The Reduced Pyridine Nucleotide Dehydrogenases of Human Erythrocytes

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Kiese (1) reported the presence of an enzyme in erythrocytes that would catalyze the reduction of methemoglobin by reduced triphosphopyridine nucleotide in the presence of methylene blue. This enzyme was purified further by Huennekens et al. (2) and reported to be active both with TPNH and, to a lesser extent, with DPNH. It was postulated that this enzyme was responsible for the physiological maintenance of hemoglobin in the red cell in the reduced state, on the assumption that some unknown electron carrier was present in red cells that could act in the same manner as methylene blue does in the assay system. Shrago and Falcone (3) purified this TPNH dehydrogenase further and reported it to be essentially free of heme protein.

A DPNH dehydrogenase with methemoglobin reductase activity has been isolated from normal human red cells (4). This enzyme is lacking in red cells of persons with hereditary methemoglobinemia (5). A second DPNH dehydrogenase, prepared from methemoglobinemic red cells, has quite different properties from the DPNH dehydrogenase of normal cells, as well as being present in lesser amount (6).

Evidence will be presented here that there are four reduced pyridine nucleotide dehydrogenases in human red cells: TPNH dehydrogenase A, TPNH dehydrogenase B, DPNH dehydrogenase I, and DPNH dehydrogenase II. The two TPNH dehydrogenases resemble each other closely, but are separable by chromatography. DPNH dehydrogenase I is the major "diaphorase" of human red cells previously described (4), while DPNH dehydrogenase II is a minor component previously purified from methemoglobinemic cells (6).

Described here are the preparation of two TPNH dehydrogenases from normal human red cells. Certain samples of these preparations contained considerable DPNH dehydrogenase II activity. The properties of these four dehydrogenases were compared and their possible role in methemoglobin reduction was estimated.

EXPERIMENTAL PROCEDURE

**Enzyme Assay**—TPNH dehydrogenase was determined at 30° in 3 ml of solution containing 0.06 μmole of 2,6-dichlorophenolindophenol, 50 μmole of Tris-HCl, pH 7.55, 1 μmole of EDTA, 500 μmole of KCl, 0.2 μmole of TPNH, and enzyme. Absorbance at 600 mμ was measured for 10 minutes. Under these conditions the rate of reaction was nearly zero order with respect to dye concentration. Rate of reaction was defined as the initial reaction rate in absorbance change per minute minus the reaction rate found for the same system without enzyme. A unit of activity was that amount of enzyme calculated to give a change in absorbance of 1 unit per minute; specific activity was units of enzyme per mg of protein.

DPNH dehydrogenase activity was determined in the same way except that KCl was not added to the system. Methemoglobin reduction was determined in 0.15 ml of solution at 38° containing 36 μg of methemoglobin, 20 μmole of potassium phosphate, pH 7.35, 2 μmole of TPNH or DPNH, and enzyme. Aliquots of 0.02 ml were withdrawn at 20-minute intervals, and methemoglobin was determined by the method of Evelyn and Malloy (7). Cytochrome c reduction was determined by measuring absorbance at 550 mμ in 3 ml of solution containing 0.12 μmole of cytochrome c, 50 μmole of Tris-HCl (pH 7.55), 500 μmole of KCl, 1 μmole of EDTA, 0.2 μmole of TPNH, and enzyme at 30°.

Purified DPNH dehydrogenase I was prepared as previously described (4). Protein was determined spectrophotometrically (8), with a correction applied for heme proteins (4). Flavin was determined by fluorophotometry (9). Methemoglobin was crystallized from human red cells after treatment with nitrite.

**Purification of Enzyme**—Cells from outdated blood from a blood bank were washed three times with an equal volume of buffered sodium chloride solution (1 volume of 0.1 M potassium phosphate, pH 7.4, plus 9 volumes of 0.9% NaCl) and were frozen until used. All subsequent operations except chromatography were carried out at 5°. All Tris-HCl buffers used were 0.01 M. Methemoglobin was determined by fluorophotometry (9). Methemoglobin was crystallized from human red cells after treatment with nitrite.

To 2 liters of washed cells were added 38 liters of water. The solution was adjusted to pH 7.4 with 2 M Tris, and 11.2 kg of (NH₄)₂SO₄ were added. The solution was passed through a Sharples centrifuge and the residue in the bowl was discarded. Then 4.2 kg of (NH₄)₂SO₄ were added to the solution, and it was again passed through the Sharples centrifuge. The residue in the bowl was dissolved in 350 ml of 0.1 M Tris-HCl. This solution was centrifuged at 30,000 × g, and the precipitate was discarded; the supernatant liquid was designated Fraction 2.

Fraction 2 was dialyzed for 5 hours in 2 liters of 0.01 M Tris-HCl; this dialysis was repeated for a second 5-hour period in fresh 0.01 M Tris-HCl. Protamine sulfate (200 ml of a 1.0% solution) was added, and the solution was diluted to 1500 ml. The precipitate was removed by centrifugation and was discarded. To the solution were added 120 g of (NH₄)₂SO₄; after centrifugation, the precipitate was discarded. Then 157 g of (NH₄)₂SO₄ were added, and the solution was centrifuged. The precipitate was dissolved in 20 ml of 0.1 M Tris-HCl to give Fraction 3.

Fraction 3 was dialyzed for 5 hours in 1 liter of 0.01 M Tris-HCl.
HCl, and the chromatography was repeated on a Ca₃(PO₄)₂ gel column, 0.5 cm in diameter and 5 cm deep. To the column were added successively 1 ml of 0.01 M Tris-HCl to give Fraction 4A.

The second TPNH dehydrogenase was treated similarly to give Fractions of 2 ml each were collected. Those fractions with specific activity greater than 5 were combined, and the enzyme was precipitated between 55 and 70% saturation with (NH₄)₂SO₄. The precipitate was dissolved in 1 ml of 0.1 M Tris-HCl to give Fraction 5B. The purification procedure is summarized in Table I.

**RESULTS**

**Differentiation of Dehydrogenases**—The two TPNH dehydrogenases were closely similar in properties except for the difference in chromatographic behavior. To test the possibility that the separation might be the result of overloading the column, two columns were prepared and half as much Fraction 3 was added to one as to the other. The recovery of enzyme from 1 volume of Fraction 3 was 11.6 units of dehydrogenase A and 23.0 units of dehydrogenase B, and from 0.5 volume of Fraction 3, 5.7 units of dehydrogenase A and 16.0 units of dehydrogenase B.

The two DPNH dehydrogenases differed not only in chromatographic behavior but also in kinetic properties. The effects of pH, temperature, and substrate concentration on the two enzymes were quite different. The ratios of activity with deaminated DPNH to that with DPNH were: DPNH dehydrogenase I, 0.97, and DPNH dehydrogenase II, 0.15. The activity of purified DPNH dehydrogenase I with TPNH was only 1% of that with DPNH (4). We were not able to prepare DPNH dehydrogenase II that was similarly free of TPNH activity, although TPNH dehydrogenase B fractions were obtained which had a ratio of activity with DPNH to that with TPNH of 0.01. This ratio in various TPNH dehydrogenase B preparations varied from 0.01 to 0.60. The data on DPNH dehydrogenase II described below were obtained on an enzyme preparation (Fraction 5B) with a DPNH/TPNH ratio of 0.48.

Both TPNH dehydrogenase fractions were red and both absorbed strongly at 410 mm. From the ratio of absorbance at 410 and 280 mm, 12% of the protein of dehydrogenase A and 47% of the protein in dehydrogenase B were calculated to be heme protein. TPNH dehydrogenase A contained no more than 1 mole of flavin per 6.8 × 10⁶ g of protein. TPNH dehydrogenase B contained 15 times this amount of flavin, but this could be ascribed to a small amount of glutathione reducetase which was present (11). Enzyme prepared by the method of Huennemken et al. (2) was similar in properties to dehydrogenase B.

**Specificity**—None of the four enzymes had dehydrogenase activity with any of the following substrates: dihydroxyacetone, glucose 6-phosphate, 6-phosphogluconate, lactate, malate, or glyceraldehyde 3-phosphate. All four dehydrogenases were good menadione reductases, but this activity was in no case ascribed to a small amount of glutathione reducetase which was present. The ratio of activity with deaminated DPNH to that with DPNH was not constant.

**Effect of pH**—The effect of pH on the B enzyme is shown in Fig. 1. The A enzyme gave results indistinguishable from those shown. The ratio of activity with DPNH compared to that with TPNH was not constant. Below pH 6.5 the enzymes were unstable, and the A enzyme was less stable than the B enzyme. TPNH dehydrogenase activity was lower when Tris buffers were used instead of phosphate.
Effect of Temperature—The effect of temperature on the B enzyme is shown in Fig. 2. The A enzyme gave similar results. DPNH dehydrogenase II activity showed little dependence on temperature with an apparent activation energy of 2700 cal per mole as compared with 5100 cal per mole for DPNH dehydrogenase I (4). The apparent activation energy of the TPNH reaction was 6900 cal per mole. Above 30°C, the enzyme was inactivated. High ionic strength and the presence of the reduced nucleotides tended to minimize inactivation. DPNH dehydrogenase activity was more stable to heat than was TPNH dehydrogenase activity.

Effect of Substrate Concentration—Change in concentration of either TPNH or dye in the ranges studied had little effect on the reaction rate of dehydrogenase B (Fig. 3). The data in Fig. 3B were fitted to the equation (12)

$$
\frac{1}{V} = \frac{1}{V_{\text{max}}} + \frac{K_{m}^{P}}{V_{\text{max}} [\text{TPNH}]} + \frac{K_{m}^{P}}{V_{\text{max}} [\text{DCI}]}
$$

where $V$ is initial velocity, the brackets indicate molar concentrations of TPNH and DCI, and $V_{\text{max}}$, $K_{m}^{P}$, and $K_{m}^{P}$ are constants. The lines were drawn in Fig. 3 with the constants taken as follows: $V_{\text{max}} = 1.10 \mu$moles per minute, $K_{m}^{P} = 5.2 \times 10^{-4}$ M, and $K_{m}^{P} = 5.8 \times 10^{-4}$ M. TPNH dehydrogenase A gave similar results.

The effects of substrate concentration on DPNH dehydrogenase II were those expected of a reaction in which a ternary complex is formed (Fig. 4). In this case, data were fitted to the equation (13)

$$
\frac{1}{V} = \frac{1}{k_{3} + k_{3}[\text{DPNH}]} + \frac{1}{k_{4}[\text{DCI}]} + \frac{k_{-3}}{k_{4}[\text{DPNH}][\text{DCI}]}
$$

The lines of Fig. 4 were drawn with the constants taken as follows: $1/k_{3} = 1.2$ minutes per $\mu$moles, $1/k_{3} = 0.2 \times 10^{-4}$ minute per $\mu$ mole, and $1/k_{3} = 2.6 \times 10^{-4}$ minute per $\mu$ mole, and $k_{-3} = 17 \mu$moles per minute. Similar results were obtained with a DPNH dehydrogenase purified from methemoglobinemic red cells (6).

Effect of Ionic Strength—Increasing ionic strength activated both TPNH dehydrogenases (Fig. 5), but inhibited both DPNH dehydrogenases. High ionic strength also inhibited the activity with TPNH of DPNH dehydrogenase I. When the activation by various salts was compared, (NH$_4$)$_2$SO$_4$ and KCl were found to be most effective.

* The abbreviation used is: DCI, 2,6-dichlorophenolindophenol.
TPNH dehydrogenase, $0.5 \times 10^{-4}$ min$^{-1}$; and DPNH dehydrogenase II, $0.6 \times 10^{-4}$ min$^{-1}$.

**DISCUSSION**

TPNH dehydrogenase B and DPNH dehydrogenase II were difficult to separate, and were at first assumed to be one enzyme. It was found, however, that competition between the nucleotides did not occur. Under standard assay conditions for DPNH dehydrogenase, the following rates of reduction, in absorbance change per minute, were observed: 67 $\mu$M TPNH, 0.114; 133 $\mu$M TPNH, 0.112; 67 $\mu$M DPNH, 0.058; and 67 $\mu$M TPNH plus 67 $\mu$M DPNH, 0.164.

The TPNH dehydrogenases of red cells are unusual among diaphorases in that they appear not to contain flavin. DPNH dehydrogenase II is unusual in its kinetic behavior, which is characteristic of the formation of a ternary complex of enzyme, DPNH, and dye. It may be assumed that the dye is a non-specific substrate, since methylene blue and ferrocyanide are also substrates. The most likely explanation of the observed enzymatic activity is that the enzyme binds DPNH and the bound nucleotide reacts more rapidly with 2,6-dichlorophenoldiophenol than does free nucleotide. The simplest formulation of the reaction is as follows.

$$E + \text{DPNH} \xrightarrow{k_1} E \cdot \text{DPNH}$$

$$E \cdot \text{DPNH} + \text{DCI} + \text{H}^+ \xrightarrow{k_2} E \cdot \text{DPN}^+ + \text{DCIH}_3$$

$$E \cdot \text{DPN}^+ \xrightarrow{k_3} E + \text{DPN}^+$$

When initial rates of reaction are considered, this formulation leads to Equation 2.

The measured rates of methemoglobin reduction by DPNH and TPNH dehydrogenases are comparable to those found in the intact red cell. In normal red cells in which hemoglobin has been oxidized to methemoglobin with nitrate, the rate of reduction of methemoglobin in the presence of glucose is from 10 to 15% per hour (13). In cells deficient in DPNH dehydrogenase, the corresponding rate of reduction is 2 to 5% per hour, indicating a 75% loss in reducing capacity. Under conditions simulating those in the red cell, the total of the first order rates of methemoglobin reduction measured for ascorbic acid, glutathione, and DPNH and TPNH dehydrogenases is $9.6 \times 10^{-4}$ min$^{-1}$, which is equivalent to 13.2% per hour. Of this total, DPNH dehydrogenase I accounts for 61%.

In methemoglobinemic individuals lacking DPNH dehydrogenase I, 15 to 45% of the total hemoglobin is present as methemoglobin (13). In persons with half the normal level of DPNH dehydrogenase (heterozygotes), no increase in methemoglobin is found (5). Thus a two-thirds loss in capacity to reduce methemoglobin in the red cell results in methemoglobinemia, while a one-third loss has no effect. Since each of the other reducing systems studied appears to comprise less than one-third of the total reducing capacity of the cell, absence of any of these systems would not be expected to cause methemoglobinemia. In fact, absence of ascorbic acid in scurvy and deficiency of TPNH in glucose 6-phosphate dehydrogenase deficiency do not cause methemoglobinemia. The only defects in reducing mechanisms of the red cell that could cause methemoglobinemia appear to be the absence of DPNH dehydrogenase I, deficiency of DPN itself, or the lack of some enzyme necessary to reduce DPN to DPNH.
SUMMARY

Four enzymes with reduced pyridine nucleotide dehydrogenase activity were demonstrated in human red cells. Two reduced triphosphopyridine nucleotide dehydrogenases with similar properties were separated by chromatography. These did not appear to contain flavin and were weak methemoglobin reductases. A reduced diphosphopyridine nucleotide dehydrogenase, previously demonstrated in methemoglobinemic red cells, was also purified from normal red cells.

The rates of reduction of methemoglobin by ascorbic acid, glutathione, and DPNH and TPNH dehydrogenases were determined. The DPNH dehydrogenase previously purified from normal red cells was calculated to account for two-thirds of the capacity of the human red cell to reduce methemoglobin. It was concluded that the only defects in reducing mechanism likely to produce methemoglobinemia are absence of DPNH dehydrogenase, of DPN, or of enzymes producing DPNH.

REFERENCES

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