Purification and Properties of Diphosphopyridine Nucleotide Diaphorase of Human Erythrocytes

Edward M. Scott and James C. McGraw

From the Arctic Health Research Center, United States Public Health Service, Anchorage, Alaska

(Received for publication, July 18, 1961)

Hereditary methemoglobinemia of the enzymatic type (1) has been shown to be due to the absence of reduced diphosphopyridine nucleotide diaphorase activity in red cells (2). The present communication describes the properties of this enzyme as prepared from normal human red cells.

METHODS

Diaphorase Assay—Six-hundredths μmole of 2,6-dichlorophenolindophenol, 50 μmole of Tris-HCl, pH 7.55, 1 μmole of EDTA, enzyme, and 0.44 μmole of DPNH in a volume of 3 ml were placed in a Beckman spectrophotometer equipped with a water jacket through which water at 25° was circulated. A control cuvette without enzyme was included, and absorbancy at 600 mp was determined for 10 minutes. Under these conditions, both the rate of the nonenzymatic reaction and that of the enzymatic reaction were very nearly first order with respect to dye concentration. Rate of reaction was defined as the apparent first order reaction constant, K, in mi-n⁻¹ minu-s the K of the control. A unit of activity was that amount of enzyme calculated to give unit value to K. Specific activity was expressed as units of activity per milligram of protein.

Protein was determined by a spectrophotometric method with a correction applied for the heme protein present. Heme protein was then calculated by the method of Warburg as units of activity per milligram of protein. Lipoamide was a gift of Dr. Lester J. Reed. Flavin was determined by the method of Burch, Bessey, and Lowry, (4). The absorption due to heme protein was sub-

determined, and the absorption due to heme protein was sub-
determined by the method of Price and Greenfield (5), and a

Calcium phosphate gel on cellulose powder was prepared according to the method of Price and Greenfield (5), and a column, 10 cm in diameter, was filled to a depth of 3 cm with the gel. To this were added successively: 50 ml of 0.01 M Tris-HCl, pH 8.65; the dialyzed enzyme solution; 1000 ml of 0.01 M Tris-HCl, pH 8.65; 500 ml of 0.05 M Tris-HCl, pH 8.65; and 1000 ml of 0.05 M Tris-HCl, pH 8.65, containing 1% (NH₄)₂SO₄. This procedure was performed at room temperature. Suction was applied to the column. The first 1500 ml of eluate contained considerable protein but no enzyme activity. The final liter of eluate was collected in 100-ml fractions, and those with a specific activity greater than 1.0 were combined as Fraction 5. To Fraction 5 were added 31.5 g of (NH₄)₂SO₄/100 ml of eluate, and the precipitate was removed by centrifuging and was discarded.

An additional 14 g of (NH₄)₂SO₄/100 ml of eluate were added, and the precipitate was dissolved in 2.5 ml of 0.1 M Tris-HCl, pH 8.65, to give Fraction 6.

At this point, five samples of Fraction 6 were combined and dialyzed for 6 hours in 2 liters of 0.01 M Tris-HCl, pH 8.65. A Ca₃(PO₄)₂ gel-cellulose column, 4 cm in diameter and 5 cm deep, was prepared, to which the following were added successively: 50 ml of 0.01 M Tris-HCl, pH 8.65; enzyme solution; 250 ml of 0.01 M Tris-HCl, pH 8.65; 250 ml of 0.05 M Tris-HCl, pH 8.65; and 250 ml of 0.05 M Tris-HCl, pH 8.65, containing 1% (NH₄)₂SO₄. The last 250 ml of eluate were collected in 25 ml portions, and those fractions with specific activity greater than 3 combined as Fraction 7.

To Fraction 7 were added 32.2 g of (NH₄)₂SO₄/100 ml of eluate, and the precipitate was removed by centrifuging and was discarded. An additional 14 g of (NH₄)₂SO₄/100 ml of eluate were added, and the precipitate was dissolved in 2.5 ml of 0.1 M Tris-HCl, pH 8.65, to give Fraction 8.

Fraction 8 was dialyzed for 6 hours against 1 liter of 0.01 M

Vol. 237, No. 1, January 1962

Printed in U.S.A.
DPNH-diaphorase of Human Erythrocytes

Vol. 237, No. 1

Purification of erythrocyte diaphorase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Treatment</th>
<th>Volume</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hemolyzed cells</td>
<td>2000</td>
<td>65 × 10^4</td>
<td>0.0016</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>(NH₄)₂SO₄</td>
<td>448</td>
<td>18,600</td>
<td>0.028</td>
<td>52.0</td>
</tr>
<tr>
<td>3</td>
<td>(NH₄)₂SO₄</td>
<td>62</td>
<td>5,200</td>
<td>0.074</td>
<td>37.6</td>
</tr>
<tr>
<td>4</td>
<td>(NH₄)₂SO₄</td>
<td>24</td>
<td>2,310</td>
<td>0.147</td>
<td>33.0</td>
</tr>
<tr>
<td>5</td>
<td>Gel-cellulose column</td>
<td>400</td>
<td>121</td>
<td>1.76</td>
<td>20.9</td>
</tr>
<tr>
<td>6</td>
<td>(NH₄)₂SO₄</td>
<td>4.4</td>
<td>70</td>
<td>2.00</td>
<td>17.9</td>
</tr>
<tr>
<td>7</td>
<td>Gel-cellulose column</td>
<td>150</td>
<td>66</td>
<td>6.92</td>
<td>9.9</td>
</tr>
<tr>
<td>8</td>
<td>(NH₄)₂SO₄</td>
<td>3.3</td>
<td>27</td>
<td>11.1</td>
<td>6.4</td>
</tr>
<tr>
<td>9</td>
<td>Gel-cellulose column</td>
<td>6.0</td>
<td>4.9</td>
<td>46.9</td>
<td>4.4</td>
</tr>
<tr>
<td>10</td>
<td>(NH₄)₂SO₄</td>
<td>1.0</td>
<td>2.8</td>
<td>53.9</td>
<td>2.9</td>
</tr>
<tr>
<td>11</td>
<td>Gel-cellulose column</td>
<td>5.0</td>
<td>1.4</td>
<td>80.6</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Five portions of Fraction 6 were combined.

Relative rates of reduction of substrates by purified enzyme

The conditions were: DPNH, 6 × 10⁻⁵ M; Tris-HCl, pH 7.55, 1.3 × 10⁻⁴ M; EDTA, 3 × 10⁻⁴ M; 2,6-dichlorophenolindophenol, 2 × 10⁻⁹ M; cytochrome c, 4 × 10⁻⁵ M; methemoglobin, 1 × 10⁻⁴ M; temperature, 25°C.

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methemoglobin</td>
<td>1.0</td>
</tr>
<tr>
<td>Oxygen</td>
<td>4.6</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>23</td>
</tr>
<tr>
<td>2,6-Dichlorophenolindophenol</td>
<td>9200</td>
</tr>
</tbody>
</table>

Tris-HCl, pH 8.65, and the Ca₃(PO₄)₂ gel-cellulose chromatography was repeated. A column, 1 cm in diameter and 5 cm deep, was prepared, and to it were added successively: 2 ml of 0.01 M Tris-HCl, pH 8.65; dialyzed enzyme; 10 ml of 0.05 M Tris-HCl, pH 8.65; 10 ml of 0.5 M Tris-HCl, pH 8.65; and 10 ml of 0.05 M Tris-HCl, pH 8.65, containing 0.5% (NH₄)₂SO₄. The last 10 ml of eluate were collected in 2 ml fractions, and those fractions with specific activity higher than 40 combined as Fraction 9. To Fraction 9 were added 34.5 g of (NH₄)₂SO₄/100 ml, and the precipitate was discarded. Then, 10.5 g of (NH₄)₂SO₄/100 ml of Fraction 9 were added, and the precipitate was dissolved in 1 ml of 0.1 M Tris-HCl, pH 8.65. (Fraction 10).

Fraction 10 was dialyzed as before and the chromatography repeated. A column, 0.5 cm in diameter and 6 cm deep, was used. To this were added successively: 1 ml of 0.01 M Tris-HCl, pH 8.65; enzyme; and 20 ml of 0.5 M Tris-HCl, pH 8.65. The enzyme was eluted in the final 4 to 6 ml with a specific activity approximately 50,000 times that in the initial hemolysate.

RESULTS

The progress of purification is shown in Table I. Until the final step, the enzyme was quite stable, and recoveries of activity in the various fractions were generally high. The presence of 10⁻² M EDTA in the buffer solutions used helped in stabilizing the enzyme. Purification procedures involving the use of organic solvents could not be applied without complete loss of activity. The enzyme was stable at a pH of 0.0, but manipulations at a pH lower than 6.5 usually led to a loss of activity. Attempts at further purification were unsuccessful because of instability.

Protein containing heme was a persistent impurity during the isolation, but the fraction of total proteins containing heme decreased as purification proceeded. In the purest preparation, heme protein, calculated as hemoglobin, was 13% of the total.

The flavin content of the enzyme increased during purification, and that of the purest fraction was 1 mole of flavin/195,000 g of nonheme protein. The flavin present resembled FAD in that fluorescence before hydrolysis with trichloroacetic acid was 13% of that found after hydrolysis (4). Sufficient heme protein was present in the purest fraction to obscure the characteristic adsorption of flavin at 450 μm. At pH 7.55 and 25°C the turnover of the enzyme was 2200 moles of DPNH oxidized per mole of flavin per minute. As shown below, this number was very much higher at lower pH values. It can be calculated from the diaphorase activity of whole hemolysates that DPNH-diaphorase cannot account for more than 5% of the flavin nor for more than 0.002% of the protein of the red cell.

Specificity—The enzyme appeared to be relatively specific for DPNH; the purified enzyme had 1.5% of the activity with TPNH that it had with DPNH. The relative reactivity with DPNH and TPNH varied in different enzyme fractions, and this variability points to the presence of a separate TPNH-diaphorase. In hemolysates of normal cells, TPNH showed between 10 and 15% of the activity observed with DPNH. The DPNH-diaphorase activity of methemoglobinemic blood was not significantly different from that of normal blood.

There was less specificity shown with respect to the electron acceptor. The relative rates of reaction of the latter are shown in Table II. The relative rates of reduction of dye and methemoglobin were comparable in magnitude to the relative rates of reduction of dye in hemolysates and of methemoglobin in whole cells (1). The enzyme had some DPNH-oxidase activity but no appreciable glutathione reductase activity. Dihydrolipoic dehydrogenase activity could not be demonstrated either with lipoic acid or lipoamide and DPNH at pH 7.5 or with dihydro- lipoic acid and DPN at pH 7.5 or pH 9.0.

Effect of pH—Both 2,6-dichlorophenolindophenol and cytochrome c are reduced by DPNH in the absence of enzyme, whereas methemoglobin does not react at the concentrations used in these experiments. The log of the nonenzymatic rate of reduction of the dye was inversely proportional to pH. The enzymatic rate of dye reduction also increased with decreasing pH (Fig. 1); phosphate buffer inhibited the reaction as compared to Tris. As shown in Fig. 2, there was no effect of pH on cytochrome c reduction between 6.3 and 8.6. In Fig. 3 it is seen that above pH 7.5, reduction of methemoglobin appeared to be independent of pH; below 7.5 the log of the rate was inversely proportional to pH.

Effect of Temperature—The nonenzymatic reduction of 2,6-dichlorophenolindophenol had a low temperature coefficient with an apparent activation energy of 3900 calories per mole. The enzymatic reduction of the dye as well as cytochrome c and methemoglobin also had relatively low temperature coefficients as shown in Fig. 4, with apparent activation energies of 5000 to 7000 calories per mole.

Effect of Substrate Concentration—The effect of varying the

Downloaded from http://www.jbc.org/ by guest on September 30, 2017
concentrations of DPNH and 2,6-dichlorophenolindophenol on initial rate of reaction is shown in Fig. 5. The results can be adequately described by the relationship (6):

\[
\frac{1}{V} = \frac{1}{V_{\text{max}}} + \frac{K_m}{[\text{DPNH}]} + \frac{K_m'}{[\text{DCP}]}
\]

where \( V \) is initial velocity, \( V_{\text{max}} \) is the maximal velocity, and \( K_m \) and \( K_m' \) are the concentrations of DPNH and dye at which one-half of the maximal rate is observed in the presence of an excess of the other substrate. \([\text{DPNH}]\) is DPNH concentration, and \([\text{DCP}]\) is concentration of 2,6-dichlorophenolindophenol. The lines in Fig. 5 were drawn from the equation:

\[
\frac{1}{V} = 4.8 \times 10^6 + \frac{3.4}{[\text{DPNH}]} + \frac{16.3}{[\text{DCP}]}
\]

the values for \( K_m \) and \( K_m' \) were \( 0.7 \times 10^{-5} \) and \( 3.40 \times 10^{-5} \) M, respectively.

Fig. 1. Effect of pH on rate of reduction of 2,6-dichlorophenolindophenol (2,6-DCP). \( V \) is the initial rate of reaction in moles per liter per minute. The conditions were the same as in Table II; all buffers were \( 1.3 \times 10^{-2} \) M. The upper curves are net enzymatic rate of reduction; the lower curve is the nonenzymatic rate.

Fig. 2. Effect of pH on rate of reduction of cytochrome c (Cyto-c). \( V \) is the initial rate of reaction in moles per liter per minute. The conditions were: DPNH, \( 1.8 \times 10^{-4} \) M; buffer, \( 0.05 \) M; EDTA, \( 6.7 \times 10^{-4} \) M; cytochrome c, \( 6 \times 10^{-4} \) M; temperature, 25°C. Rates were corrected for enzyme concentration to be comparable with those in Fig. 1. Upper line, net enzymatic reaction rate; lower curve, nonenzymatic rate.

Fig. 3. Effect of pH on rate of reduction of methemoglobin (MetHb). \( V \) is the initial rate of reaction in moles per liter per minute. The conditions were: DPNH, \( 3.0 \times 10^{-4} \) M; buffer, \( 0.067 \) M; EDTA, \( 6.7 \times 10^{-4} \) M; methemoglobin, \( 4 \times 10^{-6} \) M. Rates were corrected for enzyme concentration to be comparable to those in Figs. 1 and 2.

Fig. 4. Effect of temperature on reduction of various substrates by DPNH-diaphorase. \( V \) is initial rate expressed in moles per liter per minute. \( T_A \) is absolute temperature, the figures in parentheses are apparent activation energies. Conditions were the same as in Figs. 1 to 3; the buffer was phosphate, pH 7.4. All rates were normalized for the same enzyme concentration.

With cytochrome c as electron acceptor, the value of \( K_m \) for DPNH was not experimentally distinguishable from that found for DPNH when dye was the acceptor; \( K_m \) for cytochrome c was not appreciably different from the \( K_m \) for 2,6-dichlorophenolindophenol. When methemoglobin was used as acceptor in a similar experiment, the reaction appeared to be first order with respect to methemoglobin concentration and zero order with respect to DPNH concentration.

\textit{Inhibition Studies}—The reduction of 2,6-dichlorophenolindophenol by the enzyme was not inhibited by \( 0.01 \) M cyanide. EDTA had no effect on dye reduction, but, in its absence, the rates of reduction of both cytochrome c and methemoglobin were considerably reduced. Quinacrine (\( 10^{-4} \) M) was not inhibitory and caused only a 35% reduction of activity at a concentration of \( 10^{-4} \) M. \( p \)-Hydroxymercuribenzoate (\( 10^{-4} \) M), on the other hand, caused a 50% inhibition of the enzyme.
DISCUSSION

The physiological function of diaphorases was a matter of speculation until it was shown that they could act as dihydro-lipoic acid dehydrogenases (7). The absence of DPNH-diaphorase activity in red cells in hereditary methemoglobinemia demonstrates another definite physiological function for a diaphorase. It appears possible that other diaphorases may have a similar function in maintaining other substances in a reduced physiologically active state.

In most respects, the erythrocyte enzyme appears to resemble other diaphorases. It has more specificity with respect to reductant than to oxidant and this could be interpreted to mean that DPNH first reduces the enzyme, which in turn reduces the other substrate (6). The reduction of enzyme by DPNH appears to be sufficiently rapid under the usual conditions of measurement to give a reaction that is nearly first order with respect to oxidant concentration. This would be anticipated if the second half of the reaction consists of the reduction of the oxidant by an essentially constant amount of reduced enzyme. The first order kinetics, low degree of temperature dependence, and pH behavior suggest that there is little or no specific binding of acceptor to enzyme in the latter half of the reaction.

SUMMARY

The reduced diphosphopyridine nucleotide diaphorase of human red cells has been purified and its properties studied. The enzyme will reduce 2,6-dichlorophenolindophenol, cytochrome c, and methemoglobin but has no appreciable dihydro-lipoic dehydrogenase or glutathione reductase activity. The reaction catalyzed is interpreted in terms of a rapid reduction of the enzyme by reduced diphosphorydine nucleotide followed by a slower reduction of the acceptor.

REFERENCES
3. Warburg, O., and Christian, W., Biochem. Z., 310, 384 (1940).
Purification and Properties of Diphosphopyridine Nucleotide Diaphorase of Human Erythrocytes
Edward M. Scott and James C. McGraw


Access the most updated version of this article at http://www.jbc.org/content/237/1/249.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/237/1/249.citation.full.html#ref-list-1