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STUDY ON THE REDUCED PYRIDINE NUCLEOTIDE DEHYDROGENASE OF BOVINE ERYTHROCYTES

I. CRYSTALLIZATION AND PROPERTIES OF THE REDUCED PYRIDINE NUCLEOTIDE DEHYDROGENASE OF BOVINE ERYTHROCYTES

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SUMMARY

Reduced pyridine nucleotide dehydrogenase (NADH: (acceptor) oxidoreductase, EC 1.6.99.3) of bovine erythrocytes was purified with DEAE-cellulose chromatography, ammonium sulfate fractionation (30–65%) and DEAE-Sephadex A-50 chromatography. One major and three minor active fractions were eluted with DEAE-Sephadex A-50 chromatography.

The major active fraction was crystallized with ammonium sulfate.

The sedimentation coefficient ($s^\circ_{20,w}$) of this enzyme was 2.5 S and the molecular weight $2.9 \cdot 10^4$.

Absorption spectra showed a maximal absorbance at 406 nm and a shoulder at 450 nm, but with repeated crystallization the absorbance at 406 nm decreased. The ratio of absorbance at 406 nm against 278 nm was 0.026, which was smaller than that obtained by other workers, and the absorption spectrum and fluorescence measurements showed that this enzyme contained neither flavin nor heme.

The molar absorbance coefficient at 278 nm of this enzyme was calculated by amino acid analysis as $2.46 \cdot 10^4$.

The relative activity for dehydrogenation of NADH and NADPH was approximately 2.9/1.0 at pH 7.5.

INTRODUCTION

It has been reported that certain factors maintain hemoglobin in the reduced state in the human erythrocyte[1,2], and that deficiency of these factors brings about methemoglobinemia[3,4].

The nature of these factors, however, is still under discussion[5,6]. As one of the ways to resolve the problem, the purification and characterization of an enzyme involved seems to be plausible, but it is difficult to obtain a large amount of fresh human

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

erythrocytes from an individual source. Recently, however, Brewer *et al.*[7] reported that diaphorase (NADH: lipoamide oxidoreductase, EC 1.6.4.3) isozymes in ovine erythrocytes show properties similar to those of human diaphorase, and Shimizu and Matsuura[8] reported that purified methemoglobin reductase from blue white dolphin also showed the similarity to that of man.

Under these circumstances, we tried to purify NADH dehydrogenase (NADH: (acceptor) oxidoreductase, EC 1.6.99.3) from bovine erythrocytes, which is supposed to be the same enzyme as reductase or diaphorase, and to compare it with the human enzyme.

A description of the crystallization method for bovine NADH dehydrogenase and a comparative discussion of the physicochemical and enzymological properties are presented in the present paper.

EXPERIMENTAL PROCEDURE

Materials

Fresh bovine blood was generously provided by the Teikokuzoki Company (Tokyo). DEAE-cellulose was purchased from Brown (capacity 0.81 mequiv/g) and DEAE-Sephadex A-50 (capacity 3.5 ± 0.5 mequiv/g) from Pharmacia. 2,6-Dichlorophenolindophenol (DCIP) was purchased from Tokyo Kasei Company. NADH and NADPH were purchased from Sigma and Boehringer Mannheim, respectively.

Methods

Enzyme assay. The NADH dehydrogenase coupled with DCIP was estimated spectrophotometrically, when 2 ml of 25 mM Tris–HCl buffer solution (pH 7.5) containing 0.5 mM EDTA, 0.1 ml of 1 mM DCIP solution, 0.02 ml of 10 mM NADH solution and 0.1 ml of enzyme solution were mixed and incubated at room temperature, and the absorbance decrease of DCIP at 600 nm was measured for 1 min by a Hitachi spectrophotometer (EPU type 2).

One unit was defined according to the International Definition, and the molar absorbance coefficient of DCIP at 600 nm was $20.9 \cdot 10^3$ (ref. 9).

Protein determination. The protein concentration was determined with the assumption that a protein concentration of 1.2 mg/ml gave an absorbance of 1.0 by measuring the absorbance at 280 nm with a Hitachi spectrophotometer.

Absorption spectrum determination. The determination of absorption spectra was performed with a Hitachi automatic spectrometer (EPU type 3).

Fluorescence measurements. Fluorescence measurements were performed with a Hitachi spectrofluorimeter (type 204), using a xenon lamp as the light source. The excitation wavelength used was 450 nm[10].

RESULTS

Purification of reduced pyridine nucleotide dehydrogenase from bovine erythrocytes

Step 1. Preparation of hemolyzate. Bovine blood[1,9] was collected at the slaughter house, defibrinized by gentle shaking with glass balls and centrifuged at 7000 rev./min for 30 min after about 2 h standing. The precipitate was washed 3 times with an equal volume of 0.9% saline solution. 4.15 l of packed erythrocytes were obtained

and an equal volume of cold water was added for hemolysis. The hemolyzate was centrifuged at 8000 rev./min for 30 min to remove stroma.

Step 2. DEAE-cellulose treatment. DEAE-cellulose was equilibrated with 1 mM phosphate buffer solution (pH 7.0) by the method of Huennekens *et al.*[11].

It was poured into glass filters to make short fat columns (15.6 cm × 10 cm), and about 4.2 l of the hemolyzate were poured on each column. The columns were washed with ice-cold 1 mM sodium phosphate buffer solution (pH 7.0) with gentle suction until the eluate became almost colorless. Then the enzyme was eluted from the DEAE-cellulose with 3 l of 250 mM citrate buffer solution (pH 5.2) containing 530 g of ammonium sulfate (corresponding to 30% saturation) without suction until the eluate became colorless.

Step 3. Ammonium sulfate fractionation. To 2.7 l of the above eluate, 540 g of ammonium sulfate powder were added with stirring (corresponding to 65% saturation). After standing for 6 h, the precipitate was collected by centrifugation at 7000 rev./min for 30 min. It was dissolved with a minimal volume (about 175 ml) of 5 mM sodium phosphate buffer solution (pH 7.0). The solution was dialyzed in a visking tube against 5 l of 5 mM sodium phosphate buffer solution (pH 7.0) for 6 h, during which time the buffer was changed 3 times. A small amount of precipitate which was formed during the dialysis was removed by centrifugation at 7000 rev./min for 30 min.

Step 4. DEAE-Sephadex A-50 chromatography. The dialyzed enzyme solution (175 ml) was subjected to DEAE-Sephadex A-50 chromatography. The column (4.8 cm × 35 cm) was equilibrated with 10 mM sodium phosphate buffer solution (pH 7.0). After loading the sample, the elution was continued with the same buffer solution. Three peaks were eluted with 10 mM sodium phosphate buffer solution (pH 7.0). The first peak was mainly hemoglobin and had no activity, while the later two peaks (Ia and Ib) showed enzyme activity. Subsequently two minor active peaks

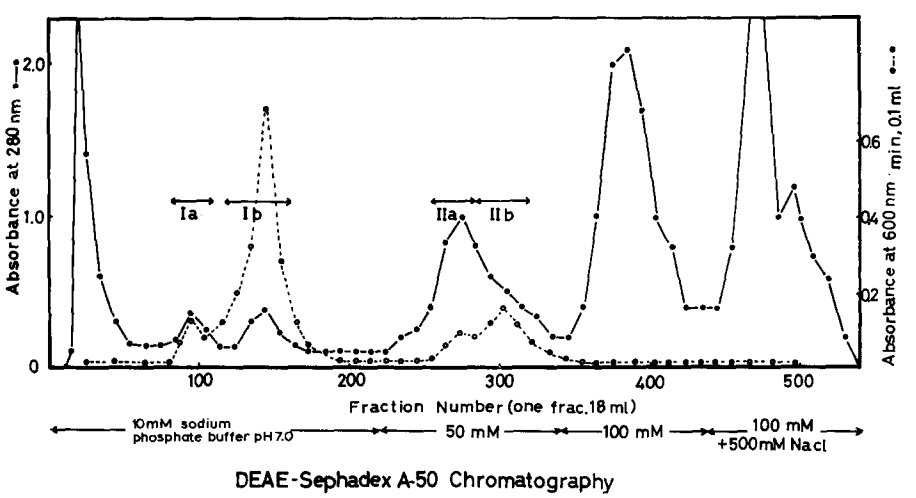

Fig. 1. Elution patterns from DEAE-Sephadex A-50 chromatography. Protein (7 g) was applied to a 4.8 cm × 35 cm column. Each tube contained 18 ml of eluate and the flow rate was about 0.57 ml/min. ●—●, Absorbance at 280 nm; ○---○, NADH–DCIP enzyme activity.

(IIa and IIb) were eluted with 50 mM sodium phosphate buffer. The elution pattern is shown in Fig. 1. The total recoveries of protein and enzymatic activity were 100% and 100.9%, respectively. The recoveries of the enzyme activity in the respective fractions were 7.2% (Ia), 65.4% (Ib), 8.3% (IIa) and 20% (IIb).

Step 5. Concentration. These fractions were precipitated by the addition of of ammonium sulfate to 65% saturation. The precipitate was dissolved in the minimal volume of 0.1 M sodium phosphate buffer solution (pH 7.0) to give a solution containing 15–20 mg of protein per ml and stored in a refrigerator.

Step 6. Crystallization. The major active fraction (Ib) was processed for crystallization. Finely powdered ammonium sulfate (about 1.5 g) was continuously added to the concentrated enzyme solution (10 ml) until a faint permanent turbidity appeared. Saturated ammonium sulfate solution (about 3 ml) was added slowly with stirring and the mixture was placed in a refrigerator. Crystallization usually began after about 12 h from the last addition of ammonium sulfate solution. Slightly yellowish crystals came out from the reddish-orange solution and the crystallization was complete within a week.

Step 7. Recrystallization. The crystals were dissolved in the minimum volume

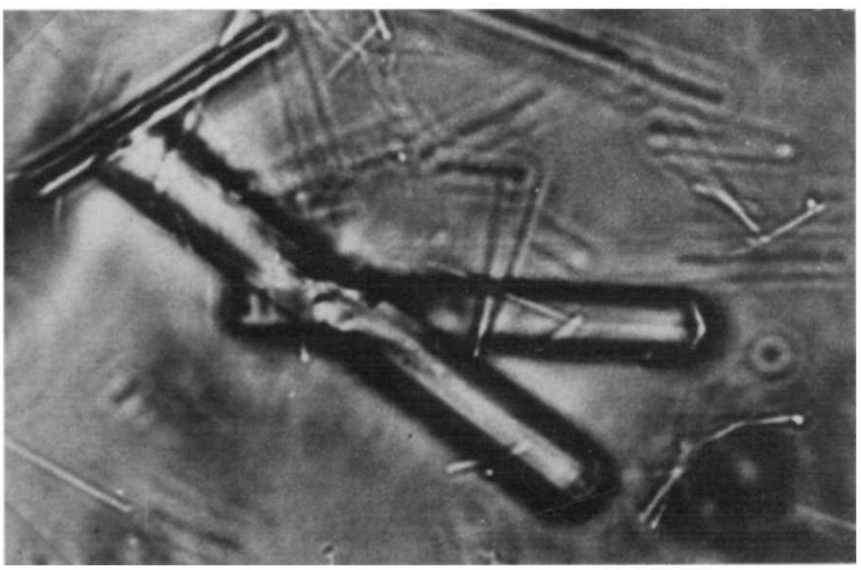

Fig. 2. Photomicrographs of crystals of reduced pyridine nucleotide dehydrogenase. (Magnification × 300).

of 0.1 M sodium phosphate buffer solution (pH 7.0). The ammonium sulfate powder and the saturated ammonium sulfate solution were added as described in the crystallization method, and few seed crystals of the enzyme were added. The mixture was kept in a refrigerator. Fig. 2 shows a photograph of crystals of the enzyme.

The purification procedure is summarized in Table I.

Properties of the enzyme

Stability. No decrease in enzyme activity was observed in crystals kept in

TABLE I

PURIFICATION OF REDUCED PYRIDINE NUCLEOTIDE DEHYDROGENASE

Fraction	*Vol. (ml)*	*Protein ($A_{280\ nm}$)*	*Specific activity**	*Yield (%)*
Hemolyzate	8300	260	0.0002	100
DEAE-cellulose eluate	2700	15.7	0.01	104
30–65% $(NH_4)_2SO_4$ ppt	175	40	0.055	93
DEAE- Ia	400	0.29	0.24	
-Sephadex Ib	1460	0.195	0.89	94
chromato- IIa	460	0.8	0.087	
graphy IIb	1000	0.49	0.16	
30–65% $(NH_4)_2SO_4$ ppt	10	18.6	1.45	65
Crystallization	7	10.6	2.46	44
Two crystallizations	5	10.4	2.61	32.9
Three crystallizations	4	8	2.74	21.2

* Total units/vol. per ml/$A_{280\ nm}$.

aliquots in a refrigerator for several months. The presence of salts increased the stability of the enzyme. Prolonged dialysis and low concentrations of enzyme caused loss of activity.

Substrate specificity of reduced pyridine nucleotides. The activity with NADH was higher than with NADPH, and the relative activity for NADH and NADPH was 2.9:1 in 25 mM Tris–HCl buffer solution containing 0.5 mM EDTA (pH 7.5).

Ultraviolet and visible absorption spectra of the enzyme. In Fig. 3 the absorption spectra at various stages of purification are shown. Samples are from before the

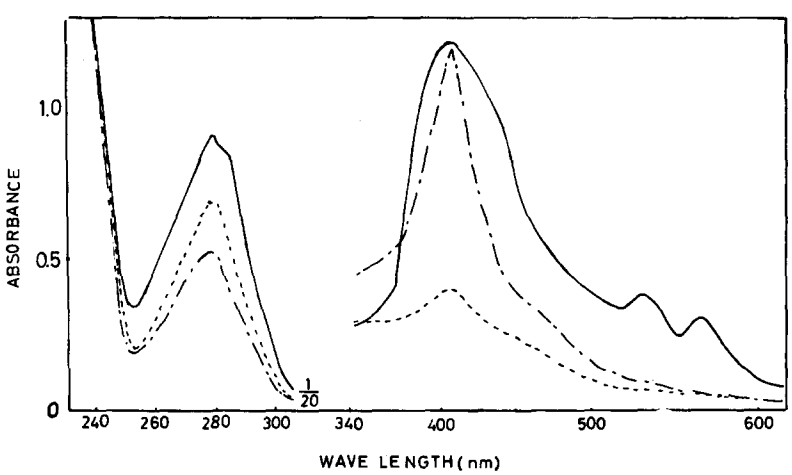

Fig. 3. Absorption spectra at the various stages of crystallization of the enzyme in 0.1 M sodium phosphate buffer solution (pH 7.0). The concentration of ultraviolet spectra is 1/20 of visible spectra. ———, before crystallization; — — —, the once crystallized enzyme; — — —, the 3 times crystallized enzyme.

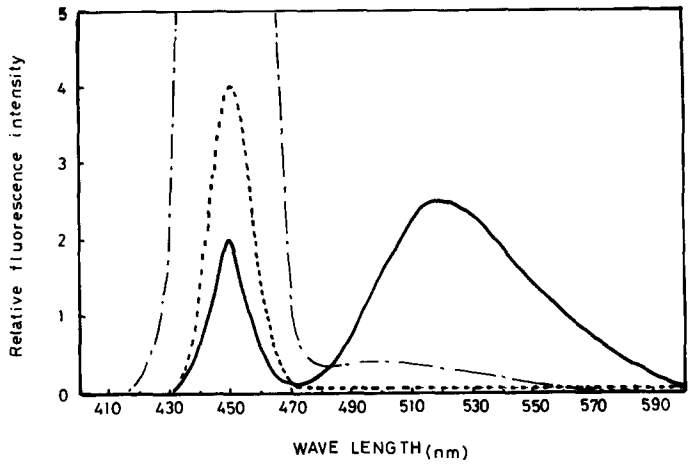

Fig. 4. Fluorescence spectrum of riboflavin and the recrystallized enzyme solution. The excitation wave length was 450 nm. The concentration of riboflavin and the enzyme was 1 μM and 10 μM in 0.1 M Tris–HCl buffer solution (pH 7.0), respectively. ———, 1 μM riboflavin; — — —, 10 μM enzyme solution; — - —, 10 μM enzyme solution, 10-fold multiplication.

crystallization, the once crystallized enzyme in sodium phosphate buffer solution (pH 7.0) and the 3 times crystallized enzyme in the same buffer solution. With repeated crystallization the absorbance at 406 nm decreased significantly, but the specific activity of 4 times crystallized enzyme was almost the same as that of 3 times crystallized enzyme.

The enzyme's visible range absorption spectrum did not show any significant changes after reduction with NADH or dithionite.

Fluorescence spectrum of the enzyme. The amount of flavin was analyzed by the fluorimetric method, which uses fluorescence at 520 nm with excitation at 450 nm. In Fig. 4, the fluorescence spectrum of 10 μM recrystallized enzyme solution is shown and compared with that of the solution of 1 μM riboflavin. Relative emission at 520 nm indicated less than 0.01 mole of flavin (as riboflavin) in 1 mole of the enzyme.

Sedimentation velocity. The 3 times crystallized enzyme appeared to be homogeneous in the ultracentrifuge (Fig. 5). A logarithmic plot of changes in the distance from the axis of rotation against time intervals was linear. From the results the sedimentation coefficient ($s^\circ_{20,w}$) in sodium phosphate buffer solution (pH 7.0) was calculated as 2.5 S.

Molecular weight. The molecular weight of the enzyme was estimated by the sedimentation equilibrium method[12]. The Rayleigh interference optical system was employed. A plot of the concentration on a log scale against the square of the radius from the center of the rotor showed an almost linear relationship. Assuming a partial specific volume of 0.75 per g, the apparent average molecular weight was $2.9 \cdot 10^4$.

Amino acid composition. The 0.6 ml of the 3 times crystallized enzyme (absorbance 1.77 at 278 nm) was placed in a test tube and evaporated to dryness. 1.0 ml of distilled 6 M HCl was added to the sample. It was sealed under reduced pressure and then hydrolyzed for 24 h at 108 °C. In Table II the amino acid composition is

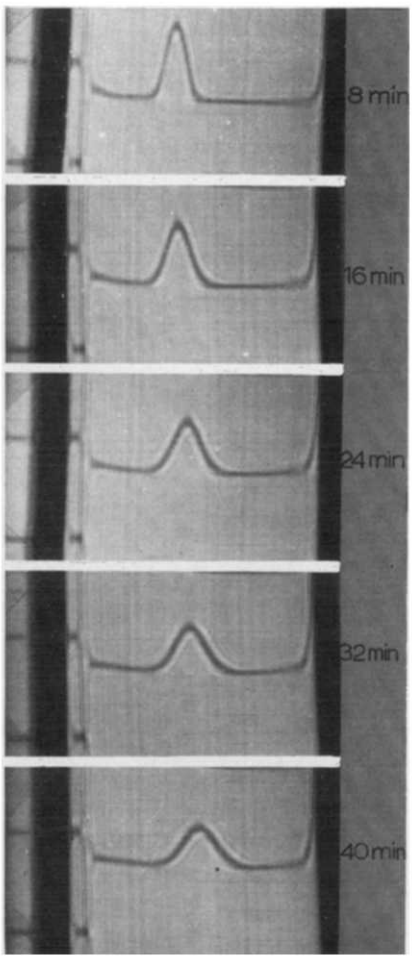

Fig. 5. Sedimentation patterns of reduced pyridine nucleotide dehydrogenase. The photographs were taken (top to botton) at 8-min intervals after 59 780 rev./min at 20 °C. The concentration of the enzyme was about 5 mg/ml in 0.1 M sodium phosphate buffer solution (pH 7.0).

shown as ratios (these are uncorrected values). Tryptophan was estimated by the method of Spies and Chambers[13].

From the amino acid analysis and the absorbance, the molar absorbance coefficient at 278 nm of this enzyme was calculated as $2.46 \cdot 10^4$.

DISCUSSION

For the preparation of the human enzyme there are various descriptions of the range of ammonium sulfate concentration appropriate for fractionation, probably due to the low concentration of this enzyme. The present enzyme, though extracted from a bovine source, almost corresponds in precipitation range to the range used by Scott and MacGraw[14].

TABLE II

AMINO ACID COMPOSITION OF REDUCED PYRIDINE NUCLEOTIDE DEHYDROGENASE OF BOVINE ERYTHROCYTES

Residue	*Moles/mole protein**	
	As calcd from amino acid	*To nearest integer*
Lysine	15.32	15
Histidine	14.80	15
Arginine	10.72	11
Asparatic acid	27.06	27
Threonine	20.94	21
Serine	10.21	10
Glutamic acid	18.90	19
Proline	12.26	12
Glycine	19.40	19
Alanine	24.00	24
Cystine (half)	1.89	2
Valine	38.81	39
Methione	6.64	7
Isoleucine	8.17	8
Leucine	19.92	20
Tyrosine	7.15	7
Phenylalanine	4.03	4
Tryptophan*	1.70	2
Total number of residues		262

* Estimated by the dimethylaminobenzaldehyde method[13].

On the multiplicity of forms of the human enzyme, there are various reports. Scott and MacGraw[14] reported that there were four forms as indicated by hydroxylapatite chromatography, NADH dehydrogenase I and II and NADPH dehydrogenase A and B[4]. Shrago *et al.*[15] reported that two forms were separated by DEAE-cellulose column chromatography and were eluted at 5 mM and 20 mM potassium phosphate buffer solution, respectively. Kajita[6] reported the existence of three forms as indicated by DEAE-cellulose column chromatography since they eluted successively as the gradient increased from 3 to 30 mM phosphate buffer solution (pH 7.0). In the present investigation, we carefully excluded the dilution effects as far as possible, since extreme dilution leads to denaturation and then the activity of this enzyme is extremely low. Chromatography on DEAE-Sephadex A-50 gave one major and three minor active fractions. The ratio of the amount of Ia to Ib and of IIa to IIb was not the same in various preparations. It would be of interest to clarify whether this behavior is due to individual differences or to artifacts.

The activity ratios for dehydrogenation of NADH and of NADPH vary with the enzyme preparation and the method of estimation. The human enzymes prepared by Scott and MacGraw[14] were specific for either NADH or NADPH when the indophenol method was used. The ratio of NADH to NADPH dehydrogenation for the enzyme prepared by Kajita *et al.*[6] was 1.2–1.4 and for that prepared by Hegesh and Avron[5], it was 27, but the enzyme prepared from sheep was almost specific for NADH[7]. In the present investigation, the ratio was 2.9. These values varied in individuals and in species. It would also be of interest to clarify whether these different values implied differences in the nature of the enzyme or not.

The spectra of the enzyme before crystallization showed high absorbance at 406 nm, 540 nm and 577 nm, suggesting the presence of the heme group as a co-factor[16], and absorbance at 450 nm suggesting the presence of flavin. But with the first recrystallization, the absorbance at 540 nm and 577 nm disappeared and the absorbance at 406 nm and 450 nm decreased greatly. The absorbance at 406 nm decreased further with repeated recrystallization.

The reduction of the enzyme by dithionite did not effect these absorbances. These facts do not suggest that the absorbance at 406 nm is related to the enzyme reaction or that flavin or heme is essential to enzyme catalysis. Even if this absorbance were due to cofactors such as flavin or heme, the amount of flavin or heme was much less than one mole per one mole of enzyme. On the other hand, Scott and MacGraw[5] reported for human enzyme that the FAD content increased with purification, but Hegesh and Avron[5] and Kajita *et al.*[6] reported that flavin and heme did not appear to be involved. In this study, the fluorimetric analysis of flavin did not indicate the existence of flavin in the enzyme.

There is another fact indicating that the crystallized enzyme contained no cofactors which have an absorbance at 278 nm. Based on the amino acid analysis and absorbance at 278 nm, the molar absorbance coefficient at 278 nm of this enzyme was calculated as $2.46 \cdot 10^4$. The amino acid analysis of this enzyme revealed that 1 mole of the enzyme contained 7 moles of tyrosine and 2 moles of tryptophan. The molar absorbance coefficient of tyrosine is $1.42 \cdot 10^3$ and of tryptophan, $5.60 \cdot 10^3$ (ref. 17). The summation of the absorbance for seven tyrosines and two tryptophans gave a molar absorbance of $2.11 \cdot 10^4$, which corresponded reasonably to the observed molecular absorbance of the enzyme.

This fact indicates that there are no contributions by cofactors to the molecular absorbance of the enzyme.

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