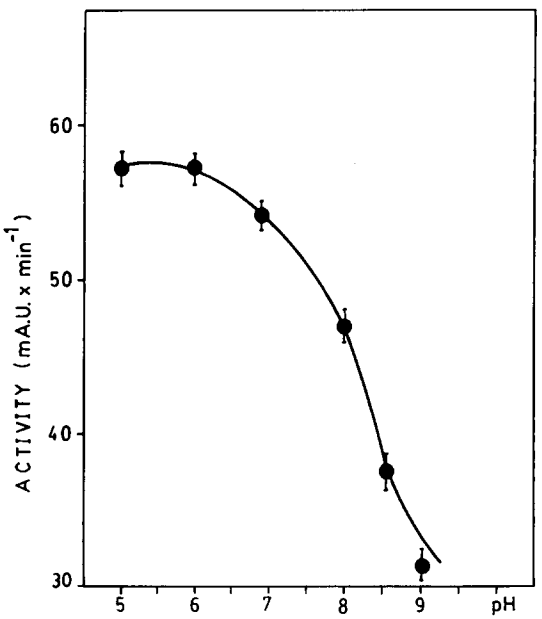

Fig. 1. Effect of pH on ferricyanide activity of plasma membrane vesicles isolated form Ehrlich cells. Activity was assayed at different pH values, as described in Materials and methods. Maximum specific activity in the figure was 930±56 nmol/min per mg protein.

DEPC is somewhat unstable in aqueous media, especially at higher pH; for this reason, it is better to use it at acidic or near-neutral pH values [11]. The reaction involves the carboxyethylation of one of the imidazole nitrogens of histidine. This modification can be reversed by hydroxylamine under the appropiate conditions [9]. On the other hand, when DEPC reacts twice with the same histidine residue, the doubly derivatized adduct is not reversible upon hydroxylamine treatment [9]. In any case, DEPC has been successfully used to demonstrate the involvement of histidine residues at the active site of proteins [10,12–16].

Table 1 shows the effect of DEPC preincubation on Ehrlich cell plasma membrane ferricyanide reductase activity. DEPC concentrations higher than 1 mM induced a significant inhibition, very similar to that previously described for folate transport in intestinal brush border membrane [16].

However, from these data alone an involvement of histidine residues at the active site of ferricyanide reductase cannot be concluded because DEPC can occasionally also react with tyrosine, serine, or cysteine. In fact, the use of specific chemical modifiers of amino acids indicated that cysteine [7] and tyrosine (Medina et al., unpublished results) could also be implicated in the active site of NADH–ferricyanide oxidoreductase activity.

3.3. Rose bengal treatment

To confirm the involvement of histidine in NADH–ferricyanide oxidoreductase activity, the photooxidation of the enzyme was carried out by using the dye Rose bengal under illumination [11,13,15,17]. Fig. 2 shows that photooxidation produced a clear and pronounced inhibition of ferricyanide reductase activity even at low concentrations of Rose bengal (1 μg/ml). Control vesicles preincubated with Rose bengal in darkness showed no inhibition in their ferricyanide reductase activity. In the absence of Rose bengal, the activity was not modified by illumination; this result rules out the possibility of unspecific photoinactivation of the plasma membrane redox system. Even though the photooxidation in the presence of Rose bengal is not fully specific for histidine residues [11,18], histidine seems to be the only amino acid that clearly reacts with low concentrations of both DEPC and Rose bengal [11].

Table 1
Effect of pretreatment of Ehrlich cell plasma membrane vesicles with different concentrations of DEPC on ferricyanide reductase activity

DEPC (mM)	Activity (%)
0	100 ± 16
1	81 ± 8
2.5	61 ± 3 *
5	32 ± 9 *

The experiments were carried out as described in Material and methods. Data are given as percentages of activity taking control values (780 nmol/min per mg protein) as 100% and they are means ± S.D. of three different determinations. Statistical significance values were calculated using Student's *t*-test (* $P < 0.01$).

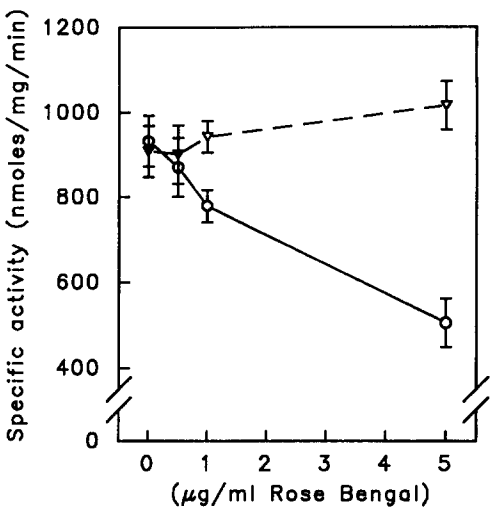

Fig. 2. Inactivation of ferricyanide reductase by Rose bengal. Vesicles were treated with Rose bengal at the indicated concentrations as described in Materials and methods, either in the light (○) or in the dark (△). Data are means ± S.D. of three different experiments.

3.4. Effect of Zn^{2+}

Histidine has been described as a primary amino acid residue involved in protein metal binding sites [19]. Zinc ions are among the most common metal ions present in naturally occurring metalloproteins. The ions favour co-ordination geometries, inducing reversible conformational changes which alter the catalytic properties. Both activating and inhibitory effects have been described for Zn^{2+} ions. Thus, Zn^{2+} is essential for the activity of a number of metalloproteins, such as metallothionein [20]. On the other hand, Zn^{2+} at concentrations in the micromolar range inhibits soybean hypocotyl plasma membrane redox activity [21]. It is noteworthy that Zn^{2+} has been also described as a blocker of H^+-channels associated with a NADPH oxidase activity of human neutrophil plasma membrane [22,23].

Fig. 3 shows that Zn^{2+} induced a pronounced and significant inhibition of Ehrlich ascites tumour cell plasma membrane ferricyanide reductase activity at concentrations higher than 5 μM; 15 μM $ZnCl_2$ produced 75% of inhibition, and inhibition was complete in the presence of 20 μM $ZnCl_2$; this effect was fully reversible when zinc-treated vesicles were incubated with a chelating agent. It should be kept in mind that in the procedure used to isolate plasma membrane

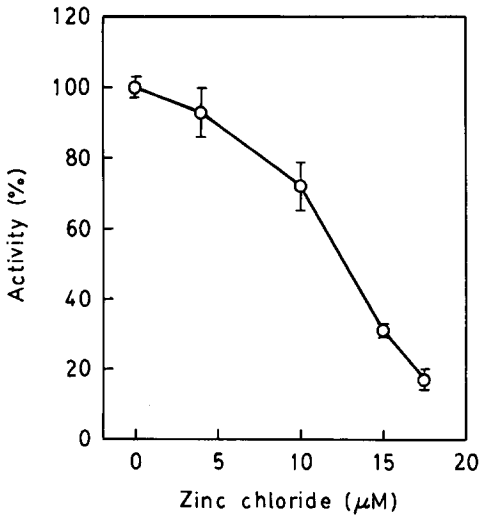

Fig. 3. Inhibitory effect of $ZnCl_2$ on ferricyanide reductase activity of Ehrlich cell plasma membrane. Activity was assayed as described in Materials and methods. Data are given as means ± S.D. of three different experiments. 100% of activity was 973 ± 25 nmol/min per mg protein.

vesicles [8], cell homogenization was carried out in 1 mM $ZnCl_2$; zinc is utilized as a stabilizer of membrane preventing its disintigration on further manipulation [24]. Zn^{2+} ions were sequestered by EDTA in a later step. In fact, NADH–ferricyanide oxidoreductase activity could not be detected in the homogenate before Zn^{2+} chelation by EDTA, and after EDTA chelation full activity was restored; furthermore, solubilized and partially purified ferricyanide reductase was also inhibited by Zn^{2+}, and this inhibition was also reversed by EDTA (authors' unpublished results). Therefore, it can be concluded that the inhibitory effect of Zn^{2+} is reversible, as that reported for plasma membrane Na^+,K^+-ATPase [24]. The reversible inhibition caused by Zn^{2+} ions points to a possible involvement of histidine residues at the active site of ferricyanide reductase. It is noteworthy that this reversible inhibition was studied at pH 7.4; under these pH conditions, assuming an apparent pK_a value of 8.5, approx. 7.5% of total histidine residues are calculated to be deprotonated, and they can easily bind Zn^{2+} ions. Consequently, in the presence of Zn^{2+} ions the acid-base equilibrium of histidine residues should move toward the formation of metalloprotein complex with the lost of the catalytic activity. The inhibition of Ehrlich cell ferricyanide reductase by Zn^{2+} strengthens the hypothesis of a proton channel regulated by Zn^{2+}, connected with a plasma membrane oxidoreductase as that proposed for human neutrophils [22,23].

3.5. Concluding remarks

In the present report it is demonstrated that: (i) ferricyanide reductase is more active at mild acidic or neutral than at basic pH values (Fig. 1); (ii) DEPC treatment inhibits the redox activity (Table 1); (iii) preincubation with Rose bengal under illumination produces a photooxidation that decreases the activity (Fig. 2); and (iv) Zn^{2+} ions strongly inhibit the activity (Fig. 3). None of these data taken separately could be considered a definite evidence for the involvement of histidine residues in the active site of ferricyanide reductase; however, the coincidence of all of them strongly suggests that histidine residues are essential for Ehrlich cell plasma membrane ferricyanide reductase activity.

4. Acknowledgements

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