Methemoglobin Reductase (Cytochrome b5 Reductase) Deficiency in Congenital Methemoglobinemia

By Takeshi Kitao, Yoshiki Sugita, Yoshimasa Yoneyama, and Kenichi Hattori

Two NADH diaphorases, diaphorase I and II, were isolated from normal red cells and congenital methemoglobinemic red cells by CM-cellulose and DE 32 column chromatography. For methemoglobinemic sample, activities of diaphorase I and diaphorase II were 80% and less than 5% of those for the normal red cells, respectively. Only diaphorase II showed cytochrome b5 reductase activity. The cytochrome b5 reductase deficiency seems to manifest methemoglobinemia through the decrease in the enzymatic reduction of cytochrome b5 and subsequent nonenzymatic reduction of methemoglobin by the reduced cytochrome b5. The methemoglobinemic diaphorase II was found similar to the normal enzyme with respect to Kₘ for the dye and NADH, heat stability, effect of pH, and electrophoretic pattern. The ratio of the diaphorase activity to the cytochrome b5 reductase activity was the same for both enzymes. Although the production of an abnormal enzyme molecule can not be excluded, it is possible that in this case the rate of enzyme formation is decreased.

CONGENITAL METHEMOGLOBINEMIA usually results from deficiency of a red cell enzyme activity that reduces methemoglobin to hemoglobin. Scott demonstrated first that patients with this condition were deficient in red cell NADH diaphorase activity. There are a few reports that cytochrome b5 was a very effective electron acceptor for NADH diaphorase in red cells, and rapid reduction of methemoglobin in vitro was observed in the presence of cytochrome b5, a phenomenon which was explained by enzymatic reduction of cytochrome b5 and subsequent nonenzymatic reduction of methemoglobin by the reduced cytochrome b5. The NADH diaphorase studied by Scott might be identical to the cytochrome b5 reductase. This paper describes the parallel fractionation of NADH diaphorases from normal and methemoglobinemic red cells by chromatographic methods and the comparative studies of the properties of the enzymes.

MATERIALS AND METHODS

Blood specimens obtained from the patient and normal subjects were anticoagulated with heparin. Cytochrome b5 was purified from rabbit liver according to the method of Omura and Takesue. The NADH diaphorase activity was assayed at 600 nm with a Cary model 14 recording photometer. A millimolar extinction coefficient of 20.1 mM⁻¹ cm⁻¹ for the oxidized 2,6-dichlorophenolindophenol was used in calculation. The assay system contained 50 μmoles of Tris-HCl buffer, pH 7.4, 0.2 μmole of 2,6-dichlorophenolindophenol, 1 μmole of EDTA, 0.4 μmole of NADH, and enzyme in a total volume of 3 ml. The reaction was started by the addition of NADH. The assay for cytochrome b5 reductase was measured at 556 nm with a Cary recording photometer and calculated with 17.8 mM⁻¹ cm⁻¹ for the difference in millimolar extinction coefficient between the oxidized and reduced cytochrome b5. The standard assay mixture contained 35 μmoles of phosphate buffer, pH 7.0, 0.1 μmole of cytochrome b5, 1.3 μmole of...
EDTA, 0.3 μ mole of NADH, and enzyme in a total volume of 2.1 ml. The reaction was started by the addition of NADH. Acrylamide disc gel electrophoresis was carried out at pH 8.3 according to the method of Davis7 and stained for the diaphorase activity according to the method of Kaplan.8

CASE REPORT

The propositus, T.M., a Japanese woman aged 36, was admitted to our hospital because of convulsion and paresis. Cyanosis of lips, nail beds, conjunctivas, and oral mucosa was noted. Except for cyanosis, physical examination including neurologic evaluation revealed no abnormalities. The patient was of a low mentality that was possibly compatible with her cultural deprivations. The EEG findings revealed frequent slow waves appearing in the left temporal region. The concentration of methemoglobin in her blood was 33%. Hemoglobin electrophoresis was normal. The activity of red cell NADH diaphorase was about 1 of normal red cells; this will be discussed later in "Discussion" section. The administration of ascorbic acid maintained the methemoglobin concentration between 4% and 10%. The patient's mother, sister, and her two children enjoyed good health, were not cyanotic, and showed no evidence of mental retardation. The father of propositus could not be examined because of his early death. The pedigree of the family is shown in Fig. 1.

RESULTS

Diaphorases were separated by the method of Kuma et al.9 The hemoglobin was oxidized to methemoglobin in red cells by adding 5 equivalents of solid sodium nitrite to the whole blood and the cells were washed four times with 0.9% saline. The packed cells were hemolyzed by adding an equal volume of cold distilled water. The hemolysate was shaken with an equal volume of cold ethyl ether and was centrifuged at 6000 rpm for 10 min to separate the stroma. The hemolysate was dialyzed against 0.01 M sodium phosphate buffer, pH 6.8, and applied to a CM-cellulose column equilibrated with the same buffer. Elution was performed with 0.05 M sodium phosphate buffer, pH 6.0. While hemoglobin was adsorbed at the top of the column under these conditions, yellow solution of nonhemoglobin proteins was eluted.

The concentrated nonhemoglobin proteins from the CM-cellulose column was dialyzed against 0.01 M sodium phosphate buffer, pH 7.5, and applied to the DE 32 column which had been equilibrated with the same buffer. A linear gradient elution was performed from 0.01 to 0.1 M phosphate buffer, pH 7.5.

Elution pattern with the preparation from 60 ml of normal red cells is shown in Fig. 2. Two peaks of NADH diaphorase activity were separated and called here diaphorase I and II.
Fig. 2. Fractionation of NADH-diaphorases by DE 32 column chromatography. A column (1.5 x 15 cm) was developed with a linear gradient device from 0.01 to 0.1 M of sodium phosphate buffer pH 7.5 with 250 ml of each buffer, and 10-ml fractions were collected. Samples from 60 ml of methemoglobinemic red cells and 60 ml of normal red cells were applied. Activity was assayed as described in the text (o-----o), and absorbance measured at 280 nm (- - - - - ).

The elution profile starting from 60 ml of methemoglobinemic red cells is shown in Fig. 2. For methemoglobinemic samples, activities of diaphorase I and II were 80% and less than 5% of those for the normal red cells, respectively. Fractions of methemoglobinemic diaphorase II were combined and concentrated. Both the diaphorase II preparations, from normal and methemoglobinemic red cells, showed cytochrome b₅ reductase activity with NADH as an electron donor, but the diaphorase I preparations, normal and methemoglobinemic, showed no cytochrome b₅ reductase activity. The ratio of diaphorase activity to cytochrome b₅ reductase activity, assayed as described in “Materials and Methods,” was 0.167 for normal diaphorase II and 0.172 for the methemoglobinemic enzyme.

The Km for 2,6-dichlorophenolindophenol and NADH with diaphorase I were 1.66 x 10⁻⁵ M and 9.1 x 10⁻⁵ M, respectively, for both of the normal and the methemoglobinemic preparations. The Km for the dye and NADH with diaphorase II were 6.7 x 10⁻⁵ M and 3.3 x 10⁻⁶ M, respectively, for both of these preparations. The heat stability (Fig. 3) and the effect of pH on enzyme activity of diaphorase I and II were quite identical for the normal and
Fig. 4. Acrylamide disc gel electrophoretic patterns of diaphorase II fraction from the normal (left) and methemoglobinemic subject (right). Electrophoresis was performed at pH 8.3 and the diaphorase activity was detected by staining with 3-(4,4-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT).

the methemoglobinemic preparations. As shown in Fig. 4 no difference was seen in the acrylamide disc gel electrophoretic patterns of diaphorase II between the normal and methemoglobinemic, although the methemoglobinemic sample, which contained only the weak activity and highly concentrated for the electrophoresis, showed a faint band of activity above the major band, which may be due to the small contamination of diaphorase I.

DISCUSSION

Of the human erythrocyte enzymes with reduced pyridine nucleotide dehydrogenase activity, the major NADH dehydrogenase isolated by Scott is particularly interesting because of its absence from erythrocytes of congenital methemoglobinemic patient. Although catalysis by this enzyme of the direct reaction between pyridine nucleotide and methemoglobin was very slow, this rate was presumed to be sufficient to satisfy the normal needs of the red cells. Sugita et al. obtained highly purified reduced pyridine nucleotide dehydrogenase from human erythrocytes, and the enzyme was considered to be the same as Scott's dehydrogenase I. The maximum methemoglobin reductase activity of their enzyme preparation was about 0.3% of its maximum diaphorase activity, and direct methemoglobin reduction by the enzyme can account for only 9% of the rate of methemoglobin reduction in nitrite-treated erythrocytes in which about 5% of total hemoglobin is reduced per hour.

Various hemoproteins were tested as electron acceptor for NADH, and cytochrome b5 was the most effective; therefore the enzyme was defined as a cytochrome b5 reductase. Passon et al. also purified cytochrome b5 reductase from human erythrocytes, and its properties suggested that their enzyme was
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the same as NADH dehydrogenase I isolated by Scott et al.5 Whereas the erythrocyte cytochrome b5 reductase catalyzed a very slow reaction between NADH and methemoglobin, the reaction was relatively rapid when erythrocyte cytochrome b5 was added, and it was reported that the methemoglobin reduction in the presence of cytochrome b5 was approximately the same as the rate of cytochrome b5 reduction.3

In our case, the activity of red cell NADH diaphorase was 17% of normal cells; this might be inconsistent with the very low value reported by Scott2 for homozygous deficiency. Scott later showed that there were four kinds of diaphorases in human red cells,5 but the effect of genetic defect on each of these enzymes was not clear. Kuma et al.6 reported the simple isolation method of two NADH-dependent diaphorases, diaphorase A and C, by CM-cellulose and DEAE-Sephadex column chromatography. They showed that the diaphorase A activity in the methemoglobinemic red cells was less than 3% of the normal, while diaphorase C activity was the same as that in the normal erythrocytes, the total diaphorase activity of the patient’s red cells being about one-fourth of normal. Kawakita et al.13 studied 15 cases of the homozygous methemoglobinemia in Okinawa Island in Japan, and the average NADH diaphorase activity was 7.0 U (range, 2.4–11.6) against the normal average of 51.4 U. Our case is considered to belong to those which show low values of diaphorase activity.

Our results that only diaphorase II has cytochrome b5 reductase activity and that only this enzyme activity is deficient in our patient strongly support the hypothesis that cytochrome b5 reductase plays a major role in physiologic methemoglobin reduction in human erythrocytes. The plausible electron transfer reactions are summarized as the following sequence.

\[ \text{NADH} \rightarrow \text{cytochrome b}_5 \text{ reductase} \rightarrow \text{cytochrome b}_5 \rightarrow \text{methemoglobin} \]

Various abnormal electrophoretic mobility of residual methemoglobin reductase are reported by several investigators,8,14-16 suggesting the heterogeneity of the enzyme defect. Schwartz et al.17 reported that the deficient enzyme activity in a Puerto Rican variant was due to its instability. The methemoglobinemic enzyme we have studied, however, was similar to the normal one in all the properties so far tested. Catalytic properties, namely the Km for the dye and NADH, effect of pH, and the ratio of diaphorase activity to cytochrome b5 reductase activity, are all exactly the same for both enzymes. Heat stability and electrophoretic property did not indicate a difference between the two enzymes. Although the possibility of silent mutation of amino acids cannot be excluded, these results suggest that the low enzyme activity may be due to the decrease in the amount of enzyme protein rather than to the production of a mutant enzyme protein.

Cytochrome b5 exists especially in high amount in the endoplasmic reticulum of mammalian liver cells. Cytochrome b5 reductase from erythrocyte is distinct from that obtained from other tissues in that the former is a soluble enzyme and does not contain flavin as a coenzyme,5 whereas the latter is a particulate flavoprotein enzyme.18 It would be interesting to investigate whether liver cytochrome b5 reductase is deficient in the congenital methemoglobinemia.
REFERENCES

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