Journal of Supramolecular Structure 9:1–14 (1978)

Isolation and Partial Characterization of Human Erythrocyte Membrane NADH: (Acceptor) Oxidoreductase

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The NADH: (acceptor) oxidoreductase (EC 1.6.99.3) was isolated from human erythrocyte ghosts by a procedure including Triton X-100 solubilization, affinity chromatography on an NAD^+-Sepharose 4B column, ammonium sulfate precipitation, and isoelectric focusing. This enzyme preparation was characterized by a single band on the urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by a single precipitin line with its corresponding antiserum on double diffusion and immunoelectrophoresis. A 103-fold purification indicates that the oxidoreductase represents approximately 1% of the ghost protein mass. The specific activity of the purified enzyme was 112 units/mg protein. The pH optimum was 6.8 and the isoelectric point, pI, was 6.6. The oxidoreductase has a specificity for NADH as a cofactor. The NADPH was ineffective as a reducing agent. The enzyme activity was strongly temperature-dependent, displaying maximal activity between 35 and 40°C. The energy of activation was 4.9 kcal. The enzyme activity was inhibited by sulfhydryl reagents, anionic detergents, and divalent ions. The amino acid composition of the purified enzyme is characterized by the presence of all common amino acids including half-cystine and tryptophan. The results of carbohydrate and lipid analyses indicated that the oxidoreductase is a glycolipoprotein with fucose, galactose, mannose, and glucosamine as the sugar components and cholesterol and sphingomyelin as the lipid constituents. The apparent subunit molecular weight estimated by urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the absence and presence of 2-mercaptoethanol was 40,000. The antiserum completely inhibited the enzymic activity at the equivalence point. We suggest that the membrane-bound NADH: (acceptor) oxidoreductase might be a transmembrane protein.

Key words: NADH: (acceptor) oxidoreductase, erythrocytes, membranes, glycolipoprotein

Abbreviations: NAD – nicotinamide adenine dinucleotide: NADH – dihydronicotinamide adenine dinucleotide; NADPH – dihydronicotinamide adenine dinucleotide phosphate.

Received April 24, 1978; accepted June 7, 1978.

0091-7419/78/0901-0001$02.60

Zamudio and co-workers [1, 2] have demonstrated that human red cells contain a membrane-bound NADH: (acceptor) oxidoreductase (EC 1.6.99.3) which is capable of oxidizing NADH in the presence of electron acceptors such as ferricyanide, cytochrome C, and 2,6-dichlorophenolindophenol. The high specificity of this enzymic activity towards NADH and not NADPH, succinate, or lactate has been utilized as one of the main criteria for its differentiation from a soluble NADH: (acceptor) oxidoreductase (NADH diaphorase) of human erythrocytes [3]. Ultracentrifugation of sonicated ghosts and freeze-drying had no effect on the release of this enzymic activity. The enzyme was released, however, by successive extractions of erythrocyte ghosts with anhydrous and aqueous butanol [2]. Although results of these studies have shown that red blood cells contain enzymes of the electron transfer system, the physiologic role of the membrane-bound NADH: (acceptor) oxidoreductase has not been established.

To study the molecular and kinetic properties of human erythrocyte membrane NADH: (acceptor) oxidoreductase and to explore its possible physiologic role, we have isolated a purified enzyme preparation by a procedure combining affinity chromatography on an NAD^+-Sepharose 4B column, ammonium sulfate precipitation, and isoelectric focusing. This paper describes the isolation procedure and presents a partial characterization of an electrophoretically and immunochemically homogeneous NADH: (acceptor) oxidoreductase from human erythrocyte ghosts.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co, St Louis, Missouri.

Preparation of NAD^+-Sepharose 4B

The activation of Sepharose 4B and the preparation of 6-aminohexanoyl Sepharose 4B were performed according to the procedure described by Cuatrecasas [4]. One hundred milliliters of packed Sepharose 4B suspended in 100 ml of distilled water were mixed with 25 g of cyanogen bromide by constant stirring at 20°C. The pH was maintained at 11.0 by the addition of 8 M NaOH. After reaching a constant pH, the activated Sepharose 4B was washed with cold distilled water and 1 mM HCl, mixed with 100 ml of 0.5 M 6-aminohexanoic acid (AH), pH 10, and allowed to react for 16 h at 4°C. After completion of the reaction, the AH-Sepharose 4B was washed with 500 ml of 0.5 M Nacl and 2–3 liters of distilled water. The washed AH-Sepharose 4B was added to 100 ml of 1% aqueous NAD^+ solution and the pH of the suspension was adjusted to 4.7 with 1 M HCl. Five hundred milligrams of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC, Bio-Rad, Richmond, California) dissolved in 3 ml of water was added dropwise over a period of 5 min at 4°C. The reaction mixture was maintained at this temperature for 16 h. The pH was readjusted to 4.7 with 1 M HCl, and after the addition of another 500 mg of EDAC under identical conditions, the suspension was kept at 4°C for 16 h. The resulting NAD^+-Sepharose 4B was washed exhaustively with 0.5 M NaCl and distilled water. The NAD^+-Sepharose 4B contained approximately 1–2 μmoles of NAD^+ per ml of packed Sepharose 4B as determined by phosphorus assay according to the procedure of Gerlach and Deuticke [5].

Isoelectric Focusing

Isoelectric focusing was performed on an LKB 8101 electrofocusing column (LKB Instrument, Inc, Bromma, Sweden) with LKB ampholine, pH 5–8. Light solution consisted of 48 ml of the sample (40–60 mg protein) dissolved in 1% Triton X-100 and 2 ml of 40% carrier ampholine solution. Heavy solution consisted of 36 ml of 1% Triton X-100, 21 g sucrose, and 2 ml of 40% carrier ampholine solution. Heavy and light solutions were applied to the column by a gradient apparatus. The focusing was performed at 4°C. The voltage was gradually increased to maintain the power output at less than 1 W in order to prevent excess heat formation. After reaching 500 V the focusing was continued for an additional two days.

Enzyme Assay

The enzyme activity was measured according to the procedure described by Zamudio, Cellino, and Canessa-Fischer [2] utilizing potassium ferricyanide as substrate. A unit of enzymic activity was defined as the amount of enzyme required to reduce 1 μmole of potassium ferricyanide per min at 37°C.

Preparation of Antiserum

A monospecific antiserum to the oxidoreductase was prepared by immunizing white New Zealand rabbits with the purified enzyme at weekly intervals for four weeks. One milliliter of the purified enzyme solution (0.2–0.5 mg/ml) in Triton X-100 (0.5–1%) was mixed with an equal volume of complete Freund's adjuvant (Difco Lab, Detroit, Michigan) and injected intraperitoneally. The rabbits were bled by cardiac puncture. Precipitating antibodies were produced four weeks after the initial injection.

Immunodiffusion and Immunoelectrophoresis

Double immunodiffusion [6] and immunoelectrophoresis [7] were performed on glass slides (25 × 75 mm) coated with 1% agarose using barbital buffer, pH 8.6, ionic strength 0.1. Plates were allowed to develop for 24–30 h, washed several times with 0.15 M NaCl and distilled water, and dried at room temperature. Plates were stained for protein with Amido Black 10B.

Agar Electrophoresis

Agar electrophoresis was carried out in 1% agar gels employing barbital buffer (pH 8.6), ionic strength 0.1. Electrophoresis was performed for 40 min at 7.5 V/cm. The protein was stained with Amido Black 10B. The enzyme activity was detected according to the procedure described by Kaplan and Beutler [8] using 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) as staining reagent.

Polyacrylamide Gel Electrophoresis

Enzyme preparations were electrophoresed into 5.6% acrylamide gels in the presence of 1% sodium dodecyl sulfate and 8 M urea according to a previously described procedure [9].

Analytical Methods

Protein samples for the amino acid analyses were hydrolyzed with constant-boiling HCl in evacuated, sealed tubes at 110°C for 24, 48, and 72 h. Amino acid analyses and

performic acid oxidation were carried out as previously described [10]. Tryptophan was determined according to the procedure of Liu and Chang [11].

Neutral sugars and hexosamines were determined by a modification [12] of the gas-liquid chromatographic procedure of Griggs et al [13].

Qualitative analysis of neutral lipids and phospholipids was carried out by thin-layer chromatography according to a previously described procedure [14]. For this analysis, samples were extracted by chloroform/methanol (1:1, v/v). Neutral lipids and phospholipids were identified by comparison with reference compounds in the same solvent systems.

Protein content was determined by a previously reported modification [15] of Lowry's procedure [16] using bovine serum albumin as standard.

The concentration of NaCl was estimated by using a conductivity meter (Radiometer, Copenhagen, Denmark).

RESULTS

Isolation and Purification of NADH: (Acceptor) Oxidoreductase

Erythrocyte ghosts were prepared from freshly drawn blood of human donors by the method of Dodge, Mitchell, and Hanshaw [17]. From each unit of blood (450 ml), 120–150 ml of packed ghosts was obtained; the protein concentration of the essentially hemoglobin-free ghosts was 3–4 mg/ml.

All steps of the isolation procedure were carried out at 0–5°C. The purpose of the initial step in the purification procedure was to remove the extrinsic membrane proteins. Packed erythrocyte ghosts (300–400 ml) isolated from 3 units of blood were extracted sequentially with 8 volumes of 1 mM ethylenediaminetetraacetic acid (EDTA) pH 7.4 and 8 volumes of 0.1 M EDTA, pH 7.4. These extraction steps, monitored by polyacrylamide gel electrophoresis, resulted in the removal of spectrin [18], glyceraldehyde-3-phosphate dehydrogenase [19] and other minor extrinsic membrane proteins. The two extraction mixtures were centrifuged in a Beckman J-21B centrifuge with a JA-10 rotor at 10,000 rpm for 3 h and 1 h, respectively, and the supernatant fractions were discarded. The remaining infranatant fraction (approximately 300–400 ml) was extracted by the addition of Triton X-100 (0.5 ml Triton X-100 per 9.5 ml of the infranatant fraction) under stirring for 1 h at 4°C. The resulting suspension was centrifuged in a Spinco L-5-50 centrifuge at 105,000 g for 3 h. The pellets were resuspended in 200 ml of 5% Triton X-100–5 mM sodium phosphate, and the suspension was centrifuged in the same manner. The supernatant fractions (approximately 500 ml) were combined and used as the starting material (fraction R-3) for affinity chromatography.

NAD^+-Sepharose 4B (150 ml) was mixed with Sephadex G-25 (25 ml) to improve the flow rate and then packed in a column with a bed size of 4.1 × 15 cm. The column was washed exhaustively with 1 M NaCl and equilibrated with 0.2% Triton X-100–10 mM sodium phosphate, pH 7.5 (equilibration buffer). The Triton X-100 soluble fraction R-3 was then chromatographed through the NAD^+-Sepharose 4B column. The column was washed successively with 200 ml of equilibration buffer and with 500 ml of the same buffer containing 0.1 M NaCl. The retained enzyme was eluted with a linear NaCl-NADH gradient consisting of 250 ml of 1 M NaCl and 250 ml of 4 M NaCl and 0.5 mM NADH in the equilibration buffer. The elution pattern is shown in Fig 1. Fractions containing enzymic activity were combined (peak III) and concentrated to approximately 200–250 ml by pressure filtration (PM-10 membrane filter, Amicon Corp, Lexington, Massachusetts).

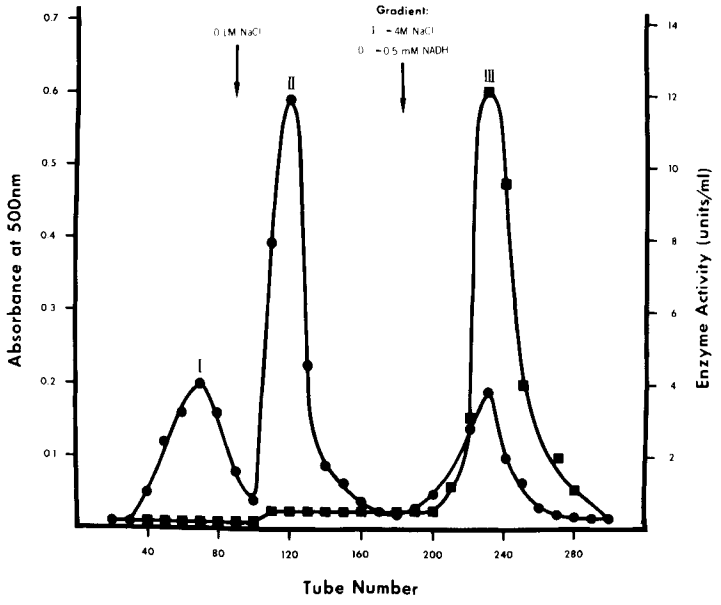

Fig 1. Affinity chromatography of human erythrocyte membrane NADH:(acceptor) oxidoreductase. Protein (•—•); enzyme activity (•—•). Solvent: 0.2% Triton X-100 (v/v), 10 mM sodium phosphate, pH 7.4. The first arrow indicates the beginning of elution with 0.1 M NaCl, while the second arrow indicates the starting point of linear gradient elution. The fraction volume was 5 ml.

Twenty-five grams of $(NH_4)_2SO_4$ was added to each 100 ml of concentrated peak III fraction with stirring at 4°C. The resulting turbid solution was centrifuged in a Sorvall HB-4 swinging bucket rotor at 10,000 rpm. The pellet which formed at the top of the centrifuge tube was removed with a wide-blade spatula and suspended in 40 ml of 5 mM sodium phosphate containing 1% Triton X-100, pH 7.4. After this suspension was stirred for 1 h at 4°C, the insoluble material was separated by centrifugation in a Sorvall SS-34 rotor at 15,000 rpm. The soluble fraction containing enzyme activity was dialyzed for 2 h against 5 liters of 0.5% Triton X-100–5 mM sodium phosphate, pH 7.4. A prolonged dialysis was avoided to prevent the loss of enzyme activity.

The dialyzed fraction was brought up to a volume of 48 ml by addition of 1% Triton X-100–5 mM sodium phosphate, pH 7.4, and used for isoelectric focusing (Fig 2) as described in Materials and Methods. The enzyme focused at pH 6.6, while the non-enzymic proteins formed precipitates in a region below pH 5.5. To remove the contaminating ampholine, the combined fractions with enzymic activity were chromatographed sequentially on Bio-Rad AG 501-X8 (bed size 3 × 10 cm) and NAD^+-Sepharose 4B (bed size 2.5 × 12 cm) columns. The unretained fractions with enzyme activity were eluted from the Bio-Rad column with the equilibration buffer, pH 6.8. The remaining ampholine was eluted from the NAD^+-Sepharose 4B column with 500 ml of equilibration buffer, pH 6.8, and the enzyme was eluted with a linear gradient formed by mixing 150 ml of 1 M NaCl and 150 ml of 4 M NaCl in the same buffer. Fractions containing the enzyme activity were collected and concentrated to a small volume (3–5 ml) by pressure dialysis. A summary of the purification procedure is shown in Table I. The purification of the oxidoreductase can be completed in 7–10 days with an average yield of 2–5 mg of a purified enzyme

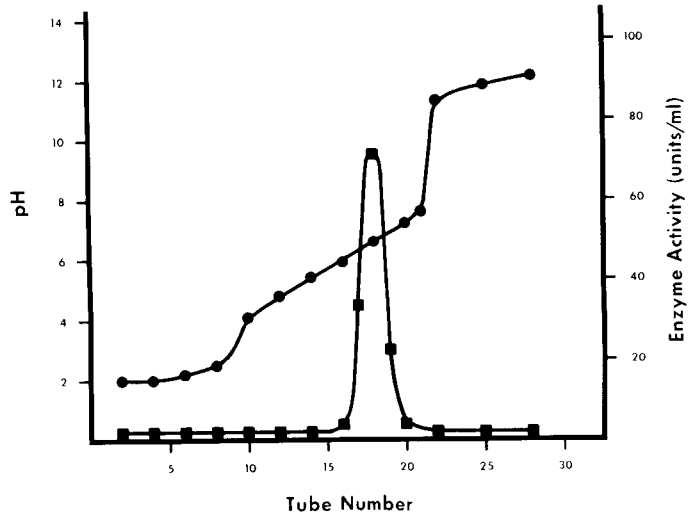

Fig 2. Isoelectric focusing of human erythrocyte membrane NADH:(acceptor) oxidoreductase. The enzyme preparation dissolved in 1% Triton X-100 was focused on a column (LKB 8101, 110 ml) with LKB carrier ampholine at pH range 5–8 as described in Materials and Methods. Enzyme activity (■—■); pH (●—●).

TABLE I. Purification of Human Erythrocyte Membrane NADH:(Acceptor) Oxidoreductase

	Total protein (mg)	Enzyme activity (units)	Specific activity (units/mg)	Purification (X-fold)	Yield (%)
Ghosts	1,180	1,283	1.09	1	100
Triton X-100-solubilized fraction R-3	736	1,150	1.56	1.4	89.8
First-affinity chromatography	194	1,015	5.23	4.8	79.1
Ammonium sulfate precipitation	45	623	13.8	12.7	48.5
Isoelectric focusing	–	592	–	–	46.1
Second-affinity chromatography	4.2	502	112	103	39.1

preparation per three units of blood. The second affinity chromatography on NAD^+-Sepharose 4B resulted in a maximal 103-fold purification of enzyme from erythrocyte ghosts; the yield was 40% of the initial enzyme activity.

Characterization of NADH: (Acceptor) Oxidoreductase

The purified enzyme preparation showed a single band on polyacrylamide gel electrophoresis (Fig 3). The mobility of the enzyme band was unchanged in the presence of

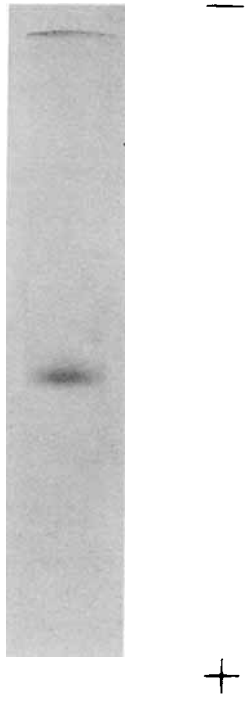

Fig 3. Urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified human erythrocyte membrane NADH:(acceptor) oxidoreductase. The enzyme was dissolved in a solution of sodium dodecyl sulfate, sodium phosphate, urea, and β-mercaptoethanol as previously described [9]. Protein was stained with Coomassie Brilliant Blue.

β-mercaptoethanol. The molecular weight determination of NADH:(acceptor) oxidoreductase by urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis both in the presence and absence of β-mercaptoethanol gave an apparent molecular weight of 40,000 ± 1,000.

On agar gel electrophoresis, the enzyme moved toward the cathode (Fig 4) and on agarose gel electrophoresis toward the anode (Fig 5). As shown in Fig 4, there was an overlap between the enzyme bands stained for the protein and enzymic activity after 40 min of electrophoresis on agar gels. The cathodal mobility of NADH:(acceptor) oxidoreductase was due most probably to the osmoelectrophoretic effect.

On immunoelectrophoresis and double-diffusion analysis the purified enzyme showed single precipitin lines with its antiserum (Fig 5). The Triton X-100-solubilized ghosts also gave a single precipitin line when examined by immunoelectrophoresis or immunodiffusion against the antiserum to NADH:(acceptor) oxidoreductase (Fig 5).

The purified enzyme is unstable in buffer solutions of low ionic strength. However, a stable enzyme preparation can be maintained for several months when kept frozen in the presence of sodium chloride at a concentration greater than 0.1 M. Ampholine seems to have a stabilizing effect on the enzyme as judged from the high recovery of enzymic activity by isoelectric focusing. The isoelectric point of NADH:(acceptor) oxidoreductase was found to be at pH 6.6.

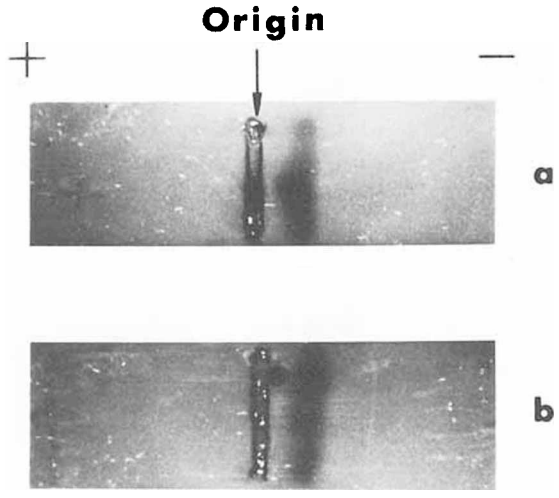

Fig 4. Electrophoretic pattern of purified human erythrocyte membrane NADH:(acceptor) oxidoreductase in 1% agar gel. Agar gels were stained for protein with Amido Black 10B (pattern a) and for enzyme activity with Thiazolyl Tetrazolium (MTT) (pattern b).

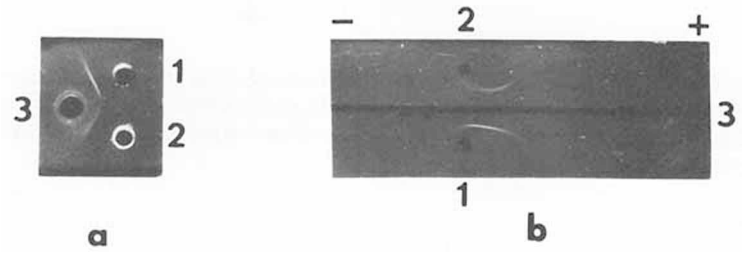

Fig 5. Immunodiffusion (pattern a) and immunoelectrophoresis (pattern b) of the purified human erythrocyte membrane NADH:(acceptor) oxidoreductase (1) and ghosts (2) against the antiserum to purified oxidoreductase (3). The supporting medium was 1% agarose gel.

The pH optimum of the enzyme activity was in the 6–8 range (Fig 6). The maximal activity was found at pH 6.8 with an unusual trough occurring near pH 6.6.

Results in Table II show the effects of various reagents and inhibitors on the enzyme activity. Both p-hydroxymercuribenzoate (1 mM) and 5,5′-dithiobis-2-nitrobenzoate (10 mM) inhibited the enzyme activity completely. On the other hand, neither EDTA (10 mM) nor sodium cyanide had any inhibitory effect. Divalent ions such as Ca^{++} or Mg^{++} partially inhibited the enzyme activity at 10 mM concentration. There was only a slight inhibition of enzyme activity in the presence of 2 M urea, but an almost complete inhibition in the presence of 4 M urea. Anionic detergents, sodium dodecyl sulfate, and sodium deoxycholate, only slightly inhibited the enzyme activity at 1 mM, but exhibited more than 90% inhibitory effect at 10 mM.

The NADH:(acceptor) oxidoreductase had a specificity for NADH as a cofactor. The NADPH was ineffective as a reducing agent.

The enzyme activity was strongly temperature-dependent, displaying maximal activity between 35 and 40°C. However, it lost 60% of the activity at 45°C and became inactive at 50°C after an incubation period of 5 min. The energy of activation was 4.9 kcal.

Determination of the amino acid composition of NADH:(acceptor) oxidoreductase indicated the presence of all common amino acids including half-cystine and tryptophan (Table III).

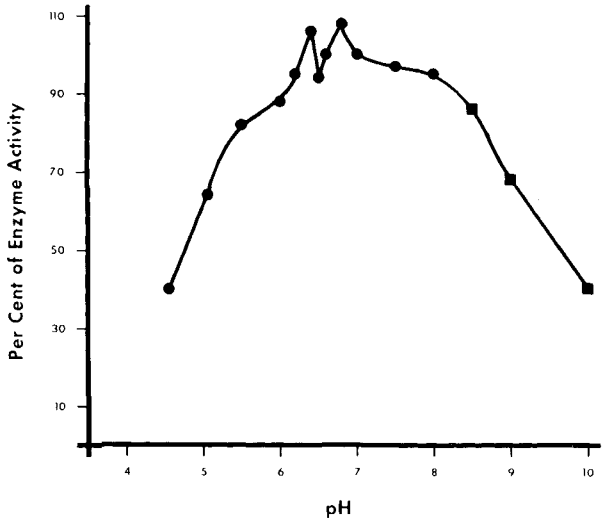

Fig. 6. pH-activity curve of purified human erythrocyte membrane NADH:(acceptor) oxidoreductase. The enzyme was incubated in the presence of either 0.1 M sodium phosphate (•—•) or 0.1 M Tris-HCl (▪—▪) for 10 min at 37°C. The reaction was started by the addition of NADH. The activity of enzyme at pH 7.0 was defined as 100%.

TABLE II. Effects of Additives on the Activity of Purified Human Erythrocyte Membrane NADH:(Acceptor) Oxidoreductase

Compound	Concentration	Percentage of control activity
p-Hydroxymercuribenzoate	1 mM	0
5,5'-Dithiobis-2-nitrobenzoate	1 mM	3
	10 mM	0
Urea	2 M	84
	4 M	2
$MgCl_2$	10 mM	63
$CaCl_2$	10 mM	66
NaCN	10 mM	105
EDTA	10 mM	101
Sodium deoxycholate	1 mM	81
	10 mM	4
Sodium dodecyl sulfate	1 mM	94
	10 mM	5

Assays were performed as described in Methods. Reaction was started by the addition of NADH, after the enzyme had been incubated with the additives for 5 min at 37°C.

TABLE III. Amino Acid Composition of Purified Human Erythrocyte Membrane NADH:(Acceptor) Oxidoreductase

Amino acid	mole %
Lysine	4.82
Histidine	1.92
Arginine	4.43
Aspartic acid	6.17
Threonine	5.59
Serine	6.29
Glutamic acid	8.91
Proline	5.22
Glycine	8.66
Alanine	8.20
Half-cystine	1.02
Valine	7.16
Methionine	1.90
Isoleucine	6.40
Leucine	13.18
Tyrosine	2.19
Phenylalanine	5.90
Tryptophan	1.97

Analysis of two enzyme preparations. Values for serine, threonine, and tyrosine were obtained by linear extrapolation to zero time of average recoveries from 24-, 48-, and 72-h hydrolyses. Values for valine and isoleucine represent average recoveries obtained after 72-h hydrolysis. Half-cystine was determined after performic acid oxidation. Tryptophan was determined according to the procedure of Liu and Chang [11].

Carbohydrate analysis demonstrated that NADH:(acceptor) oxidoreductase is a glycoprotein containing fucose, mannose, galactose, and glucosamine as the sugar components (Table IV). Although the sialic acid content has not been determined, the absence of galactosamine suggests that the enzyme preparation was not contaminated by glycophorin [20] or acetylcholinesterase [21].

Examination of the chloroform/methanol extract of the purified enzyme preparation by thin-layer chromatography revealed sphingomyelin as the major phospholipid and cholesterol as the major neutral lipid. Quantitative determination of these two lipid components has not yet been done.

Interaction of NADH:(Acceptor) Oxidoreductase With Its Antiserum

Results of a study on the interaction between a purified preparation of the oxidoreductase and its antiserum showed that antibodies completely inhibited the enzyme activity at or above the equivalence point (Fig 7). Under such experimental conditions, there was no measurable enzymic activity in the incubation mixture or in the supernatant fraction after separation of immunoprecipitate; inhibition of enzyme activity was not observed in the serum from nonimmunized rabbits (Fig 7). These results indicating that the antigen-antibody complex of NADH:(acceptor) oxidoreductase lacked enzymic activity were confirmed by a direct assay of the activity of washed immunoprecipitates.

TABLE IV. Carbohydrate Composition of Purified Human Erythrocyte Membrane NADH:(Acceptor) Oxidoreductase

Carbohydrates	% Weight
L-Fucose	0.44
D-Galactose	2.05
D-Mannose	0.59
D-Glucosamine	2.63

Analysis of two enzyme preparations. The enzyme samples were hydrolyzed with 1 M HCl in sealed tubes for 4 h at 100°C for determination of neutral sugars, and with 4 M HCl in sealed tubes for 6 h at 100°C for estimation of hexosamines.

DISCUSSION

Results of this study have confirmed the original observation by Zamudio and co-workers [1, 2] of a membrane-bound NADH:(acceptor) oxidoreductase in human red blood cells and provided a procedure for the isolation of an electrophoretically and immunochemically homogeneous enzyme preparation. A 103-fold purification indicates that NADH:(acceptor) oxidoreductase represents approximately 1% of the ghost protein mass. The affinity chromatography on the NAD^+-Sepharose 4B collumn resulted in only a partially purified enzyme preparation. This was probably due to some nonspecific adsorption and protein-protein interactions. The binding of oxidoreductase to the NAD^+-

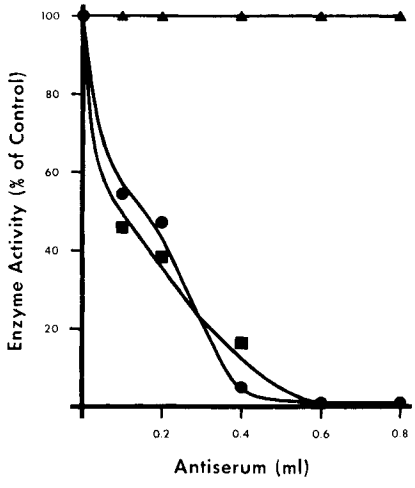

Fig 7. Inhibition by antibodies of NADH:(acceptor) oxidoreductase activity. The aliquots of enzyme solution (0.2 ml) containing 17.8 units of enzymic activity were incubated with increasing amounts of antiserum for 1 h at room temperature. The final volume of incubation mixture was adjusted to 1 ml with a buffer containing 0.15 M NaCl and 10 mM sodium phosphate, pH 7.4. Aliquots (0.1 ml) of the enzyme-antibody suspension (■—■) and supernatant fractions (●—●) after centrifugation were used for assaying the enzyme activity. Nonimmunized rabbit serum (▲—▲) served as control.

immobilized matrix was also dependent on the pH value of the buffer. Kinetic studies showed that the dissociation constant of the NAD^+-oxidoreductase binary complex was 2.73 mM at pH 7.4 and 0.2 mM at pH 6.0 in the presence of 10 mM sodium phosphate. Unfortunately, both the specific and nonspecific binding increased at lower pH values. It is therefore essential to determine the optimal pH for each freshly prepared NAD^+-Sepharose 4B column in order to attain a minimum nonspecific and maximum specific binding. The precipitation of oxidoreductase by $(NH_4)_2SO_4$ was found to be a very efficient way of further purifying the enzyme. Upon addition of $(NH_4)_2SO_4$, the insoluble enzyme was recovered from the top of the supernatant fraction after centrifugation. The flotation of the enzyme was due most probably to its interaction with lipids or detergent. Because of difficulties in measuring the protein concentration of the isoelectric focusing eluates in the presence of Triton X-100 and ampholine, it was not possible to assess directly the extent of purification achieved by isoelectric focusing. However, a substantial amount of protein was removed as precipitates at a pH value lower than 5.5. Simple dialysis of the eluates was insufficient to remove the ampholine, most probably because it seems to form large micelles with the detergent. Even the application of a procedure by Brown and Green [22] resulted in only partial removal of ampholine. Its complete removal was achieved by affinity chromatography of the eluates on an NAD^+-Sepharose 4B column.

Results of quantitative carbohydrate analysis indicate that NADH: (acceptor) oxidoreductase is a glycoprotein. If additional quantitative studies confirm the qualitative identification of cholesterol and sphingomyelin as the integral lipid components, the oxidoreductase should be considered a glycolipoprotein. In a recent report from this laboratory, Niday, Wang, and Alaupovic [21] have provided evidence that human erythrocyte acetylcholinesterase is also a glycolipoprotein. The potentially significant feature of the lipid moieties of these two membrane-bound enzymes is the specificity of the phospholipid composition. While both enzymes share cholesterol as the neutral lipid component, acetylcholinesterase contains phosphatidyl serine [21] and NADH:(acceptor) oxidoreductase sphingomyelin as the main phospholipids. The significance of this specificity is not known. It has been suggested, however, that phospholipids may play an important role as activators for acetylcholinesterase [23].

The purified enzyme preparation (2 mg protein per milliliter, 0.05 M Tris-HCl, pH 7.4) is colorless and shows no absorption bands in the visible wave range. Since flavoproteins would give absorption peaks at this protein concentration, we have concluded that the NADH:(acceptor) oxidoreductase is not a FAD-enzyme as suggested by Zamudio and associates [1, 2].

The subunit molecular weight of NADH:(acceptor) oxidoreductase is 40,000. Since both the reduced and nonreduced enzyme forms have the same molecular weight, this finding argues against the presence of an interchain disulfide linkage in the enzyme molecule. However, the actual number of subunit polypeptide chains present in the native enzyme is not known.

The enzyme was not inhibited by a chelating agent, suggesting that metal ions may not be required for the activity. However, the maintenance of high ionic strength was essential for enzyme stability. The inhibitory effect of a sulfhydryl reagent suggests that the $-SH$ group may be involved in the catalytic process.

The finding that the enzyme-antibody complex lacks enzymic activity suggests that the antibodies bind at or close to the active site of the enzyme. An alternative explanation

is that the binding of antibodies to the antigenic site causes an alteration in the conformation of the enzyme, converting it into an inactive form.

Zamudio, Cellino, and Canessa-Fischer [2] suggested on the basis of histochemical evidence that NADH:(acceptor) oxidoreductase might be localized on the inner surface of the erythrocyte membrane. Similarly, Kant and Steck [24, 25] have concluded on the basis of their studies with the resealed inside-out and right-side-out vesicles of erythrocyte ghosts that the enzyme is accessible on the cytoplasmic side of the membrane. If the assumption that all glycoprotein and glycolipid sugars are located on the external surface of the membrane is correct, then the present finding of the carbohydrate moiety suggests that NADH:(acceptor) oxidoreductase might be a transmembrane protein with the catalytic site localized at the inner surface and the carbohydrate moiety at the external surface of the membrane [26].

Hultquist and Passon [27] have suggested that the reduction of methemoglobin by the soluble NADH-methemoglobin reductase is a slow process which can be enhanced by the addition of cytochrome B_5. The physiologic role of the membrane-bound NADH:(acceptor) oxidoreductase has not been established. Our present working hypothesis is that its main function is to catalyze directly the reduction of methemoglobin. This hypothesis is currently under investigation.

ACKNOWLEDGMENTS

This study was supported by the resource of the Oklahoma Medical Research Foundation.

The authors are grateful to Dr E. Niday for valuable criticisms and assistance in the preparation of this manuscript. We thank Mr C.W. Gorden and Mr R.K. Burns for their skillful technical assistance, and Mrs M. Farmer and Mr R.K. Burns for the typing of this manuscript and for the preparation of the illustrations.

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