A Transmembranous NADH-Dehydrogenase in Human Erythrocyte Membranes

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Abstract

Evidence is presented for a transmembranous NADH-dehydrogenase in human erythrocyte plasma membrane. We suggest that this enzyme is responsible for the ferricyanide reduction by intact cells. This NADH-dehydrogenase is distinctly different from the NADH-cytochrome b_5 reductase on the cytoplasmic side of the membrane. Pretreatment of erythrocytes with the nonpenetrating inhibitor diazobenzene sulfonate (DABS) results in a 35% loss of NADH-ferricyanide reductase activity in the isolated plasma membrane. Since NADH and ferricyanide are both impermeable, the transmembrane enzyme can only be assayed in open membrane sheets with both surfaces exposed, and not in closed vesicles. The transmembrane dehydrogenase has affinity constants of 90 μM for NADH and 125 μM for ferricyanide. It is inhibited by p-chloromercuribenzoate, bathophenanthroline sulfonate, and chlorpromazine.

Key Words: Transmembranous NADH-dehydrogenase; enzyme kinetics; human erythrocyte; plasma membrane.

Introduction

NADH-dehydrogenase activity in the plasma membrane from hepatocytes and adipocytes has been proposed to help control the response of the cell to hormones which act at the cell surface (Löw and Crane, 1976; Löw *et al.,* 1978; Gayda *et al.,* 1977; Goldenberg, 1982). A similar enzyme which may provide the energy for selective amino acid transport has been identified in Ehrlich ascites cell plasma membranes (Garcia-Sancho *et al.,* 1977; Kilberg and Christensen, 1979).

NADH-dehydrogenase activity is also shown to be present in mammalian erythrocyte plasma membranes (Zamudio and Canessa, 1966). This

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dehydrogenase activity has generally been interpreted to be related to the soluble NADH-methemoglobin reductase which is found in erythrocytes (Scott *et al.*, 1965; Choury *et al.*, 1981), and has been shown to be a modified form of NADH cytochrome b_5 reductase (Choury *et al.*, 1981; Passon and Hultquist, 1972; Gato-Tamura *et al.*, 1976; Kitajima *et al.*, 1981). Recently Wang and Alaupovic (1978) have isolated a NADH-ferricyanide reductase from erythrocyte membranes which they describe as a transmembrane protein different from NADH-cytochrome b_5 reductase.

The study of the plasma membrane enzyme from the mature human erythrocyte is greatly facilitated because of the lack of internal membranes. Well-developed procedures are available for the preparation of membranes with both surfaces exposed to the incubation media, or of sealed vesicles with known, outside or inside, surface exposure (Steck and Kant, 1974). The NADH-cytochrome c reductase is oriented exclusively toward the inner surface of the erythrocyte membrane (Kant and Steck, 1972).

In this paper we demonstrate a transmembranous localization of a NADH-ferricyanide dehydrogenase by using oriented erythrocyte membrane vesicles. With membranes from diazobenzene sulfonate-exposed erythrocytes it is shown that this enzyme activity is separated from the NADH-cytochrome b_5 reductase.

Materials and Methods

Membrane Orientation

Erythrocyte membranes were prepared from blood obtained from volunteers. The open membranes, right-side-out and inside-out vesicles were prepared as described by Steck and Kant (1974) and separated by using a dextran gradient. The integrity of the closed vesicles was controlled according to the methods of Steck and Kant (1974) by determination of acetylcholinesterase, which is exposed on the membrane exterior, glyceraldehyde-3-phosphate dehydrogenase, and NADH-cytochrome c reductase, exclusively located on the interior surface. Full activity of these marker enzymes was revealed by treatment with 0.02% Triton X-100 for acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase. Saponin (0.005%) was used for NADH-cytochrome c reductase, since this activity was severely impaired by Triton X-100.

Synthesis of Diazobenzene Sulfonate (DABS) and Treatment of Erythrocytes

The diazonium salt of sulfanilic acid was used to selectively inhibit enzymes with active sites facing the external surface of the cells (Bender *et al.*,

1971). The diazonium group is highly reactive and forms covalent bonds with sulfhydryl, amino, and hydroxyl groups of proteins and lipids. The negative charge on its sulfonate group prevents the small molecule from entering intact cells (Berg, 1969).

Sulfanilic acid diazonium salt was freshly prepared before use (De Pierre and Karnovsky, 1974): $NaNO_2$, 23 mg, and sulfanilic acid, 39 mg, were dissolved in 2 ml cold water. HCl (12 M), 50 μl, was added and the mixture was kept on ice. The diazonium salt precipitated when the inside surface of the glass tube was scratched. The aged (15 min) precipitate was washed with cold water and dissolved in 10 ml phosphate-buffered saline (PBS), pH 8.0, to approximately 20 mM, thereafter kept on ice and used within 30 min.

A suspension of 40% (v/v) intact erythrocytes was incubated in 37°C with the selected concentrations of DABS in PBS for different periods of time. The excess of DABS was removed by extensive washing with ice-cold PBS, and open membranes were prepared. The effectiveness of the treatment was checked by measuring the activity of acetylcholinesterase (Bender *et al.,* 1971). The NADH-cytochrome *c* reductase activity was determined as an intracellular indicator to make sure that DABS had not penetrated the cells.

Enzyme Assays

The NADH-ferricyanide reductase activity was determined spectrophotometrically by following the decrease in absorbance at 420 nm. The assay was carried out at 22°C using 0.17 mM NADH and 0.35 mM $K_3Fe(CN)_6$. Na-phosphate buffer (100 mM, pH 7.0) was used for all orientation studies. NADH-cytochrome *c* reductase activity was determined by following the absorbance at 550 nm as previously described (Crane and Löw, 1976).

The consistent lack of succinate-dehydrogenase activity in the erythrocyte ghost preparation shows that the dehydrogenase activity does not arise from contaminating mitochondria from other types of blood cells.

Results

Orientations in the Membrane

The orientation of the NADH-ferricyanide reductase in the membrane was studied by comparing the activity observed in open membranes with activity in closed membrane ghosts with external or internal surface exposure as defined by Steck and Kant (1974). The inverted vesicles show a low acetylcholinesterase activity, a high glyceraldehyde-3-phosphate dehydrogenase and NADH-cytochrome *c* reductase activity (Fig. 1). The vesicles with exposed external surface show a low activity for these dehydrogenases and a high activity for acetylcholinesterase. When the vesicles are treated with

acetylcholinesterase

μmol/min,mg protein

NADH-cytochrome C reductase

glyceraldehyde-3-phosphate dehydrogenase
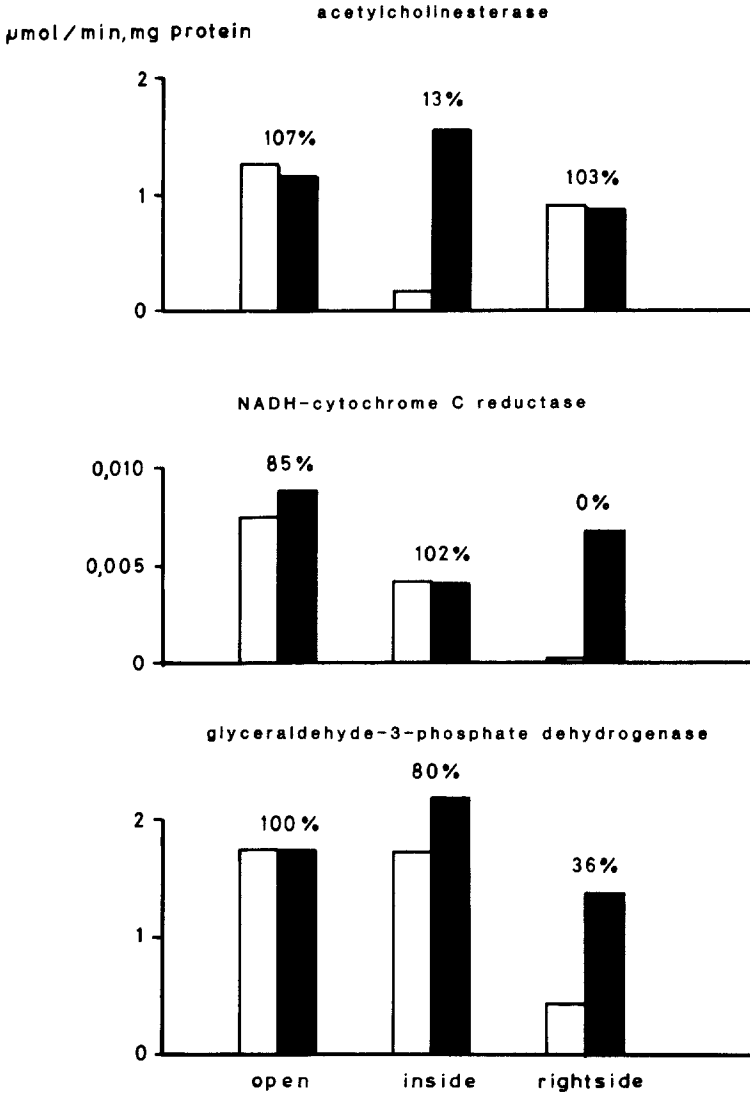

open   inside   rightside

Fig. 1. Accessibility of marker enzymes in oriented membrane vesicles and open membranes from human erythrocytes. The accessibility is given in percent of full activity revealed by detergents. Oriented ghosts and vesicles prepared by the method of Steck and Kant (1974), and enzyme activity measured according to the same authors. Detergents used to open the oriented membranes: 0.02% Triton X-100 for acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase, and 0.005% saponin for NADH-cytochrome c reductase. Assays in 100 mM Na-phosphate buffer, pH 7.0. (□) No detergent; (■) with detergent.

detergent they are opened and all of the activity is revealed. Open membranes show maximum activity with all the marker enzymes without saponin or Triton treatment.

The NADH-ferricyanide reductase activity in sealed vesicles with exposed internal or external membrane surfaces is compared to the activity in open membranes before and after detergent treatment (Fig. 2). The ionic strength is maintained at a high level. Inside-out vesicles show approximately one-half of the activity which can be revealed by detergent treatment. In right-side-out ghosts, only a low activity is observed. Open membranes have maximum activity also without detergent treatment. It thus appears that at least one-half of the total NADH-ferricyanide reductase activity requires exposure of both sides of the membrane. This is in contrast to the NADH-cytochrome c reductase which shows 75–100% of its maximum activity in inverted vesicles.

Freezing and thawing of the closed membrane ghosts give the same effects as detergent treatment. The NADH-ferricyanide reductase activity increases in right-side-out ghosts, indicating a rupture of the membrane (Table I). Inverted vesicles show a 33% increase of NADH-ferricyanide reductase after freezing. Freezing and thawing have no effect on the enzyme activities in the open membranes. The NADH-ferricyanide reductase retains

NADH -ferricyanide reductase
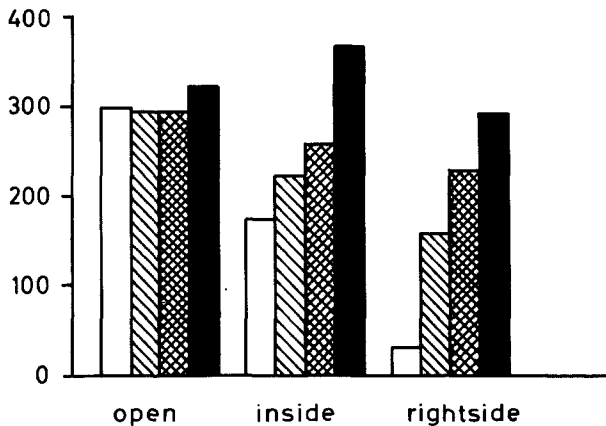nmol/min, mg protein

Fig. 2.   NADH-ferricyanide reductase activity in human erythrocyte membranes with different surface exposure and the effect of Triton X-100 treatment. Assays in 100 mM Na-phosphate buffer, pH 7.0, as described in Materials and Methods. (☐) No detergent added; (〰) 0.017% Triton X-100; (▨) 0.033% Triton X-100; (■) 0.133% Triton X-100.

Table I.   Effect of Freezing and Thawing on NADH-Ferricyanide Reductase Activity Using Oriented Ghosts from Human Erythrocytes[a]

Membrane orientation	NADH-ferricyanide reductase (nmol/min · mg protein)	
	Unfrozen	Frozen
Open ghosts	345	355
Right-side-out	78	393
Inside-out	180	240

[a] Assays in 100 mM Na-phosphate buffer, pH 7.0.

most of its activity on storage for two days at 3°C. Up to 20% of the activity may be lost after freezing for 6 days. This is in contrast to the lability of the microsomal NADH-ferricyanide dehydrogenase (Masuda *et al.,* 1973).

The interaction of DABS with the erythrocyte membrane is dependent on the protein amount present, the reagent concentration, time of incubation, and the temperature. Open membranes are isolated from the cells after preincubation with different concentrations of DABS for 20 min. Inhibition of
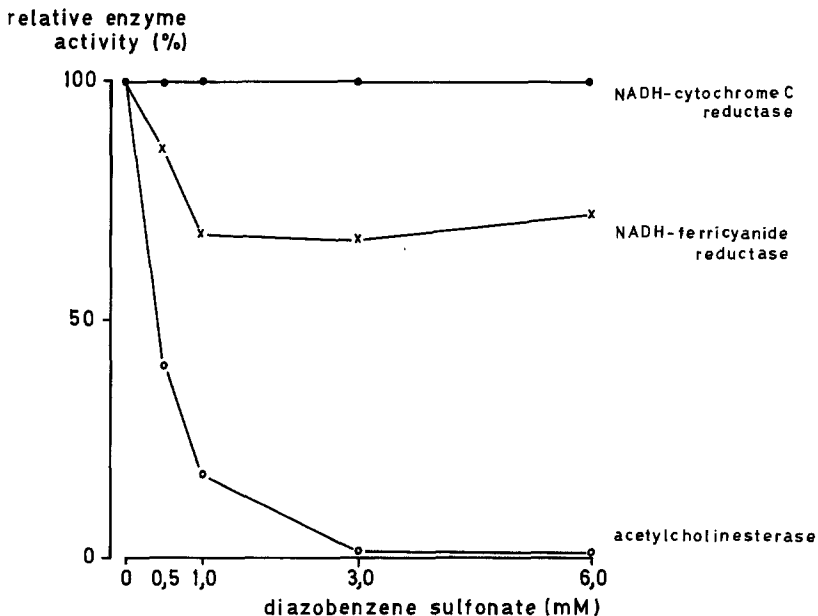

Fig. 3.   NADH-ferricyanide reductase activity in open membranes prepared from human erythrocytes after pretreatment with diazobenzene sulfonate. Diazobenzene sulfonate treatment of intact erythrocytes was performed in PBS 37°C for 10 min as described in Materials and Methods. Acetylcholinesterase and NADH-cytochrome *c* reductase activity was determined as described elsewhere. The activity of NADH-cytochrome *c* reductase was set to 100%. The enzyme activities were calculated relative to the NADH-cytochrome *c* activity in each preparation.

acetylcholinesterase was rapid and effective (Fig. 3). If a membrane is treated too extensively with the diazonium reagent, its permeability barrier may be broken down. The intracellular indicator NADH-cytochrome c reductase was, however, not affected even after as much as 60 min incubation of cells with 3 mM DABS, whereas the NADH-ferricyanide reductase activity was reduced to 35%.

Affinity for NADH

The NADH-ferricyanide reductase activity in open erythrocyte membranes has a K_m for NADH of 90 μM (Table II), which is higher than the K_m observed for the NADH-cytochrome b_5 reductase in liver membranes or that purified from erythrocyte membrane (Wang and Alaupovic, 1978; Spatz and Strittmatter, 1973). The inverted vesicles of erythrocyte membranes show a K_m for NADH of 36 μM. With increase of NADH concentration the ferricyanide reductase activity with inverted vesicles shows additional increase which changes the direction of the K_m plot. This increased activity might be interpreted as an additional low-affinity site for NADH (280 μM) (Fig. 4). It is not clear if this site is present on open membrane or is obscured by the higher-affinity reaction.

Table II. Kinetic Constants for NADH with Ferricyanide as Acceptor Using Oriented Human Erythrocyte Ghosts[a]

Membrane orientation	NADH		References
	K_m (μM)	V_{max} (nmol/min · mg protein)	
Erythrocyte ghosts: Open	90	1750	
Right-side-out	17	270	
	2000	4000	
Inside-out	36	667	
	280	1818	
Purified erythrocyte plasma membrane dehydrogenase	14	115000	Wang (1980)
Liver plasma membrane	28	1070	Masuda et al. (1973)
Ascites cell plasma membrane	30	1000	Kilberg and Christensen (1979)
Liver microsomes	20	4660	Masuda et al. (1973)
Purified erythrocyte plasma membrane NADH-cytochrome b_5 reductase	6.4		Wang and Alaupovic (1978)
Liver NADH-cytochrome b_5 reductase (soluble)	2.5		Spatz and Strittmatter (1973)
Liver NADH-cytochrome b_5 reductase (holoenzyme)	6		Spatz and Strittmatter (1973)
Ascites tumor cell (whole cell)	4000		Cherry et al. (1981)

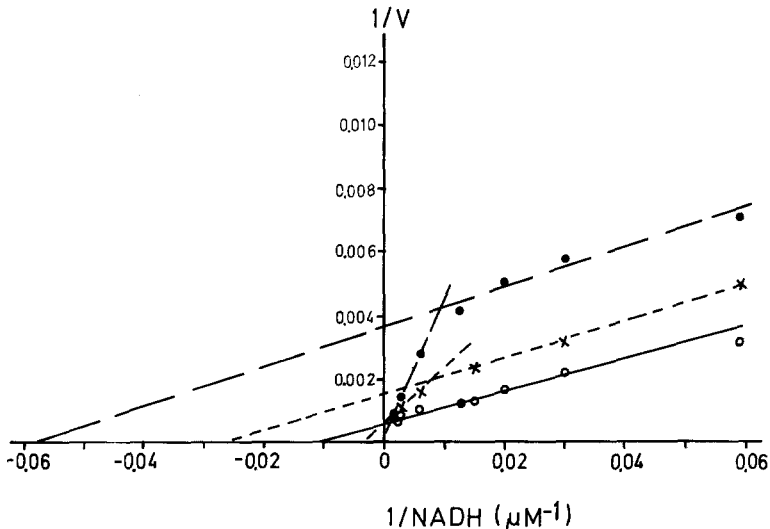[a]Erythrocyte ghost assays in 100 mM Na-phosphate buffer, pH 7.0.

Fig. 4. Lineweaver–Burk plot of NADH-ferricyanide reductase activities in oriented human erythrocyte membranes with variation of NADH concentration. The reaction mixture consisted of 100 mM Na-phosphate buffer, pH 7.0, and 0.7 mM $K_3Fe(CN)_6$ (saturated concentration). Velocity expressed as nmol/min · mg protein. Background subtracted. For K_m and V_{max} values see Table II. (O) Open membrane; (●) right-side-out ghost; (x) inside-out vesicles.

The closed vesicles with external surface exposed show a very low NADH-ferricyanide reductase activity. It is also possible that this low activity represents a slight substrate penetration to internal sites.

The observations of NADH-ferricyanide reductase activity indicate the existence of three different sites of NADH oxidation which are associated with the open, right-side-out and inverted membrane orientations, respectively. No activity could be demonstrated when NADPH was used as substrate.

Affinity for Ferricyanide

The erythrocyte open membranes have a linear Lineweaver–Burk plot for ferricyanide concentration (Fig. 5) which gives a K_m value of 125 μM (Table III). This is the same as reported by Zamudio et al. (1969). The sealed inverted vesicles show an additional stimulation of activity at higher ferricyanide concentrations which changes the slope of the Lineweaver–Burk plot and gives a second apparent K_m of 200 μM in addition to a high-affinity site of 24 μM. This low-affinity site also appears in liver and ascites cell plasma membranes (Table III). The very low NADH-ferricyanide reductase activity
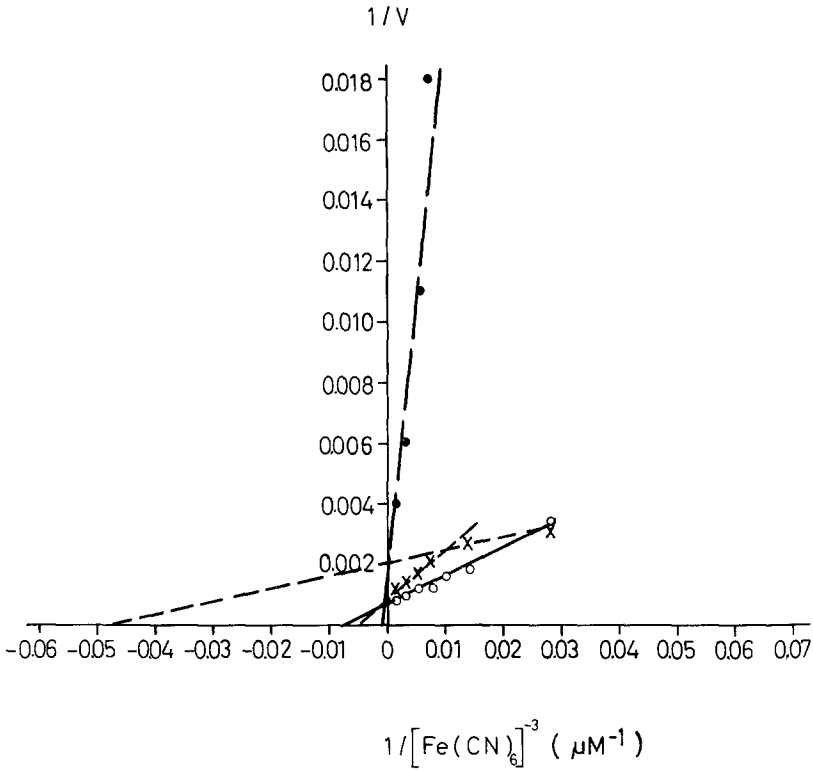
Fig. 5. Lineweaver–Burk plot of NADH-ferricyanide reductase activities in oriented ghosts from human erythrocytes with variation of $K_3Fe(CN)_6$ concentration. The reaction mixture consisted of 100 mM Na-phosphate buffer, pH 7.0, and 0.33 mM NADH (saturated concentration). Velocity expressed as nmol/min · mg protein. Background subtracted. For K_m and V_{max} values see Table III. (O) Open membrane; (●) right-side-out ghost; (x) inside-out vesicles.

of right-side-out ghosts appears to show lack of saturation and the Lineweaver–Burk plot indicates a first-order reaction. The apparent K_m would be more than 2000 μM if saturation could occur. Thus, there are different affinity sites for ferricyanide reduction revealed in open, right-side-out and inside-out erythrocyte membrane vesicles.

Effects of Salts

The NADH-ferricyanide reductase activity of both open membranes and inverted vesicles is strongly stimulated by salts in low ionic buffer. Both monovalent and divalent ions are effective. The NADH-cytochrome c reduc-

Table III. Kinetic Constants for Ferricyanide for the NADH-Ferricyanide Reductase Activity of Oriented Human Erythrocyte Ghosts[a]

Membrane orientation	Ferricyanide		References
	K_m (μM)	V_{max} (nmol/min · mg protein)	
Erythrocyte ghosts: Open			
Right-side-out	125	1538	Zamudio et al. (1969)
Inside-out	>2000	1000	Wang (1980)
	24	500	Huang et al. (1979)
	200	1250	
Erythrocyte open ghost	125		
Purified erythrocyte ferricyanide reductase	49		
Liver plasma membrane	17		
	200		
Ascites cell plasma membrane	220		Kilberg and Christensen (1979)
Liver microsomes	200		Huang et al. (1979)
Purified erythrocyte plasma membrane			
NADH-cytochrome b_5 reductase	5.0		Wang and Alaupovic (1978)

[a]Erythrocyte ghost assays in 100 mM Na-phosphate buffer, pH 7.0.

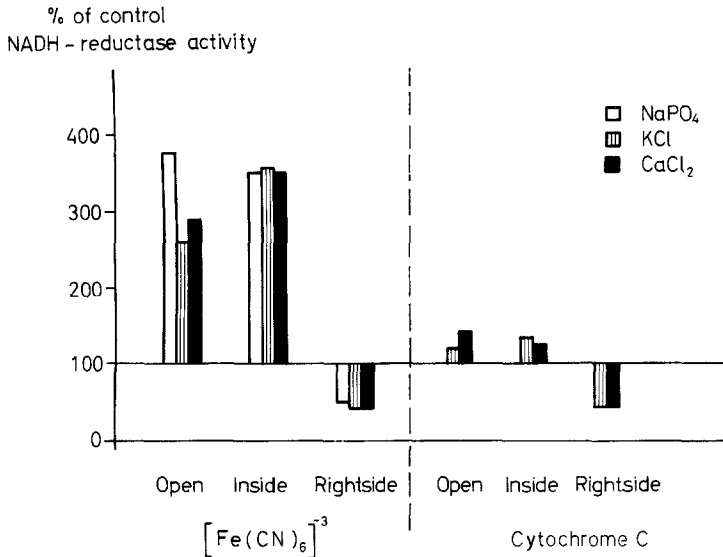% of control
NADH - reductase activity

Fig. 6. Effect of salt on NADH-ferricyanide reductase activity and on NADH-cytochrome c reductase activity in open membranes, right-side-out and inside-out vesicles from human erythrocytes. Assay run in 1 mM Na-phosphate buffer, pH 7.0. This activity is the control value set at 100%. The effect of added salt is indicated by the bars. (▥) Addition of 10 mM KCl; (■) addition of 0.33 mM $CaCl_2$; (□) 100 mM Na-phosphate buffer, pH 7.0.

tase activity of open membranes and inverted vesicles shows much less stimulation by salts (Fig. 6).

Salts cause resealing in right-side-out ghosts after opening in low ionic buffers. Since there is no further stimulation of NADH-ferricyanide activity by adding additional salt after complete closing, it appears that the weak NADH-ferricyanide reductase activity on the outside of the erythrocyte membrane is not stimulated by salt.

Effects of Inhibitors

The NADH-ferricyanide reductase in open membranes is very sensitive to the nonpenetrating inhibitor p-chloromercuribenzoate (PCMB) which reacts with sulfhydryl groups (Åkerström *et al.*, 1976) (Table IV). The inside-out vesicles have about the same sensitivity, whereas the activity in right-side-out vesicles is less sensitive. Although the cytochrome c reductase activity also is inhibited by PCMB, there is a distinct difference in the degree of inhibition between the activities; the cytochrome c reductase is less sensitive (Table IV).

The transition metal chelator bathophenanthroline sulfonate, which is an

Table IV. Inhibitors of NADH-Ferricyanide Reductase in Oriented Human Erythrocyte Ghosts[a]

Inhibitor	Concentration (M)	NADH-ferricyanide reductase			NADH-cytochrome c reductase
		Open	Right-side-out	Inside-out	Open
p-Chloromercuribenzoate	5×10^{-7}	66	50	71	28
p-Chloromercuribenzoate	2×10^{-6}	100	88	94	80
Bathophenanthroline sulfonate	3×10^{-3}	79	100	94	26
Chlorpromazine	5×10^{-4}	100	0	100	50

[a] Assays in 1 mM Na-phosphate buffer, pH 7.0, with 0.33 mM $CaCl_2$ added.

impermeable inhibitor of nonheme iron enzymes (Harmon and Crane, 1976), is a good inhibitor of the external and internal NADH-ferricyanide reductase activities, but gives less inhibition of the transmembrane enzyme. NADH-cytochrome c reductase activity in open membranes is only inhibited to 26% by bathophenanthroline sulfonate. Thus, this inhibitor separates the NADH-ferricyanide dehydrogenase from NADH-cytochrome c reductase.

Chlorpromazine, an agent which is known to have effects on calcium function and on membrane structure, also affects the NADH-ferricyanide reductase. It gives complete inhibition of the dehydrogenase activity in both open membranes and inside-out vesicles, but has no effect on the external dehydrogenase activity (Table IV).

Discussion

The NADH-dehydrogenases in thoroughly washed erythrocyte plasma membranes can react with a variety of oxidizing agents including ferricyanide, cytochrome c, 2,6 -dichlorophenolindophenol, and monodehydroascorbate (Zamudio and Canessa, 1966; Goldenberg, 1980; Jarasch et al., 1979). The NADH-ferricyanide reductase activity is higher whereas the NADH-cytochrome c reductase activity and the NADH-monodehydroascorbate reductase activity in erythrocyte membranes is much lower than has been reported in other plasma membranes (Masuda et al., 1973; Goldenberg, 1980; Goldenberg et al., 1979). Among these acceptors ferricyanide and cytochrome c are of special interest because they do not penetrate the membrane. Therefore, they can be used to decide the orientation of the reducing site of the dehydrogenase in the membrane.

Ferricyanide reduction on the outside of intact erythrocytes was observed by Dormandy and Zarday (1965) by potentiometric measurements and by Mishra and Passow (1969) by spectrophotometric methods. The external reduction of ferricyanide was also correlated to an increased ATP formation (Dormandy and Zarday, 1965; Manyai and Szekely, 1954) and to changes in external proton concentration (Dormandy and Zarday, 1965). Additionally, hepatocytes have been shown to reduce extracellular ferricyanide (Clark et al., 1981). A transmembrane dehydrogenase has been proposed to be responsible for the external ferricyanide reduction by whole cells (Mishra and Passow, 1969).

Since plasma membranes are impermeable to NADH and ferricyanide, this dehydrogenase, which uses internal NADH to reduce external ferricyanide, should only be detected in open membranes with both sides of the membrane exposed and should not be observed in sealed vesicles with only exterior or interior surfaces exposed.

It is clear, when NADH-ferricyanide reductase is measured in membrane preparations with different surfaces exposure, that the open membranes show more activity than inverted vesicles, and that the exterior surface has very little activity. Addition of detergent to the open membrane does not increase the NADH-ferricyanide reductase activity, when assayed under high ionic strength conditions, whereas addition of detergent to sealed vesicles causes more than a 50% stimulation of reductase activity. The stimulation is not based on detergent activation of the enzyme, because freezing and thawing have the same effect.

The monosided enzyme activities used as marker enzymes for the inside surface (cytochrome c reductase and glyceraldehyde-3-phosphate dehydrogenase) and for the outside surface (acetylcholinesterase) show no increase in activity after detergent treatment of sealed preparations with the relevant surface exposed.

The low absolute value of the NADH-cytochrome c reductase activity found with inside-out vesicles has been a consistent observation in our studies. This can most likely be related to the procedures required to prepare the inside-out vesicles, e.g., alkaline and low ionic strength treatment. The fact remains that the cytochrome c reductase activity (which is the marker for the inside of the membrane) shows no increase when external sites are exposed by addition of detergent. The change in the cytochrome c reductase activity during the preparation made it important to follow another inside marker, namely the glyceraldehyde-3-phosphate dehydrogenase, where we got a good recovery of activity. Moreover, this marker enzyme shows no increase of activity when inverted vesicles are treated with detergent.

The 35% inhibition of the NADH-ferricyanide dehydrogenase by DABS treatment of the intact erythrocytes indicates that a large part of the enzyme must be exposed at the external cell surface. This demonstrates a distinct difference between the transmembranous NADH-ferricyanide dehydrogenase and the internal NADH-cytochrome b_5 reductase. The residual 65% activity of the NADH-ferricyanide dehydrogenase which is not influenced by DABS treatment would represent the NADH-cytochrome b_5 reductase restricted to the inner surface.

In open membranes isolated from diazobenzene sulfonate-treated pig erythrocytes, it has been reported by MacKellar, (1981) that the NADH-ferricyanide activity is inhibited to 30%. With inverted vesicles prepared from the same cell no effect was found.

The glycoprotein with NADH-dehydrogenase activity, which Wang and Alaupovic (1978) isolated from erythrocytes, has properties somewhat different from the NADH-cytochrome b_5 reductase, so it is more likely that this is the transmembrane NADH-ferricyanide reductase.

The NADH-ferricyanide reductase on the exterior surface of the mem-

brane has an extremely low affinity for NADH and ferricyanide. This activity does not show simple zero-order kinetics, so it is not clear what type of reaction is involved. It might indeed be the result of a small leak of NADH through the membrane. The presence of an external NADH-ferricyanide reductase type activity which requires a high level of substrate has been observed when NADH and ferricyanide are added to intact tumor cells (Cherry *et al.*, 1981). Oxidation of external NADH has been observed on human platelets (Finazzi-Agro *et al.*, 1982) and with protoplasts (Lin, 1982a), and a NADH-oxidase has been extracted from the outside surface of plant cells (Lin, 1982b).

The transmembrane enzyme is less sensitive to the metal chelator bathophenanthroline sulfonate and more sensitive to the sulfhydryl reagent PCMB than the interior and exterior enzyme. The reduction of ferricyanide by intact erythrocytes has been reported to be inhibited by PCMB, probably indicating an inhibition of a transmembrane dehydrogenase (Arese *et al.*, 1972). This inhibition is observed in conditions where PCMB does not enter the cells and indicates that the sulfhydryl site of the redox carrier is on the external surface of the cell.

Chlorpromazine is known to inhibit the function of calmodulin in membranes (Nelson *et al.*, 1982). The external NADH-ferricyanide dehydro-genase is not affected whereas the activity on open membranes and inverted vesicles is completely inhibited by chlorpromazine.

The salt effects might, in part, be related to a screening of negative charges on the surface of the membrane which would prevent close approach of the highly negative ferricyanide ion. This effect would be proportional to ionic strength. On the other hand, since divalent cations are more effective than expected from ionic strength considerations, this effect may be related to earlier reported findings (Zamudio and Canessa, 1966; Löw *et al.*, 1979) that sonication as well as detergent treatment can lead to increased activity when assayed under low ionic conditions. Since an activation of ferricyanide reductase can also be seen by the addition of protonophoric agents (Wang and Alaupovic, 1978; Löw *et al.*, 1979), we believe that a possible explanation of this salt activation might be a breakdown of an intramembranous proton gradient caused by the protonogenic nature of the NADH-ferricyanide reductase activity.

The ferricyanide reductase in the erythrocyte membrane seems to be nucleotide specific. In contrast to other plasma membranes, to endoplasmic reticulum, and to the soluble enzyme (Crane *et al.*, 1979), we have not been able to observe any NADPH-dehydrogenase activity in our ghost prepara-tions. In the intact erythrocyte Arese *et al.* (1972) have, on the other hand, shown that ferricyanide induces a greater decline in cellular NADPH concentration than in that of NADH and also a very marked decrease in the reduced glutathione concentration. This indicates that in the intact cell a

transdehydrogenase converts NADPH to NADH, which then is the substrate for the transmembrane ferricyanide reductase.

The presence of a transmembrane dehydrogenase which can reduce the impermeable 1,4-naphthoquinone-2-sulfonate would also be a basis for the effects of this quinone on erythrocyte function since it could oxidize cytoplasmic substrates without penetrating the membrane (McMahon and Stern, 1979).

Thus, it is apparent that there are at least two NADH-dehydrogenases in the erythrocyte plasma membrane. One is transmembranous, and its activity as an NADH-ferricyanide reductase requires exposure of both surfaces of the membrane. The other is the NADH-cytochrome b_5 reductase which is located exclusively on the inside of the membrane. Finally, ferricyanide reduction by NADH is observed on the exterior of the membrane.

The stimulation of the NADH-ferricyanide dehydrogenase in closed membrane ghosts by detergent or by freezing and thawing and the inhibition by DABS treatment in conjunction with the evidence that ferricyanide is reduced by intact cells, show the existence of a transmembranous NADH-ferricyanide dehydrogenase.

ACKNOWLEDGMENTS

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