Properties and Reaction Mechanism of DT Diaphorase from Rat Liver

SYUN HOSODA, WATARU KAKAMURA, AND KAZUKO HAYASHI

From the Laboratory of Pathology, Aichi Cancer Center Research Institute, Nagoya, Japan

SUMMARY

DT diaphorase was purified to homogeneity from rat liver and characterized. The molecular weight of the enzyme was calculated to be $5.0 \pm 0.06 \times 10^4$ from sedimentation equilibrium experiments and to be $4.8 \times 10^4$ by thin layer gel filtration method using Sephadex G-200. The identity of FAD as a prosthetic group was confirmed by o-amino acid oxidase test. It was found that 1 mole of FAD was present per mole of enzyme.

The interaction of the enzyme with NADPH and K$_3$ in the presence and absence of bovine serum albumin and dicumarol were also studied by steady state and stopped flow kinetic methods, together with binding experiments using [$^4$C]K$_3$ and [14C]dicumarol. The probable reaction sequences of the enzyme, including the pyridine nucleotide-dependent reduction of K$_3$ mainly by a ping-pong type mechanism with a rate-limiting step at the point of dissociation of the enzyme-reduced K$_3$ complex, are proposed. There is evidence that the native form of enzyme has two independent binding sites for K$_3$ and dicumarol in its active center, forming the partially active enzyme-K$_3$ complex reducible by NADPH but not oxidizable by K$_3$ and the fully inactive enzyme-dicumarol complex not reducible by NADPH. The reduced species is capable of binding only K$_3$. It was also demonstrated that bovine serum albumin competes with the native enzyme for K$_3$ and dicumarol, probably through the ability of this simple protein to associate with these substances, preventing the formation of enzyme-K$_3$ and enzyme-dicumarol complexes, and leading to the apparent increase in enzyme activity.

In this paper we report some physicochemical properties of DT diaphorase purified from rat liver, together with steady state and stopped flow kinetic studies of the interaction of the enzyme with NADPH and K$_3$ in the presence and absence of serum albumin as well as of dicumarol. It is proposed that DT diaphorase catalyzes the pyridine nucleotide-dependent reduction of K$_3$ mainly by a ping-pong type mechanism in which the oxidized enzyme is reduced by NADPH to produce the free reduced species. This type of mechanism is indicated schematically in Equations 1, 2, 3, and 4.

\[
E \cdot FAD + NADPH \xrightarrow{k_{+1}} E \cdot FADH_2 - NADP \quad (1)
\]

\[
E \cdot FADH_2 - NADP \xrightarrow{k_{+2}} E \cdot FADH_2 + NADP \quad (2)
\]

\[
E \cdot FADH_2 + K_3 \xrightarrow{k_{+3}} E \cdot FAD - K_3H_2 \quad (3)
\]

\[
E \cdot FAD - K_3H_2 \xrightarrow{k_{+4}} E \cdot FAD + K_3H_2 \quad (4)
\]

It will be shown that in the reaction sequences of DT diaphorase, the step in Equation 4 is rate-limiting. In addition, we present some evidence that both K$_3$ and dicumarol can bind to the native enzyme at equimolar concentration through two different binding sites to form the enzyme-K$_3$ complex and the enzyme-dicumarol complex, respectively, whereas the reduced species is capable of binding only K$_3$. It was also demonstrated that bovine serum albumin appears to lead to an increase in enzyme activity by facilitating the dissociation of these complexes.

In 1958, a flavoprotein which catalyzes the oxidation of NADH and NADPH by various dyes and quinones was found in rat liver by Ernster and Navazio (1) who named the enzyme DT diaphorase (EC 1.6.99.2, reduced NAD(P):acceptor oxidoreductase) from its almost equal activity with NADH(DPNH) and NADPH(TPNH). Subsequent studies (2, 3) disclosed that this enzyme closely resembles "vitamin K$_3$ reductase" from ox liver and ox "brain diaphorase," described by Marki and Martius (4, 5) and Giuditta and Strecker (6), respectively.

It has been shown that the enzyme purified from several mammalian sources is characterized by three distinctive enzymic properties. These consist of an immediate disappearance of the yellow color in oxidized flavoprotein after adding excess NAD(P)H, a marked increase in enzyme activity produced by bovine serum albumin, and a powerful specific inhibition by dicumarol (2, 4, 6). Further clarification of these aspects, however, has not been accomplished, since no precise reaction mechanism of this enzyme had been established.
EXPERIMENTAL PROCEDURE

Materials—NADP, NADPH, FAD, cytochrome c, and d-amino acid oxidase were purchased from Boehringer, vitamin K₁ (menadione, 2-methyl-1,4-naphthoquinone), dicumarol, and crystallized bovine serum albumin from Sigma, crystallized egg albumin from Nutritional Biochemicals Corporation, 2-[14C]methyl-1,4-naphthoquinone (8.8 mCi per mole) from The Radiochemical Centre, and [methylen-14C]dicumarol (3.64 mCi per mole) from New England Nuclear. CM-cellulose and DEAE-cellulose were obtained from Brown, hydroxylapatite from Bio-Rad, cellulose acetate in a gel form (Cellogel) from Chemetron (Milan, Italy), and Sephadex G-200 from Pharmacia. The reduced vitamin K₃ (K₃H₂) was prepared according to the procedure of Fieser (7).

Enzyme Assay—DT diaphorase activity was determined spectrophotometrically by following the decrease in absorbance of NADPH at 340 nm. All determinations were made at room temperature with cuvettes having a 1-cm light path. The assay solution contained 100 μmol of Tris-HCl buffer, pH 7.4, 0.04 to 0.2 μmol of NADPH, 0.0008 to 0.16 μmol of K₃, 0 to 2 mg of bovine serum albumin, 1 mg of cytochrome c, and 2-μl volumes of the enzyme and [methylene-14C]dicumarol (3.64 mCi per mole) from New England Nuclear. All determinations were made at room temperature.

Specific activity is expressed as units of enzymic activity per mg of protein. The protein concentrations were determined by the Lowry-Folin method (8).

Ultracentrifugal Measurements—Sedimentation velocity and diffusion and sedimentation equilibrium experiments were performed in the analytical ultracentrifuge equipped with phase plate schlieren system and RTIC temperature-control system. The diffusion coefficient was estimated from the spreading of the boundary of 8 μg of protein per ml at 12,590 rpm according to the method of Ehrenberg (9). For sedimentation equilibrium experiments a schlieren optical system was used with 8-mm column in a filled Upon double-sector cell. The schlieren patterns were photographed with the phase plate angle at 85°, 80°, 75°, and 65°.

Thin Layer Gel Filtration—The thin layer gel filtration for cotimation of approximate molecular weight of purified DT diaphorase was performed according to Radola (10) using Sephadex G-200 (superfine) and Pharmacia TLG apparatus.

Cellogel Electrophoresis—Cellogel was equilibrated with 20 mM Tris-HCl buffer, pH 8.0. After applying each 0.5 ml sample on the original line of the gel, electrophoresis was performed horizontally at 4°C with a constant voltage of 200 volts. The gel was stained with a freshly prepared mixture composed of 1.3 mM NADH, 0.24 mM 2,3'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-3,3'-dimethoxy-4,4'-diphenylene) dietrazoium chloride, 0.1 mM 2,6-dichlorophenol, and 2 mM bovine serum albumin in 0.1 M Tris-HCl buffer, pH 8.0. The gel was developed at room temperature after applying the staining mixture. The enzyme activity appeared as a reddish blue band.

Stopped Flow Kinetic Experiments—Stopped flow experiments were performed with a Yanaa SPS-I stopped flow spectrophotometer. The details of this apparatus have been described elsewhere (11). The light path length of the observation chamber was 10 mm and the dead time was about 2.0 ms. The temperature was controlled at 15°C.

Binding Experiments—DT diaphorase at 1 μM concentration in terms of the enzyme-bound FAD was incubated for 20 min at 30°C in the dark with 0.2 mM [14C]K₃, 10 μM [14C]dicumarol, or both in 10 mM potassium phosphate buffer, pH 7.0. After incubation, each 0.5 ml of sample chilled in ice was directly applied to a column, 0.9 × 15 cm, of Sephadex G-25 (coarse) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, and was eluted with the same buffer. The gel filtration was carried out at 4°C and fractions of 1 ml each were collected. To another 0.5 ml of sample at room temperature was added 1 mg of NADPH. After 1 min the reaction mixtures were quickly chilled in ice and applied to the Sephadex column in the same manner as in the former gel filtration. Under these conditions the enzyme was eluted between fractions 3 and 5 with the peak at fraction 4 and K₃ between fractions 7 and 20 with the peak at fraction 11, but no dicumarol was eluted because of its strong affinity for Sephadex. However enzyme-bound dicumarol was eluted in the void volume of a column. A 0.5 ml aliquot of eluate from each fraction was transferred to a vial containing 4.5 ml of the dioxane scintillator, composed of 300 ml of 1.4-bis(4-methyl-5-phenyloxazolyl)benzene, 0.7 g of 2,5-diphenyloxazole, 100 g of naphthalene, and 1 liter of dioxane. The radioactivity was measured in a liquid scintillation counter. The amounts of [14C]K₃ and [14C]dicumarol bound to enzyme were calculated from the radioactivity of authentic samples at known concentrations.

For comparison bovine serum albumin and egg albumin were used instead of the enzyme.

Purification—All manipulations were performed at 4°C. The analytical details of a typical purification are shown in Table I. Rat liver (1,000 g) was homogenized in a Waring Blender with 3 volumes of Hanks’ solution. The homogenization was repeated in a Teflon homogenizer. The homogenate was centrifuged for 60 min at 150,000 × g. Powdered ammonium sulfate was added to the clear pinkish supernatant until 0.5 saturation was attained. The precipitate was removed by centrifugation and additional ammonium sulfate was added to the supernatant until 0.7 saturation was attained. The precipitate was dissolved in 10 mM potassium phosphate buffer, pH 7.0, and extensively dialyzed against the same buffer solution. Each one-third of the dialyzed enzyme solution was applied to a column, 5 × 45 cm, of CM-cellulose equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The enzyme activity was eluted with the same buffer. The active fractions were divided into three equal portions, and each portion was concentrated to 1 ml. The specific activity and yield are shown in Table I. A unit of activity is the amount of enzyme which oxidizes 1 μmol of NADPH per min.

Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Whole homogenate</td>
<td>3,350</td>
<td>243,000</td>
<td>60,750</td>
<td>0.25</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2. 100,000 × g supernatant</td>
<td>2,680</td>
<td>129,500</td>
<td>54,390</td>
<td>0.42</td>
<td>1.7</td>
<td>90</td>
</tr>
<tr>
<td>3. (NH₄)₂SO₄ precipitate (50 to 70%)</td>
<td>300</td>
<td>24,660</td>
<td>35,017</td>
<td>1</td>
<td>1.4</td>
<td>58</td>
</tr>
<tr>
<td>4. CM-cellulose eluate</td>
<td>208</td>
<td>13,700</td>
<td>27,337</td>
<td>2.0</td>
<td>8</td>
<td>45</td>
</tr>
<tr>
<td>5. DEAE-cellulose eluate</td>
<td>415</td>
<td>7,540</td>
<td>20,890</td>
<td>3.5</td>
<td>14</td>
<td>43</td>
</tr>
<tr>
<td>6. (NH₄)₂SO₄ precipitate (70%)</td>
<td>110</td>
<td>4,450</td>
<td>24,030</td>
<td>5.4</td>
<td>22</td>
<td>40</td>
</tr>
<tr>
<td>7. 1st hydroxyapatite eluate</td>
<td>684</td>
<td>1,170</td>
<td>15,710</td>
<td>13.4</td>
<td>54</td>
<td>26</td>
</tr>
<tr>
<td>8. 2nd hydroxyapatite eluate</td>
<td>224</td>
<td>388</td>
<td>12,216</td>
<td>31.5</td>
<td>126</td>
<td>20</td>
</tr>
<tr>
<td>9. 3rd hydroxyapatite eluate</td>
<td>128</td>
<td>225</td>
<td>9,528</td>
<td>42.3</td>
<td>159</td>
<td>16</td>
</tr>
<tr>
<td>10. 4th hydroxyapatite eluate</td>
<td>80</td>
<td>91</td>
<td>7,801</td>
<td>80.2</td>
<td>345</td>
<td>13</td>
</tr>
<tr>
<td>11. 5th hydroxyapatite eluate</td>
<td>40</td>
<td>42</td>
<td>5,762</td>
<td>137.2</td>
<td>550</td>
<td>10</td>
</tr>
<tr>
<td>12. 6th hydroxyapatite eluate</td>
<td>20</td>
<td>15</td>
<td>3,645</td>
<td>242.8</td>
<td>971</td>
<td>6</td>
</tr>
</tbody>
</table>

* Protein concentration was estimated by absorbance at 280 nm with the value of ε₂₈₀ of 1.0 for a solution containing 1 mg of protein per ml.
was applied to a column, 5 × 45 cm, of DEAE-cellulose equilibrated with the same phosphate buffer. The column was eluted by a linear gradient from 0.01 to 0.3 M phosphate buffer, pH 7.0. The enzyme fractions with a higher activity were collected and powdered ammonium sulfate was added to the enzyme solution until 0.7 saturation was attained. The precipitate redissolved in 10 mM phosphate buffer, pH 7.0, was dialyzed against the same buffer solution. Thereafter, each 1/4th volume of the enzyme solution was applied to a column, 1 × 12 cm, of hydroxylapatite (5 g) equilibrated with 10 mM phosphate buffer, pH 7.0, and the column was developed with a linear gradient made from 200 ml of the equilibration buffer and 200 ml of 0.3 M phosphate buffer, pH 7.0. Fractions of 4 ml each were collected at a flow rate of 20 ml per hour. The active fractions from 8 columns were combined and concentrated by ultrafiltration. The concentrated enzyme solution was divided into four equal portions. Each portion was loaded again onto the same size column and a similar run was performed. The active fractions were combined and concentrated. The third hydroxylapatite chromatography using two columns was carried out in exactly the same manner as the previous run. When this procedure was repeated once, the protein and enzyme activity were eluted almost concurrently between fractions 35 and 45. After this stage the chromatography was performed by loading the enzyme onto one column of the same size.

RESULTS

Properties

Molecular Weight—The enzyme sedimented as a single symmetrical boundary. The sedimentation coefficient of native protein is slightly dependent upon protein concentration, decreasing from 3.8 to 3.6 S as protein concentration is increased. By extrapolation, the $s_{20w}$ value of 3.84 was obtained. The diffusion coefficient was $7.37 \pm 0.07 \times 10^{-7}$ cm$^2$ s$^{-1}$ at a protein concentration of 8 mg per ml. From the value of $s_{20w}$ and $D_{20w}$, the molecular weight of native DT diaphorase was calculated to be $5.0 \pm 0.1 \times 10^4$. In the sedimentation equilibrium measurements the plot of ln ($Z$)/$r$ versus $r^2$ was linear. A graph of the results of one of these experiments is shown in Fig. 1. The average molecular weight of the native enzyme is calculated to be $5.0 \pm 0.06 \times 10^4$. The molecular weight of DT diaphorase was also estimated to be $4.8 \times 10^4$ from its relative migration distance using Sephadex G-200 according to the method of Radola (10).

Absorption Spectra—The purified enzyme exhibits absorption maxima at 278, 379, and 440 nm with shoulders at 430 and 480 nm. The specific absorption coefficients of the enzyme (5 mg per ml) at the maxima were as follows: 12.0 at 278, 0.738 at 379, and 0.856 at 450 nm. Assuming a millimolar extinction coefficient of 11.3 mM$^{-1}$ cm$^{-1}$ for FAD at 450 nm, it was calculated that 1 mole of FAD was present per 52,500 g of protein.

Prosthetic Group—The supernatant of boiled enzyme acts as a cofactor of apoenzyme of $\alpha$-amino acid oxidase prepared by the method involving dialysis against 1 M potassium bromide according to the method of Massey and Curti (12), but the activity of DT diaphorase treated similarly was not significantly influenced by adding either boiled extract or FAD, indicating strong binding of apoenzyme to cofactor. The firm association of apoenzyme with cofactor was also indicated by the discovery that even after extensive dialysis of DT diaphorase against distilled water for 5 days at 2°, the dialyzed enzyme, which revealed only half of the original enzyme activity, was not activated by either the boiled extract or FAD.

Cellogel Electrophoresis—The purified enzyme migrated toward the anode as a single protein band when subjected to Cellogel electrophoresis at pH 8.0, and the protein band corresponded with that of the enzyme activity, as shown in Fig. 2.

Stability—The enzyme with a specific activity of 242.8 units per mg of protein was diluted 1000 times with 10 mM phosphate buffer, pH 7.0, to a concentration of 0.16 PM. When the diluted enzyme was placed at 0° and 25° in the dark, none of the enzyme activity was lost at 0° for 24 hours, but 63% of the activity was lost at 25° after only 2 hours. Therefore, the thermal stability of the diluted enzyme was tested in the presence of bovine serum albumin, NADPH, or both. As shown in Table II, 0.01% bovine serum albumin (1.5 PM) prevented the loss of enzyme activity at 40°, whereas the enzyme reduced by 60% NADPH withstanded inactivation at 50°. A synergistic action against thermal inactivation of enzyme was observed after pre-treatment with both albumin and NADPH. FAD had no activating effect on the diluted enzyme.

Catalytic Activity—The maximal turnover number was calculated to be 43,270 moles of NADPH oxidized per min per mole of protein with $K_m$(NADPH) = 0.696 PM. The optimal pH for NADPH-K$\varepsilon$ reductase activity was 5.0.

The physicochemical properties of rat liver DT diaphorase described above are summarized in Table III, and compared together with those of the ox liver enzyme purified by Mäkki and Martius (4, 5). It can be seen from this table that the
that DT diaphorase activity was measured by varying the concentration of both K3 and NADPH in the presence of cytochrome c. Fig. 3 shows that when K3 concentration was raised to 80 μM, distinct competitive inhibition with NADPH was observed. Below a 2 μM concentration of K3 in the presence of cytochrome c, the inhibitory effect of K3 on enzyme activity was not observed, as shown in Fig. 4A. In the 1/e versus 1/NADPH plots at lower concentrations of K3, parallel lines were obtained. Similar parallel lines were also obtained in the 1/e versus 1/K3 plots, as shown in Fig. 4B. Bovine serum albumin could compete also with K3. Fig. 5 demonstrates clearly that at low concentrations of K3, bovine serum albumin did not enhance the enzyme activity, and the enzyme activity decreased at increased K3 concentrations was restored again by increasing albumin concentration. In the presence of a competitive inhibitor (I), the rate equation is

\[
\frac{1}{v} = \frac{1}{v_{\text{max}}} \left( K_{m(\text{NADPH})} \left( \frac{1}{K_3} \right)^{-1} \cdot \frac{1}{(\text{NADPH})^*} \cdot \frac{1}{(K_3)^*} \right) + \frac{1}{v_{\text{max}}}
\]

in which \( K_I \) is the dissociation constant of the enzyme-inhibitor complex. In the case of DT diaphorase, K3 at higher concentrations may be regarded as I. If apparent \( K_{m(\text{NADPH})} \) can be substituted for \( K_{m(\text{NADPH})} \), K3 of the enzyme-K3 complex can be calculated to be 54 μM from the slope of the line in Fig. 3, since \( K_{m(\text{NADPH})} = [1/(K_3)] \) in the above equation gives the negligible value, 0.0087. In order to obtain the velocity constant \( (k_{+2}) \) of enzyme oxidation by K3, a stopped flow kinetic experiment was also carried out. It was found, however, that the oxidation of the reduced enzyme by K3 was too fast to measure and was completed mostly within dead time. A similar rapid reaction was also observed when K3 and NADPH at equimolar concentration in the drive syringe were combined with enzyme at same concentration in the sample syringe. In another experiment, NADPH in the drive syringe was mixed with K3 and enzyme both at the same concentration and preincubated for 20 min at 15°C in the sample syringe. The results obtained are shown in Fig. 6, A and B. The enzyme reaction proceeded rapidly at a rate comparable with that produced NADPH alone, but the reoxidation of reduced enzyme by K3 became much more sluggish, taking 2½ min for the complete oxidation. In Fig. 6B it should be noted that the reoxidation of reduced en-

**Table II**

**Thermal inactivation of DT diaphorase under various conditions**

The enzyme containing 2.5 μg of protein per ml of 10 mM potassium phosphate buffer, pH 7.0, was pretreated in the presence of 0.01% bovine serum albumin, 50 μM NADPH, or both compounds for 5 min at varied temperatures. Reaction mixture and conditions same as in Table I, except that the reaction was started by final addition of K3.

<table>
<thead>
<tr>
<th>Pretreatment of enzyme</th>
<th>Activity at 0°</th>
<th>37°</th>
<th>50°</th>
<th>60°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin</td>
<td>100</td>
<td>75</td>
<td>55</td>
<td>12</td>
</tr>
<tr>
<td>NADPH</td>
<td>100</td>
<td>96</td>
<td>75</td>
<td>37</td>
</tr>
<tr>
<td>NADPH + bovine serum albumin</td>
<td>100</td>
<td>100</td>
<td>95</td>
<td>75</td>
</tr>
</tbody>
</table>

**Table III**

**Properties of DT diaphorases purified from liver of rat and ox**

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th>Ox*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight (10^4)</td>
<td>5.0</td>
<td>5.2</td>
</tr>
<tr>
<td>Cofactor</td>
<td>FAD</td>
<td>FAD</td>
</tr>
<tr>
<td>Type and number</td>
<td>Strong</td>
<td>Moderate</td>
</tr>
<tr>
<td>Binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turnover number</td>
<td>43,270</td>
<td>44,000</td>
</tr>
<tr>
<td>Km(K3) (μM)</td>
<td>0.696</td>
<td>4.0</td>
</tr>
<tr>
<td>pH optimum</td>
<td>5.0</td>
<td>5.8</td>
</tr>
<tr>
<td>Electrophoretic behavior</td>
<td>Anodal enzyme</td>
<td>Cathodal enzyme</td>
</tr>
</tbody>
</table>

*Quoted from the results reported by Marki and Martius (4, 5).

differences between the rat liver enzyme and that from ox liver are small and not significant except for the electrophoretic behavior.

**Reaction Mechanism**

**Reduction of DT Diaphorase by NADPH**—Addition of a small molar excess of NADPH to the enzyme solution immediately bleached the 379 and 449 nm bands. They were restored gradually under aerobic conditions. The course of the oxidation followed first order kinetics and the first order velocity constant for the reoxidation of reduced enzyme by dissolved oxygen was calculated to be 4.4 × 10⁻⁸ s⁻¹. From the initial slope of the reoxidation of reduced enzyme, the oxidative activity of DT diaphorase was found to be only 1.5 nmols of oxygen reduced per min per mg of protein. In the absorption spectrum of reduced enzyme there was no appearance of absorbance in the 500- to 800-nm region. The kinetics of reduction of the enzyme-bound FAD of DT diaphorase by NADPH in the presence of cytochrome c indicates that cytochrome c did not act as hydrogen carrier from DT diaphorase but was reduced readily by reduced K3, so that DT diaphorase activity was measured by varying the concentration of both K3 and NADPH in the presence of cytochrome c. Fig. 3 shows that when K3 concentration was raised to 80 μM, distinct competitive inhibition with NADPH was observed.
FIG. 4. A, effect of NADPH concentration on DT diaphorase in the absence and presence of NADP. Reaction mixture and conditions are the same as in Fig. 3, except that 0.026 μg of enzyme protein was used. O, 2 μM Ks; □, 2 μM Ks in the presence of 1 mM NADP; ■, 0.4 μM Ks. B, effect of Ks concentration on DT diaphorase in the absence and presence of NADP. Reaction mixture and conditions are the same as in A except that indicated concentrations of Ks were used. O, 100 μM NADPH; △, 50 μM NADPH; ▲, 50 μM NADPH in the presence of 1 mM NADP; □, 20 μM NADPH.

Fig. 5. Effect of bovine serum albumin on DT diaphorase activity at lower Ks concentrations. Reaction was started by adding 0.1 ml of 1.2 mM NADPH to 1.9 ml of reaction mixture containing 100 μmoles of Tris-HCl buffer, pH 7.4, 0.036 μg of enzyme protein, 1 mg of cytochrome c, and indicated concentrations of bovine serum albumin with added Ks. ■, 10 μM Ks; ▲, 8 μM Ks; O, 6 μM Ks; □, 4 μM Ks.

Effect of NADP on DT Diaphorase Activity—Fig. 4A shows that in the 1/v versus 1/NADPH plots the lines do not converge even in the presence of excess NADP, indicating that NADP does not compete with NADPH for the oxidized enzyme. As shown in Fig. 4B, however, in the 1/v versus 1/Ks plots, the line in the presence of added NADP crosses that in the absence of added NADP, suggesting the possible competition of NADP with Ks for the reduced enzyme.

Effect of Dicumarol on DT Diaphorase—The inhibition by dicumarol was competitive with respect to NADPH, as has been reported (2), and Ks was found to be 47.5 μM. As shown in Fig. 7, however, in the 1/v versus 1/Ks plots, the line in the presence of added NADP crosses that in the absence of added NADP, suggesting the possible competition of NADP with Ks for the reduced enzyme.

Binding of [14C]Ks and [14C]Dicumarol by DT Diaphorase—In order to demonstrate the actual formation of the enzyme Ks complex and the enzyme-dicumarol complex, binding experiments using [14C]Ks and [14C]dicumarol were carried out by gel filtration through a column of Sephadex G-25. The results of these experiments are given in Table IV. It can be seen that almost exactly 1 mole of dicumarol was bound per mole of en-
Fig. 6. A, photograph of stopped flow oscilloscope trace at 450 nm on mixing DT diaphorase with NADPH in the presence of $K_2$. Enzyme (15.2 $\mu$m) preincubated with $K_2$ at same concentration at 15° for 20 min in sample syringe was combined with same concentration of NADPH in drive syringe. $B$, reoxidation of reduced enzyme in A by $K_2$. Changes in transmittance at 450 nm were followed with a recorder connected with a Yanaco SPS-I stopped flow spectrophotometer.

Fig. 7. Effect of bovine serum albumin on DT diaphorase in the absence (○) and presence (■) of dicumarol. Reaction was started by adding 0.1 ml of 0.8 mM $K_3$ to 1.9 ml of reaction mixture containing 100 $\mu$moles of Tris-HCl buffer, pH 7.4, 0.051 $\mu$g of enzyme protein, 60 $\mu$m NADPH, and indicated concentrations of albumin used.

Fig. 8. Reduction of DT diaphorase preincubated with dicumarol by NADPH. Enzyme (13 $\mu$m) preincubated with equimolar dicumarol at 15° for 20 min in a sample syringe was combined with the same concentration of NADPH in a drive syringe. Changes in transmittance at 450 nm were illustrated in Fig. 6B.

TABLE IV

<table>
<thead>
<tr>
<th>Enzyme treatment</th>
<th>Radioactivity</th>
<th>$[^{14}C]K_2$ or $[^{14}C] dicumarol bound/mole protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{14}C]K_2$</td>
<td>3040</td>
<td>0.78</td>
</tr>
<tr>
<td>NADPH after treatment with $[^{14}C]K_2$</td>
<td>3160</td>
<td>0.81</td>
</tr>
<tr>
<td>$[^{14}C] dicumarol$</td>
<td>1980</td>
<td>0.99</td>
</tr>
<tr>
<td>NADPH after treatment with $[^{14}C] dicumarol$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$[^{14}C]K_2$ and $[^{14}C]dicumarol$</td>
<td>5080</td>
<td>0</td>
</tr>
<tr>
<td>NADPH after treatment with $[^{14}C]K_2$ and $[^{14}C] dicumarol$</td>
<td>3100</td>
<td></td>
</tr>
</tbody>
</table>
started by adding both 10 μl of enzyme (0.019 pg of protein) and 0.1 ml of 1.2 mM NADPH to 1.9 ml of reaction mixture containing 100 μmoles of potassium phosphate buffer, pH 6.2, 0.5 μmole of EDTA, 0.08 μmole of K2, 200 μg of bovine serum albumin, and indicated concentrations of reduced K3.

Reduced K3 on the reaction rate was examined at pH 6.2, where the auto-oxidation rate of reduced K3 was very low (13). Fig. 9 shows that the relative activity of DT diaphorase decreased linearly by increasing the reduced K3 concentration.

**DISCUSSION**

The major part of the present work pertains to the interactions of DT diaphorase with K3 and NADPH in the presence and absence of bovine serum albumin and dicumarol. In the NADPH-K3 reactions under catalytic conditions, the results obtained can be interpreted in terms of the reaction sequences of DT diaphorase as shown in Equations 1 to 4. In Equation 1 the native enzyme was very rapidly and fully reduced by NADPH, whereas, when the native enzyme was preincubated with K3 at a relatively higher concentration, the rate of K3 reduction was suppressed considerably (Figs. 3 and 6B). These observations indicate that the first substrate combining with the native enzyme is NADPH but not K3. Equation 2 can be justified by the following two observations. First, the absorption spectrum of the fully reduced enzyme was accompanied by no appearance of absorption in the 500- to 800-nm region. Second, even in the presence of excess NADP, the lines in the 1/ν versus 1/NADP plots do not converge (Fig. 4A), but those in the 1/ν versus 1/K3 plots do (Fig. 4B). Thus, it is reasonable to expect that the fully reduced enzyme in Equation 1 catalyzes the reduction of K3 mainly by a ping-pong type mechanism as shown in Equations 2 and 3. In Equation 3 the velocity constant (k14) between the reduced enzyme and K3 was found to be much higher than k−1 by stopped flow kinetic experiments. Here, if the apparent Km(NADPH) can be substituted for Km(NADPH), k14 can be calculated to be 1146 s−1 from Km(NADPH) = (k−1 + k14/k14), in which k14 ≫ k−1. With a similar assumption, k43 was calculated to be 1.66 × 10^9 M−1 s−1 from Km(K3) = (k−4 + k43/k43), in which k43 ≫ k−4. It is clear that Equation 4 is rate limiting in the over-all reaction of DT diaphorase. The significant inhibition of enzyme activity in the presence of the reduced K3 may also account for this low k14 value (Fig. 9).

Another important aspect of DT diaphorase is concerned with the interaction of substrate (K3) or inhibitor (dicumarol) with the oxidized enzyme. In the steady state and stopped flow kinetic experiments the prior incubation of the native enzyme with either K3 or dicumarol gives some evidence for the possible formation of both a partially active complex and a fully inactive complex, respectively, leading us to the concepts as indicated in Equations 6, 7, and 8.

\[
\begin{align*}
E \cdot \text{FAD} & \xrightarrow{\text{NADPH}} E \cdot \text{FADH}_2 \xrightarrow{K_3} E \cdot \text{FAD} \\
E \cdot \text{FAD} & \xrightarrow{\text{Albumin}} E \cdot \text{FAD} - K_3 \\
E \cdot \text{FAD} & \xrightarrow{\text{Dicumarol}} E \cdot \text{FAD} - \text{Dicumarol} \\
E \cdot \text{FAD} & \xrightarrow{\text{Dicumarol}} E \cdot \text{FAD} - \text{Dicumarol} \\
\end{align*}
\]

Equation 6 is an abbreviation of Equations 1, 2, 3, and 4. The differences among Equations 6, 7, and 8 involve the following facts. In Equation 7 the enzyme-K3 complex is reducible by NADPH but the reduced complex is not oxidized by K3, indicating the partially active species. In Equation 8 none of the enzyme-dicumarol complex is reducible by NADPH, indicating the fully inactive species. It can be mentioned as evidence for Equation 7 that the native enzyme preincubated with K3 at the same concentration was reduced rapidly by NADPH as a rate almost comparable with that of the enzyme in the absence of K3, whereas the subsequent oxidation of reduced enzyme became much more sluggish and followed apparently zero order kinetics (Fig. 4). The indication is that the reaction depends only on dissociation of the reduced enzyme-K3 complex.

Direct evidence for the formation of the enzyme-K3 and enzyme-dicumarol complex was obtained from the binding experiments using [14C]K3 and [14C]dicumarol. These experiments show that one mole of dicumarol was bound per mole of enzyme, though the binding ratio of K3 to enzyme was somewhat lower (Table IV). Another interesting aspect of the binding study is that while the native enzyme can bind both K3 and dicumarol, the reduced form of the enzyme binds only K3 (Table IV).

This means that the native DT diaphorase has two independent binding sites for K3 and dicumarol in its active center and in the reduced form of the enzyme the site binding dicumarol disappears, whereas the site binding K3 remains.

Of particular interest in relation to the marked enzyme inhibition by either K3 or dicumarol is the observation that bovine serum albumin counteracts this inhibition which proved to be competitive with respect to added serum albumin (Fig. 7). Several papers have stressed the association of K3 and dicumarol with serum protein fractions, especially with albumin (14, 15); therefore, the binding experiments were also carried out using [14C]K3 and [14C]dicumarol. It was demonstrated that bovine serum albumin is capable of binding either K3 or dicumarol, although the affinity of K3 for this simple protein was much smaller than that of dicumarol. Equations 7 and 8 indicate that bovine serum albumin facilitates the dissociation of the enzyme-K3 and enzyme-dicumarol complexes.
remain unclear. We feel that there may be significant conformational differences between the native enzyme and the reduced one since the reduced enzyme is more stable than the oxidized enzyme at the elevated temperatures (Table II) and reveals higher fluorescence polarization (0.130) than native enzyme (0.106).1

Finally, it should be pointed out that further studies should include the interaction of NADH with DT diaphorase in the presence of the various compounds mentioned above, since NADH is also a hydrogen donor, albeit a rather poor one, for this enzyme.

Acknowledgment—We want to express our appreciation to Dr. M. Nishikimi, Department of Biochemistry, Nagoya University School of Medicine, Nagoya, Japan, for help with stopped flow kinetic experiment.

REFERENCES

7. FIESER, L. F. (1940) J. Biol. Chem. 133, 301-306
10. RADOLA, B. J. (1965) J. Chromatogr. 38, 61-77

1 S. Hosoda and W. Nakamura, unpublished results.
Properties and Reaction Mechanism of DT Diaphorase from Rat Liver
Syun Hosoda, Wataru Nakamura and Kazuko Hayashi


Access the most updated version of this article at [http://www.jbc.org/content/249/20/6416](http://www.jbc.org/content/249/20/6416)

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

[Click here](http://www.jbc.org/content/249/20/6416.full.html#ref-list-1) 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/249/20/6416.full.html#ref-list-1](http://www.jbc.org/content/249/20/6416.full.html#ref-list-1)