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STUDIES ON REDUCED PYRIDINE NUCLEOTIDE DEHYDROGENASE IN BOVINE ERYTHROCYTES

II. ELECTRON ACCEPTOR SPECIFICITY OF TWO TYPES OF REDUCED PYRIDINE NUCLEOTIDE DEHYDROGENASE IN BOVINE ERYTHROCYTES

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SUMMARY

Two types of reduced pyridine nucleotide dehydrogenase have been characterized in erythrocytes with respect to electron acceptors and donors. Both enzymes have reducing activity towards dyes such as 2,6-dichlorophenolindophenol (DCIP) and methylene blue. Cytochrome b_5 and ferricyanide reductase activity are characteristic to only one of the dehydrogenases.

The reduced pyridine nucleotide specificity of the two enzymes was different. The NADH/NADPH ratio for the former enzyme was 2.9 at pH 7.5; the latter enzyme was specific for NADH only.

Methemoglobin prepared by various oxidizing methods was directly reduced by the enzyme *plus* NADH only.

Methylene blue and DCIP effected methemoglobin reduction but the effect of the dyes was different for each enzyme.

INTRODUCTION

In previous reports[1,2], it was shown that there are multiple forms of reduced pyridine nucleotide dehydrogenase in bovine erythrocytes. One of the enzymes was crystallized.

In the case of the human enzyme, Scott *et al.*[3] reported the existence of NADH dehydrogenase I, II and NADPH dehydrogenase A, B. However, the enzymes prepared by Huenneken and co-workers[4,5], although shown by chromatography to contain multiple forms, were almost identical with respect to electron donor specificity (the ratio of NADH/NADPH activity for the enzyme prepared by Kajita *et al.*[4] was

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

3.8–4.2 and for the enzyme prepared by Niethammer and Huenneken[3], 3.0–3.3). Both Hegesh and Avron[6] and Sugita *et al.*[7] purified an enzyme specific for NADH. The enzyme prepared by Sugita *et al.*[7] showed very high cytochrome b_5 reductase activity whereas Hegesh *et al.*[6] showed very high ferricyanide reductase activity in their preparation.

This investigation shows that there are two different types of reduced pyridine nucleotide dehydrogenase in bovine erythrocytes with regard to electron acceptors and donors. The reduction of methemoglobin by the enzyme has also been studied.

EXPERIMENTAL PROCEDURE

Materials

The following reagents were purchased from the commercial sources indicated; FMN, phenazine methosulfate, 2-methyl-1,4-naphthoquinone, $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, thioctic acid, $NaNO_2$, $NaNO_3$, glutathione, hemin, methylene blue (Wako Pure Chemicals), 2,6-dichlorophenolindophenol (DCIP) (Tokyo Kasai Inc.) and NADPH, NADH (Boehringer Mannheim). Cytochrome b_5 from rat liver was a gift from Dr Sugita of Kanazawa University and cytochrome *c* from horse heart muscle was a gift from Dr Yamauchi of Tokyo Metropolitan University.

Enzyme. In this study, twice recrystallized Enzyme Ib prepared by the method reported previously[1,2] was used. Enzyme IIb was used after further purification of the material described in the previous report. After chromatography on DEAE-Sephadex A-50 Enzyme IIb was concentrated by means of $(NH_4)_2SO_4$ precipitation and gel filtration on Sephadex G-75. The active peak was concentrated by addition of $(NH_4)_2SO_4$ and dialyzed against 10 mM sodium phosphate buffer solution (pH 7.0). The dialysate was purified by rechromatography on DEAE-Sephadex A-50, the eluate was concentrated with $(NH_4)_2SO_4$ and dissolved in 50 mM Tris–acetate buffer (pH 7.0). The specific activity of the purified enzyme was about $2 \cdot 10^4$ times that of the starting hemolyzate and the enzyme was almost one band on disc electrophoresis. The details of the preparation procedure and further properties of the enzyme will be reported in a following communication.

The other enzyme preparations Ia and IIa, were used at the state of purification reached after chromatography on DEAE-Sephadex A-50 and concentration of the eluate with $(NH_4)_2SO_4$.

Preparation of various types of methemoglobin. Nitrite-oxidized hemoglobin was prepared by the method of Asakura *et al.*[8] and ferricyanide-oxidized hemoglobin and anion-exchange resin (Dowex 1-X2) treated ferrihemoglobin were prepared by the method of Hegesh and Avron[6]. These methemoglobins were treated with DEAE-cellulose to remove the enzyme and dialyzed against cold distilled water to remove any supposed electron carriers of low molecular weight.

Enzyme assay

The standard assay system contained 0.1 ml of 1 mM hydrogen acceptor, 0.02 ml of 10 mM NADH, 0.1 ml of the enzyme (about 0.1–1 mg) and 2 ml of 0.1 M Tris–HCl buffer (pH 7.0) at room temperature. The reaction rate was measured by the decrease of absorbance at 340 nm in the absence of DCIP. In the presence of DCIP, the rate was measured by the decrease of absorbance at 600 nm.

The final concentration of hemoprotein (when present) was 0.025 mM for methemoglobin and cytochrome b_5 and 0.01 mM for cytochrome *c*. The reaction rate was determined by the increase in the absorbance at 576 nm for methemoglobin, 556 nm for cytochrome b_5 and 550 nm for cytochrome *c*.

The millimolar extinction coefficients were as follows: 6.22 at 340 nm (NADH, NADPH), 20.9 at 600 nm (DCIP), 11.9 at 576 nm (methemoglobin), 18.5 at 550 nm (cytochrome *c*) and 17.8 at 556 nm (cytochrome b_5).

TABLE I

SPECIFICITY OF REDUCED PYRIDINE NUCLEOTIDE DEHYDROGENASE (Ib AND IIb) FOR ELECTRON ACCEPTORS

Assay conditions as described in the text. Final concentration of electron acceptors; 0.025 mM for cytochrome *c* and methemoglobin, 0.01 mM for cytochrome b_5 and 0.05 mM for others.

Electron acceptors	*Initial rate (μmoles/min per mg)*	
	Ib	*IIb*
Phenazine methosulfate	16.2	4.26
DCIP	1.8	2.2
Methylene blue	1.1	0.1
2-Methyl,1,4-naphthoquinone	$6.7 \cdot 10^{-3}$	$5.0 \cdot 10^{-2}$
$K_3Fe(CN)_6$	$5.0 \cdot 10^{-3}$	48
Flavin mononucleotide	$3.4 \cdot 10^{-3}$	$6.4 \cdot 10^{-3}$
Hematin	$1.6 \cdot 10^{-3}$	$5.6 \cdot 10^{-3}$
Cytochrome *c*	$3.0 \cdot 10^{-4}$	$2.2 \cdot 10^{-3}$
Nitrite-treated hemoglobin	$1.9 \cdot 10^{-4}$	$3.7 \cdot 10^{-3}$
Cytochrome b_5	0	24
Thioctic acid	0	0
$NaNO_2$	0	0
Oxidized glutathione	0	0

RESULTS

Acceptor specificity of the two types of NADH dehydrogenase

The intial rates of reduction of electron acceptors are listed in Table I. Enzyme Ib reduced dyes such as phenazine methosulfate, DCIP and methylene blue. The rate of reduction of quinone, ferricyanide, flavin or hematin by the enzyme was in the order of 10^{-3} μmole/min per mg and that of nitrite-treated hemoglobin or cytochrome *c* was in the order of $1 \cdot 10^{-4}$ μmole/min per mg. Cytochrome b_5, thioctic acid, nitrite or oxidized glutathione were not reduced under the same conditions.

Purified Enzyme IIb could reduce $K_3Fe(CN)_6$ and cytochrome b_5, although DCIP reductase activity was almost the same as for Enzyme Ib. The reducing activities of Enzyme IIb towards quinone, cytochrome *c* and nitrite-treated hemoglobin were about ten times as great as those observed with Enzyme Ib but the the reduction of methylene blue was only 10% of that observed with Enzyme Ib.

The other Enzymes Ia and IIa did not reduce $K_3Fe(CN)_6$ and cytochrome b_5 and gave almost the same results as Enzyme Ib.

Electron donor (pyridine nucleotide) specificity

The pyridine nucleotide specificity for the crystallized Enzyme Ib was reported

previously[1,2], the ratio for NADH/NADPH being 2.9 at pH 7.5. For the other reduced pyridine nucleotide dehydrogenases (Enzymes Ia and IIa), the ratio was the same as for Enzyme Ib. Enzyme IIb was specific for NADH under the same conditions.

These results on electron acceptors and donors show that the enzyme fractions Ia, Ib and IIa from DEAE-Sephadex A-50 chromatography are the same enzyme but enzyme fraction IIb is different.

The reduction of methemoglobin oxidized by various methods

Methemoglobin was prepared by various methods as described in the experimental procedure. The reduction rates of methemoglobin are shown in Table II. For these measurments, about 0.1–0.2 mg of the enzyme was used and the reduction rate was measured at 30-min intervals.

TABLE II

THE REDUCTION OF METHEMOGLOBIN PREPARED BY DIFFERENT METHODS

Rate of increase in oxygenated hemoglobin was measured at 576 nm under standard assay condition in the text. The reaction mixture contained; methemoglobin, 0.025 mM; NADH, 0.1 mM; Tris–HCl, 0.1 M, pH 7.0.

Methemoglobins	*Initial rate (μmoles/min per mg)*	
	Enzyme Ib	*Enzyme IIb*
Nitrite-treated hemoglobin	$1.9 \cdot 10^{-4}$	$3.7 \cdot 10^{-3}$
Anion-exchange-treated hemoglobin	$1.3 \cdot 10^{-4}$	$1.5 \cdot 10^{-3}$
Ferricyanide-treated hemoglobin	$1.3 \cdot 10^{-3}$	$1.8 \cdot 10^{-2}$
Cyanomethemoglobin	0	0

Methemoglobin was reduced by Enzyme IIb ten times more rapidly than by Enzyme Ib. The rates of reduction of nitrite-treated and of anion-exchange resin-treated hemoglobin were almost the same for each enzyme (about 10^{-3} μmole/min per mg for Enzyme IIb and about 10^{-4} μmole/min per mg for Enzyme Ib) but ferrihemoglobin was reduced by Enzyme IIb about seven times more rapidly and by Enzyme Ib five times more rapidly than nitrite-treated hemoglobin. Cyanomethemoglobin was not reduced to oxyhemoglobin under these conditions. The rate of reduction of these methomeglobins was proportional to the enzyme concentration and both enzymes reduced methemoglobin not to reduced hemoglobin but to oxyhemoglobin.

Fig. 1 shows one example of the change of the absorption spectra from methemoglobin to oxyhemoglobin during the reduction of nitrite-treated hemoglobin by NADH *plus* Enzyme Ib (2.8 mg/2 ml). The change produced by NADH *plus* Enzyme IIb was the same as Fig. 1 at an enzyme concentration of about 1/10 that of Enzyme Ib.

The effect of electron carriers on nitrite-treated hemoglobin reduction

It is known that electron carriers such as methylene blue effect methemoglobin reduction. In this study, the nitrite-treated hemoglobin was used as one representative of methemoglobin and the effect of various electron carriers was examined. The results are shown in Table III. Methylene blue and DCIP increased the rate of reduc-

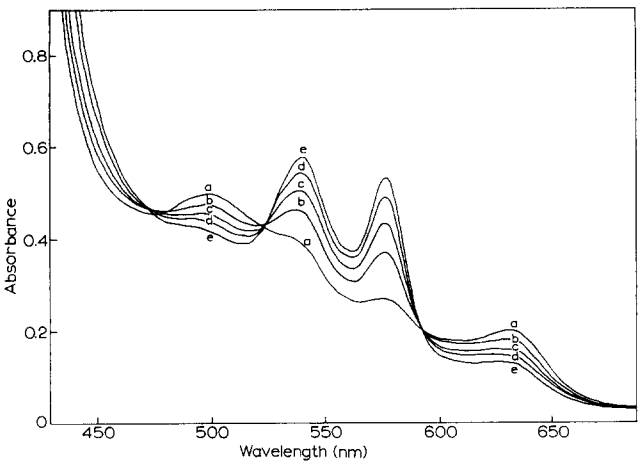

Fig. 1. The time change of the absorption spectra of the reduction of nitrite-treated hemoglobin in the absence of electron carriers. The conditions were as follows; 0.05 μmole of nitrite-treated hemoglobin, 0.2 μmole of NADH and 2.8 mg of Enzyme Ib in 2 ml of 0.1 M Tris–HCl buffer solution. Curve a, 0 min; b, 30 min; c, 60 min; d, 120 min; e, 180 min.

TABLE III

EFFECT OF VARIOUS ELECTRON CARRIERS TO THE REDUCTION OF NITRITE-TREATED HEMOGLOBIN

Increase of the reduction rate of hemoglobin was measured at 576 nm under standard assay conditions as given in the text. The reaction mixture contained: electron carriers, 5 μM; NADH, 0.1 mM; nitrite-treated hemoglobin, 0.025 mM; Tris–HCl buffer, 0.1 M, pH 7.0.

Electron carriers	*Initial rate (μmoles/min per mg)*	
	Enzyme Ib	*Enzyme IIb*
None	$1.9 \cdot 10^{-4}$	$3.7 \cdot 10^{-3}$
Methylene blue	$7.6 \cdot 10^{-1}$	$2.9 \cdot 10^{-2}$
DCIP	$3.2 \cdot 10^{-2}$	$4.2 \cdot 10^{-2}$
FMN	$2.0 \cdot 10^{-4}$	$4.2 \cdot 10^{-3}$
2-Methyl 1,4-naphthoquinone	$2.0 \cdot 10^{-4}$	$5.6 \cdot 10^{-3}$
$K_4Fe(CN)_6$	$1.9 \cdot 10^{-4}$	$7.0 \cdot 10^{-3}$
$K_3Fe(CN)_6$	0	0
Phenazine methosulfate*	$2.4 \cdot 10^{-1}$	

* See text.

tion of nitrite-treated hemoglobin by both enzymes. However, their effect on the reduction of nitrite-treated hemoglobin by Enzyme IIb was about 10% of that observed for Enzyme Ib. $K_4Fe(CN)_6$ increased the reduction rate of nitrite-treated hemoglobin by Enzyme IIb about 2-fold compared with the control (no electron carriers present) but did not effect Enzyme Ib.

In all cases methemoglobin was reduced by enzyme *plus* electron carrier not to reduced hemoglobin but to oxyhemoglobin.

Fig. 2 shows the change with time of spectra of nitrite-treated hemoglobin reduced with Enzyme Ib in the presence and absence of methylene blue.

NADH *plus* phenazine methosulfate reduced nitrite-treated hemoglobin at a rate of 0.24 μmole per min when phenazine methosulfate (0.005 μmole) and NADH

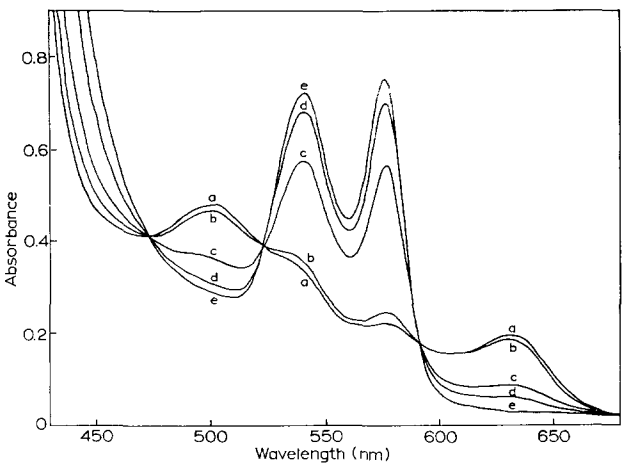

Fig. 2. The time change of the absorption spectra of the reduction of nitrite-treated hemoglobin with or without methylene blue. The conditions were as follows: 0.04 μmole of nitrite-treated hemoglobin, 0.2 μmole of NADH, 0.18 mg of Enzyme Ib and in the case of the addition methylenene blue 0.001 μmole in 2 ml of 0.1 M Tris–HCl buffer solution at room temperature. Without methyle blue: Curve a, 0 min; b, 60 min. After addition of methylene blue: Curve c, 5 min; d, 10 min; e, 20 min.

(0.2 μmole) were added in 2 ml Tris–HCl buffer solution (pH 7.0) but this reduction was not enzymatic.

DISCUSSION

This investigation shows that in bovine erythrocytes there are two types of reduced pyridine nucleotide dehydrogenase with respect to the specificity of electron (hydrogen) acceptors and donors: one is NADH (NADPH): (acceptor) oxidoreductase (EC 1.6.99.3) and the other is NADH:ferricytochrome b_5 oxidoreductase (EC 1.6.2.2).

From these results with bovine enzymes, the human reduced pyridine nucleotide dehydrogenases may also be classified into two types with respect to donor and acceptor specificity: the enzyme prepared by Huenneken and co-workers[4,5] and Shrago and Falcono[9] is NADH (NADPH):(acceptor) oxidoreductase (EC 1.6.99.3) and the enzyme prepared by Sugita *et al.*[7] and Hegesh and Avron[6] is NADH:ferricytochrome b_5 oxidoreductase (EC 1.6.2.2).

Scott[10] reported that NADH specific dehydrogenase is lacking in red cells of individuals with hereditary methemoglobinemia. This deficiency would thus be in the latter enzyme.

The enzyme in bovine erythrocytes could reduce methemoglobin directly with only NADH as electron donor. The nitrite-treated hemoglobin reducing activity of NADH oxidoreductase (Enzyme Ib) was about $1 \cdot 10^{-4}$ times that of its DCIP reductase activity. This result was similar to the value for the human enzyme prepared by Scott and Macgraw[11]. However, the methemoglobin reducing activity of the cytochrome b_5 reductase (Enzyme IIb) was $1.7 \cdot 10^{-3}$ times that obtained for the DCIP reductase activity, a result very similar to that obtained for the human enzyme pre-

pared by Sugita *et al.*[7]. Cytochrome b_5 reductase (Enzyme IIb) may be the main enzyme for methemoglobin reduction.

It was reported[8] that nitrite-treated methemoglobin in erythrocytes could be reduced at a rate of 2–2.7 μmoles/h per l red cells by various steps of sugar metabolism. In a previous report[2], the content of NADH oxidoreductase was shown to be about 32 mg/per red cells and the content of cytochrome b_5 reductase (Enzyme IIb) about 8 mg/per red cells. Thus nitrite-treated hemoglobin could be reduced at a rate of 2.14 μmoles/h per l red cells (0.34 μmole by NADH oxidoreductase (Enzyme Ib, Ia, IIa) and 1.7 μmoles by cytochrome b_5 reductase (Enzyme IIb)) at pH 7.0 and this fact may account for sufficient reduction of nitrite-treated hemoglobin in erythrocytes.

Vestling reported[12] that the reduction rate of ferrihemoglobin by ascorbic acid was higher than that of nitrite-treated hemoglobin. Hegesh and Avron[13] also reported that the reduction rate of ferrihemoglobin by the hemolyzate was more rapid than the rate for anion-exchange resin-treated hemoglobin; nitrite-treated hemoglobin was not reduced.

This investigation showed that the reduction of ferrihemoglobin by the enzyme was about 10-fold higher than anion-exchange resin-treated hemoglobin and 5–6-fold higher than nitrite-treated hemoglobin. No reduction of cyanomethemoglobin was observed. These facts suggest some kind of anion effect on the rate of reduction of methemoglobin.

Hematin was reduced more rapidly than anion-exchange resin-treated hemoglobin. It has also been observed that dyes activate methemoglobin reduction but no physiological activation factor has yet been found *in vivo*. These facts may suggest that conformational or configurational factors are involved in the reduction of methemoglobin.

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