NADPH-FLAVIN REDUCTASE IN HUMAN ERYTHROCYTES AND THE REDUCTION OF METHEMOGLOBIN THROUGH FLAVIN BY THE ENZYME

Toshitsugu Yubisui, Takasumi Matsuki, Kiyoo Tanishima*, Masazumi Takeshita*, and Yoshimasa Yoneyama

Department of Biochemistry, School of Medicine, and *Department of Medical Technology, School of Paramedicine, Kanazawa University, Kanazawa, Japan

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SUMMARY

A NADPH-dehydrogenase of human erythrocytes was exhaustively purified to a homogeneous protein judging from the electrophoresis on a polyacrylamide gel in the presence of sodium dodecyl sulfate. Studies on the specificity for the electron acceptor of this enzyme suggest that flavins serve as the natural and direct electron acceptor. The enzyme showed a broad specificty for flavins and the Michaelis constants for flavins were estimated to be 5×10^{-5} M for both FMN and riboflavin. Rapid reduction of methemoglobin by the enzyme in the presence of flavin was demonstrated, and the reduction was explained by the reduction of flavin by the enzyme, and subsequent non-enzymatic reduction of methemoglobin by the reduced flavin. The therapeutic significance of flavins was discussed with reference to the flavin reductase activity in hereditary methemoglobinemia.

INTRODUCTION

During the studies on methemoglobin reductases we found that a NADPH-dehydrogenase of human erythrocytes is well stabilized by flavin and that the purified enzyme can reduce methemoglobin rapidly in the presence of flavin (1). The flavin can be replaced by methylene blue to reduce methemoglobin by the enzyme. Further studies revealed that the enzyme we purified has flavin reductase activity other than the diaphorase activity or the methemoglobin reductase activity via methylene blue. Removal of flavin from the buffer during purification, however, resulted in an enzyme without the flavin redcuctase activity. The lability of the flavin reductase activity of the enzyme may be a major reason why many workers have failed to observe the physiological enzyme activity (2,3). In this communication we report some properties of the enzyme which apparently characterized it as the flavin reductase. We also discuss the role of flavins in erythrocytes in relation to the reduction of

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methemoglobin in hereditary methemoglobinemia.

MATERIALS AND METHODS

Normal human red cells were obtained from the central laboratory of the Tokyo Red Cross Blood Transfusion Service. β-NADH, cytochrome c, FAD and FMN were obtained from Boehringer Manheim, NADPH from Sigma Chemicals Co., and other reagents from commercial sources. Superoxide dismutase purified from cow blood according to the method of McCord and Fridovich (4) was kindly supplied by Dr. A. Tomoda in our laboratory. Frog biopterin, cow adrenodoxin, and spinach ferredoxin were also generously presented by Dr. M. Akino of Tokyo Metropolitan University, by Dr. E. Itagaki and Dr. K. Suhara of Kanazawa University, and by Dr. M. Shin of Kobe Yamate Women's College, respectively.

Spectrophotometric determinations were performed with a Hitachi spectrophotometer model 124 fitted with a recorder model 056. NAD(P)H-diaphorase activity was determined as described by Sugita et al. (5) in 2.0 ml of 50 mM phosphate buffer (pH 7.5). NAD(P)H-flavin reductase activity during the purification was determined by following the reduction of methemoglobin in the presence of flavin at 576 nm. Two ml of reaction mixture contained 100 μmoles phosphate buffer (pH 7.5), 200 nmoles NAD(P)H, 200 nmoles flavin, 1 μmole EDTA, 100 nmoles methemoglobin and an appropriate amount of enzyme. The reaction was started by the addition of enzyme and performed at 23-25°. The enzyme activity was calculated using a millimolar extinction coefficient of 11.9 at 576 nm.

Purification of enzyme --- NADPH-dehydrogenase was purified from human erythrocytes by the modified method of previous workers (5-8). Red cells were centrifuged at 1,500 X g for 5 min to remove plasma proteins and buffy coats, and then washed with isotonic saline at least three times. The washed cells were hemolyzed by adding 3 volumes of 20 μM FMN, and stroma was removed by high speed centrifugation. To the supernatant an additional 1.5 volumes of 20 μM FMN was added, and the hemolysates thus prepared were applied on a DEAE-cellulose column which had been equilibrated with 2 mM phosphate buffer (pH 7.0) containing 20 μM FMN. NADPH-methemoglobin reductase fraction eluted from the column by 100 mM phosphate buffer (pH 7.0) containing 20 μM FMN were combined, concentrated on a PM 10 Diaflo membrane, and applied on a Ultrogel AcA 54 column which had been equlibrated with 50 mM phosphate buffer (pH 7.5) containing 20 μM FMN and 1 mM EDTA. The NADPH-methemoglobin reductase fractions eluted from the column were pooled, concentrated as described above, and dialyzed against 10 mM phosphate buffer (pH 7.5) containing 20 μM FMN and 1 mM EDTA. The dialyzed enzyme was applied on a DE-32 column which had been equilibrated with the same buffer used for the dialysis. The NADPH-methemoglobin reductase was eluted from the column during the wash with the equilibrating buffer, while NADH-cytochrome b_5 reductase was eluted by the linear gradient of phosphate. The NADPH-enzyme was fractionated with ammonium sulfate, and precipitates appearing between 40 to 70% saturation were collected. Precipitates were dissolved in a minimum volume of buffer, and again applied on the Ultrogel column as described above. The NADPH-enzyme was concentrated on a PM 10 Diaflo membrane and dialyzed against 10 mM phosphate buffer (pH 6.8) containing 20 μM FMN and 1 mM EDTA. The dialyzed enzyme was applied on a CM-32 column which had been equilibrated with the same buffer used for the dialysis. By washing the column with the equlibrating buffer almost all the colored protein was eluted and the NADPH-enzyme was eluted by the linear gradient of phosphate from 10 to 100 mM (pH 6.8). The enzyme was concentrated on a membrane and again treated with Ultrogel, and then further purified by electrophresis with carrier ampholite of pH range from 7-9. The electrophoresis was performed at 4°-6° for 38-40 hrs. The enzyme was concentrated and the carrier ampholite was removed by gel filtration. Protein was determined by the method of Lowry et al. (9).

Table I

Summary of purification

Fractions	Total (a) activity	Protein (g)	Specific (b) activity	Yield (%)
1 Hemolysates	380	4070	0.093	100
2 DEAE-cellulose	116	46.3	2.51	30.5
3 Ultrogel AcA 54	73.6	10.7	6.88	19.4
4 DE-32	65.5	8.2	8.0	17.2
5 $(NH_4)_2SO_4$ (40-70%)	63.0	4.3	14.7	16.6
6 Ultrogel AcA 54	58.1	2.63	24.6	15.3
7 CM-32	16.2	0.055	296	4.3
8 Ultrogel AcA 54	11.8	0.035	333	3.1
9 Electrofocusing	6.03	0.021	281.7	1.6

a) Total activity; μmoles hemoglobin reduced / min.
b) Specific activity; nmoles hemoglobin reduced / min / mg.

The reduction of methemoglobin was followed at 576 nm in the presence of 100 μM FMN in the assay mixture as described in "METHODS".

RESULTS

Table I shows the summary of purification of a NADPH-dehydrogenase from human erythrocytes. The enzyme was purified 3,500-fold with respect to the NADPH-methemoglobin reductase in the presence of flavin, and had a specific activity of 330 nmoles methemoglobin reduced/min/mg. The ratio of NADPH to NADH-flavin reductase activity by the final enzyme was 4.5-5.0, while that of diaphorase activity was close to unity. Purity of the final enzyme was examined by electrophoresis on a polyacrylamide gel in the presence of sodium dodecyl sulfate (10), and only one distinct band was observed on the gel as shown in Figure 1. The enzyme purified in the presence of FMN had a characteristic absorption spectrum of a flavoprotein showing absorption maxima at 443, 370, and 276 nm; however, the flavin in the enzyme was easily removed by gel filtration or by electrophoresis. The resulting enzyme had an absorption maximum only at 276 nm, and the absorbance at 443 and 370 nm decreased to about 1% of those of untreated enzyme. The yield of the flavin reductase activity after electrophoresis was 50%, and after gel filtration more than 100%, respec-

Figure 1. Polyacrylamide gel electrophoresis of the flavin reductase in the presence of sodium dodecyl sulfate. Fifteen and 30 μg of the purified enzyme was applied on the left and right gels, respectively. The electrophoresis and staining of protein were performed according to the method of Weber and Osborn (10).

tively. Figure 2 shows the effects of three flavins (riboflavin, FMN and FAD), and methylene blue on the reduction of methemoglobin by the enzyme. The reduction of methemoglobin by the enzyme in the absence of an electron acrrier was only barely detectable (curve a); however, flavins as well as methylene blue greatly stimulated the reduction of methemoglobin (curves b-e). Boiled enzyme had no activity, and the NADH-cytochrome b_5 reductase was also inactive on the reduction of methemoglobin through flavin. The initial rates of methemoglobin reduction through 50 μM riboflavin or FMN were almost the same, and that through FAD was about half of these, while methylene blue showed more than two times higher activity at 0.5 μM than those through flavins. Table II

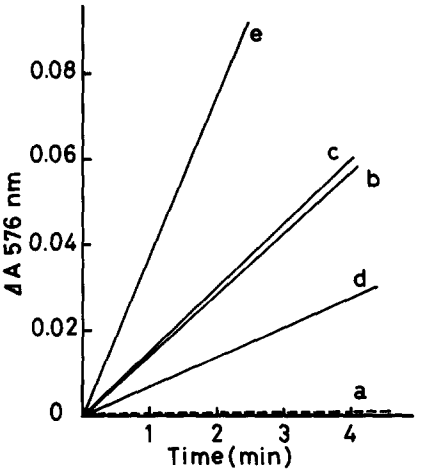

Figure 2. Reduction of methemoglobin by the flavin reductase through flavin and methylene blue. Reduction of methemoglobin by the flavin reductase was followed spectrophotometrically at 576 nm as described in "METHODS". Curve a) control (no addition of flavin or methylene blue), b) 100 nmoles FMN added, c) 100 nmoles riboflavin added, d) 100 nmoles FAD added, e) 1 nmole methylene blue added.

shows the specificity for electron acceptor of this enzyme with NADPH as an electron donor. Among the various natural substances examined, only flavins were found to serve as the efficacious electron acceptors, but hemoproteins (methemoglobin, cytochrome $\underline{b}_5$ and $\underline{c}$), nonheme iron proteins (adrenodoxin and ferredoxin), hemin, GSSG, and menadione were all inactive as an direct electron acceptor. Artificial dyes such as methylene blue or 2,6-dichlorophenolindophenol (DCIP) served as a good acceptor. Km values for flavins and NADPH of the enzyme were estimated to be about 50 μM for both riboflavin and FMN, 120 μM for FAD, and 2 μM for NADPH. The Km value for DCIP was also estimated to be about 4 μM. As the direct assay of the reduction of flavin is troublesome because the reduced flavin is quickly reoxidized by air, the flavin reductase activity was determined conveniently by following the reduction of methemoglobin in the presence of flavin. The reduction of flavin, however, was directly observed anaerobically as shown in Figure 3. Figure 3 shows the change of the absorption spectra of FMN during the reduction by the enzyme with NADPH

Table II

Specificity of the NADPH-dehydrogenase of Human Erythrocytes for Various Electron Acceptors.

Electron acceptors	Concentration (μM)	(ΔA 340 nm/min) x 5
None	-	(0)
Methemoglobin	20	(0)
Cytochrome b_5	20	(0)
Cytochrome c	20	(0)
Biopterin	20	(0)
Adrenodoxin	20	(0)
Ferredoxin	20	(0)
Hemin	20	(0), 0.002
FMN	50	0.081, 0.079
FAD	50	0.037, 0.034
Riboflavin	50	0.060, 0.051
GSSG	50	0.0008, 0.0015
Menadione	50	0.002, 0.004
Methylene blue	50	0.132, 0.115
2,6-Dichlorophenolindophenol	50	0.716, 0.720
Ferricyanide	50	0.0096, 0.003

The enzyme activity was determined by following the absorbance of NADPH at 340 nm with the electron acceptor given in the Table. For weak activity the initial rate was followed by using the expanded scale of the recorder. The absorbance change "(0)" means "no detectable change in value" within more than 7-8 min after the reaction. The enzyme (72 μg protein) of another preparation from that described in Table I was used for the assay. The specific activity was 35.5 nmoles/min/mg with FMN as an acceptor, and 194.4 nmoles/min/mg with 2,6-dichlorophenolindophenol as an acceptor.

as an electron donor. The rate of reduction was dependent on the amount of enzyme added to the mixture. These results described above apparently suggest that flavin serves as a natural and direct electron acceptor of this enzyme rather than as a tightly bound cofactor. Atebrin at 0.5 mM strongly inhibited (80% inhibition) the reduction of methemoglobin through flavin by the enzyme. Superoxide dismutase did not affect the reduction of methemoglobin through flavin by the enzyme. This result suggests that O_2^- which may be derived from the reaction of flavin with NADPH is not involved in the reduction of methemoglobin by the enzyme.

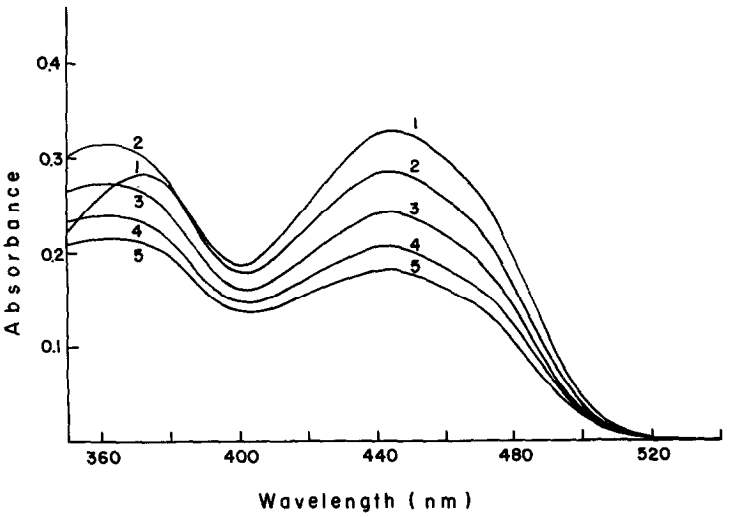

Figure 3. The time change of the absorption spectra of FMN during reduction by the flavin reductase. The reduction of FMN by the enzyme with NADPH as an electron donor was followed under anaerobic conditions. A Thunberger's tube whose main compartment contained 28 μM FMN in 1.95 ml of 50 mM phosphate buffer (pH 7.5) and 106 μg of the purified enzyme, and the side arm compartment which contained 0.05 ml NADPH was sealed with vacuum grease. The tube was evacuated carefully and the gas phase was exchanged with Q-gas (helium-isobutane (99.05 : 0.95)) at least three times. After mixing the NADPH with the reaction mixture in the cell, the reduction of FMN was followed at an appropriate interval of time as indicated. 1) Before mixing, 2) 5 min, 3) 15 min, 4) 30 min, 5) 60 min after mixing.

DISCUSSION

Many controversial results have been reported on both NADH- and NADPH-methemoglobin reductase as to whether flavin is a constituent of the enzyme(s), or whether free flavin is involved in the reduction of methemoglobin (2, 3), and those problems are not yet settled. The enzyme purified in the present studies was demonstrated to have the flavin reductase activity other than the diaphorase activity or the methemoglobin reductase activity via methylene blue. However, the flavin reductase activity was observed only with the "NADPH-dehydrogenase" whereas NADH-cytochrome $\underline{b}_5$ reductase of human erythrocytes (5-8) had no flavin reductase activity, although both the enzymes have strong diaphorase activity. It is very likely that our enzyme, which seems to correspond to the "NADPH-dehydrogenase", does not contain flavin as a cofactor, but that free flavin is the substrate of the enzyme, judging from the results of spectral and

kinetic studies. The flavin reduced by the enzyme can in turn reduce methemoglobin non-enzymatically, and the rate was found to be fast ($t_{1/2}$ = about 15 msec)[a]. The sequence of the electron transfer from NADPH to methemoglobin through flavin can be explained as follows;

NADPH ---→NADPH-flavin reductase ---→Flavin ---→Methemoglobin.

On the other hand, great interest lies in the fact that flavin reductases of some luminous bacteria (9-12), which provide the reduced flavin to their luciferase as a substrate, have closely similar properties to our enzyme with respect to the broad specificity and high Km values for flavins, and to their molecular weights. The molecular weight of our enzyme was estimated to be about 22,000 by disc electrophoresis in the presence of sodium dodecyl sulfate, and bacterial enzymes have molecular weights of about 23,000 (9-12).

Our finding that flavins, the components in erythrocytes, are involved in the overall reduction of methemoglobin by our enzyme suggests that there is a possibility that the enzyme may function depending on the concentrations of flavins in erythrocytes. Although the concentrations of flavins in erythrocytes seem to be low (13), the enzyme is expected to be activated by flavins if they are enriched in erythrocytes by some method. To the patients with herediatary methemoglobinemia, whose erythrocytes lack the NADH-cytochrome $\underline{b}_5$ reductase, the major methemoglobin reductase, methylene blue has been administered as one of the therapeutic agents to activate the NADPH-dehydrogenase. The agent, however, although greatly effective, sometimes produces side-effects in the patients. The administration of riboflavin, the permeable form of flavin through the erythrocyte membrane, to such patients will be better than methylene blue in the sense that flavin is a natural component in erythrocytes and harmless for the patients.

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a) T. Yubisui, unpublished result.

REFERENCES

1) Yubisui, T., Okamoto, H., Tanishima, K., Takeshita, M., and Yoneyama, Y. (1976) The 16th Ineternational Congress of Hematology, Abst., 85, Kyoto.
2) Jaffe, E.R. (1976) Cellular and Molecular Biology of Erythrocytes (Yoshikawa, H., and Rapaport, S., ed.) pp. 185-216, University of Tokyo Press, Tokyo.
3) Kiese, M. (1974) Methemoglobinemia: A Comprehensive Treatise, pp. 14-25. CRC Press, Cleveland, Ohio.
4) MacCord, J.M., and Fridovich, A.M. (1976) J. Biol. Chem., 244, 6049-6055.
5) Sugita, Y., Nomura, S., and Yoneyama, Y. (1971) J. Biol. Chem., 246, 6072-6078.
6) Passon, P.G., and Hultquist, D.E. (1972) Biochim. Biophys. Acta, 275, 62-73.
7) Kuma, F., and Inomata, H. (1972) J. Biol. Chem., 247, 556-560.
8) Kitao, T., Sugita, Y., Yoneyama, Y., and Hattori, K. (1974) Blood, 44, 879-884.
9) Lowty, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem., 193, 265-275.
10) Weber, K., and Osborn, M. (1976) J. Biol. Chem., 244, 4406-4412.
11) Puget, K., and Michelson, A.M. (1972) Biochimie, 54, 1197-1204.
12) Gerlo, E., and Charlier, J. (1975) Eur. J. Biochem., 57, 461-467.
13) Duane, W., and Hasting, J.W. (1975) Mol. Cell. Biochem., 6, 53-64.
14) Watanabe, H., Miura, N., Takimoto, A., and Nakamura, T. (1975) J. Biochem., 77, 1147-1155.
15) Harris, J.W., and Kellermeyer, R.W. (1972) The Red Cell (Harris, J.W., and Kellermeyer, R.W. ed.) pp.281-333, Harvard University Press, Cambridge, Massachusetts.