Further Properties of Spermidine Dehydrogenase from *Citrobacter freundii* IFO 12681

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Spermidine dehydrogenase was crystallized for the first time from the cell-free extract of *Citrobacter freundii* IFO 12681. The enzyme contained one mole of heme b as the prosthetic group and the heme was autooxidizable. Absorption maxima in the visible region were at 561, 530, 428, and 335 nm with the reduced enzyme, while only one peak at 416 nm predominated with the oxidized enzyme. No flavin prosthetic group was detected with the enzyme and PQQ was detected in acid hydrolysate of the enzyme. PQQ appeared to be bound covalently to the enzyme. Thus, the spermidine dehydrogenase of *C. freundii* has been characterized to be a quinohemoprotein. This is the first instance of amine dehydrogenase in which both heme b and PQQ are involved in enzyme activity.

There has been a controversy for these several years whether eucaryotic copper-carbonyl containing amine oxidases and methylothropic methylamine dehydrogenases really contain pyrroloquinolinet quinone (PQQ) as the cofactor. A new idea has come out11 that the amine oxidase contains 6-hydroxy-dopa, TOPA, or topaquinone in the active site instead of covalently bound PQQ. With respect to the cofactor of methylamine dehydrogenases from methylothrops, available evidence has been obtained indicating PQQ,23 pro-PQQ,36 or TTQ (tryptophan and tryptophylquinone).41 In our study on the quinohemoprotein amine dehydrogenase from *Pseudomonas* sp.,5 digested chromophore fragments activated quinoprotein apo-quinol dehydrogenase, and we proposed that a covalently bound PQQ was involved in the enzyme. Unlike methyamine dehydrogenase, pseudomonad amine dehydrogenase contains a heme c component like the other quinohemoprotein dehydrogenases.6 A similar situation is found in which methanol dehydrogenases from methylothrops and alcohol dehydrogenases from *Pseudomonas aeruginosa* and *P. putida* lack a heme component but alcohol dehydrogenases from acetic acid bacteria and *P. testosteroni* contain cytochrome c.7

In our previous report,8 the occurrence of spermidine dehydrogenases in various microorganisms was surveyed. Some enzymatic properties of the purified enzymes from *Pseudomonas aeruginosa* and *Citrobacter freundii* have also been reported, documenting that the enzymes are localized in the soluble fraction and are hemoproteins with molecular weights of about 63,000. For this paper, further characterization of the enzyme from *C. freundii* was done with respect to the nature of the cofactors.

Materials and Methods

**Chemicals.** Polyamines and *ω*-aminooctyl agarose were purchased from Sigma Chemical Co. Sep-Pak cartridge was a product of Waters Ltd. PQQ was prepared as reported previously.9

**Culture conditions.** The bacterial strain used and culture conditions were the same as reported previously.8

**Assays.** The assay of spermidine dehydrogenase was done by the method described previously8 with some modifications. It was done in a mixture containing 0.5 μmol of spermidine, 10 μmol of potassium ferricyanide, and 25 μmol of Tris-Cl buffer, pH 8.0, in a total volume of 1.0 ml. The reaction mixture containing all of this except potassium ferricyanide was incubated at 25°C for 5 min. The reaction was started by the addition of potassium ferricyanide and stopped by adding 0.5 ml of ferric sulfate-Dupanol reagent; then, 3.5 ml of water was added to the reaction mixture. A ferric sulfate-Dupanol11 reagent was composed of 5 g of Fe(SO₄)₃·nH₂O, 3 g of Dupanol (sodium dodecyl sulfate), and 95 ml of 85% phosphoric acid, making the total volume to 1 liter with distilled water. The resulting Prussian blue color was measured with a spectrophotometer at 660 nm after standing for 20 min at 25°C. One unit of enzyme activity was defined as the amount of enzyme which catalyzed oxidation of 1 μmol of spermidine per min under the conditions described above: 4.0 absorbance units equaled to 1 μmol of spermidine oxidized. Specific activity (units per mg of protein) was based on the protein estimation by a modification of the Lowry method12 with bovine serum albumin as the standard.

**Purification of enzyme.** Purification of the enzyme was done under essentially the same conditions as reported previously8 except that some modifications were used in the cell-free extract preparation and column chromatographies. Wet cells (75 g) were collected from 50 liters of culture and suspended in 300 ml of 5 mM potassium phosphate buffer, pH 7.2. Cells were disrupted by passing the cell suspension into a French pressure cell press for 2 times at 1,000 kg/cm². The cell homogenate was then treated with sonic oscillation for 5 min at 0–4°C. After 3 repetitions of sonic oscillation, the cell homogenate was centrifuged at 10,000 × g for 10 min to remove undisrupted cells. The resulting cell free extract was further centrifuged at 68,000 × g for 60 min and the resulting supernatant was used for further enzyme purification. The supernatant was put on a DEAE-cellulose column (3 × 2.5 cm), which had been equilibrated with 5 mM potassium phosphate buffer, pH 7.2. The column was washed thoroughly with the same buffer containing 0.1 M KCl. The enzyme activity was eluted by increasing KCl concentrations to 0.2 M. After a 2-times dilution of the pooled enzyme fractions with 5 mM potassium phosphate buffer, pH 7.2. The enzyme solution (5 ml) was precipitated by ultracentrifugation at 100,000 rpm (540,000 × g) for 3 hr. A rose-red precipitate was suspended and homogenized gently in a minimal volume of the same buffer and briefly centrifuged to remove insoluble materials. The enzyme solution (1.5 ml) was put on a Sephadex G-200 column (1 × 80 cm) which had been equilibrated with 50 mM potassium phosphate buffer, pH 7.2. Elution was done at a flow rate of 10 ml/hr and 5-drop (1.3 ml) fractions were collected. Under these conditions, a peak fraction appeared at fraction 80. When the elution was monitored in triplicate, a high absorbance at 280 nm
and 550 nm and enzyme activity, a symmetric elution peak came out having a constant specific activity throughout the elution peak.

**Analytical ultracentrifugation.** Estimation of purity and measurement of sedimentation velocity of the purified enzyme were done with a Hitachi model SCP85H ultracentrifuge at 60,000 rpm with schlieren optics and absorption scanning, respectively.13

**Identification of heme component.** Identification of the heme component involved in the purified enzyme was done by measuring the absorption spectrum and pyridine hemochrome spectrum. Measurement of heme b content was done from the absorption spectrum of pyridine hemochromogen using the molar extinction coefficient of 20.7 cm \(^{-1}\) nm \(^{-1}\), as reported by Falk.14

**Identification of PQQ.** Identification of PQQ in the purified enzyme was done under essentially the same conditions as described for pseudomonal amine dehydrogenase.19 Measurement of PQQ in the acid-digested fraction was done as described previously.13

**Results and Discussion**

**Purification of spermidine dehydrogenase**

The enzyme was readily purified by our previous method.21 When the cell-free extract was fractionated with ultracentrifugation at 68,000 \( \times g \) for 60 min, half of the enzyme activity was detected in the supernatant and the rest in the precipitate containing cytoplasmic membranes. The precipitate was re-homogenized by passing into a French pressure cell press and followed by sonic oscillation in the presence of high salt concentrations (0.25 M potassium phosphate buffer, pH 7.2), and spun down in an ultracentrifuge as above. However, the enzyme activity found in the membrane fraction was not recovered in the supernatant. This means that the enzyme is membrane-bound in nature and solubilized partially during cell disruption as similarly seen with other examples of membrane-bound enzymes. Solubilization of spermidine dehydrogenase from the membrane fraction and further purification are in progress. The enzyme activity collected in the supernatant from ultracentrifugation was put on a DEAE-cellulose, followed immediately by affinity chromatography on an \( \omega \)-aminooctyl agarose column. Since the size of the enzyme (63,000) is known,8 gel filtration chromatography by a long column of Sephadex G-200 (1 \( \times \) 180 cm) could be used. As expected, a symmetric elution peak came out clearly separated from some impurities that appeared at the void volume. After checking the purity of the enzyme by conventional polyacrylamide gel electrophoresis, the enzyme was crystallized using polyethylene glycol 6,000 as the crystallizing agent. Crystallization of the enzyme readily occurred after standing the enzyme solution for a few days in a refrigerator and crystals like fine spindles appeared (Fig. 1). No further increase in specific activity was observed with the crystalline preparation. This means that the purification procedure used here gave an almost

![Crystals of Spermidine Dehydrogenase from *Citrobacter freundii* IFO 12681.](image)

**Table 1.** Summary of Enzyme Purification of Spermidine Dehydrogenase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>14.920</td>
<td>2,675</td>
<td>0.18</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant</td>
<td>6.240</td>
<td>1,240</td>
<td>0.19</td>
<td>1.0</td>
<td>46</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>2.570</td>
<td>950</td>
<td>0.37</td>
<td>2.0</td>
<td>35</td>
</tr>
<tr>
<td>Aminoocetyl agarose</td>
<td>47.5</td>
<td>920</td>
<td>19.37</td>
<td>107.6</td>
<td>34</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>5.1</td>
<td>910</td>
<td>179.80</td>
<td>998.9</td>
<td>34</td>
</tr>
<tr>
<td>Crystallization</td>
<td>5.0</td>
<td>900</td>
<td>180.00</td>
<td>1,000.0</td>
<td>34</td>
</tr>
</tbody>
</table>
complete enzyme purification. A typical result of enzyme purification is summarized in Table I.

Homogeneity
The homogeneity of the crystalline enzyme was checked by both polyacrylamide gel electrophoresis and SDS-gel electrophoresis and confirmed its homogeneity as similarly seen in our previous paper. However, a small difference in electrophoretic mobility in polyacrylamide gel electrophoresis was occasionally observed between two enzyme species, the reduced form and the oxidized form. Two protein bands having enzyme activity sometimes appeared in polyacrylamide gel electrophoresis, unless the enzyme was converted to the reduced form by the addition of excess spermidine before putting it on (data not shown). On the other hand, only a single protein band was always seen in SDS-polyacrylamide gel electrophoresis irrespective of the enzyme species. These observations indicate that the heme component involved is autooxidizable. Some heterogeneity in hydroxyapatite chromatography shown in our previous paper designating as enzyme I and enzyme II may be related to coexistence of the oxidized and the reduced enzyme species, though it has not been confirmed. The crystalline enzyme showed a single sedimentation pattern in analytical ultracentrifugation. The sedimentation constant, $S_{20,w}$ of the enzyme was 4.2 s, indicating that the molecular size of the enzyme of 63,000 is quite reasonable. At the same time, it is clear that the enzyme always exists as a monomerically dispersed form. There were no difference in sedimentation rate between the oxidized enzyme and the reduced enzyme, both of which always gave a constant sedimentation rate.

Absorption spectra
Our previous study indicated the presence of a heme component in the enzyme. In this study, wavelengths of the characteristic peaks were surveyed to know the molecular species of the heme concerned. As shown in Fig. 2, absorption maxima were found at 561, 530, 428, and 335 nm in the visible region with the reduced enzyme, indicating the presence of heme b. On the other hand, with the oxidized enzyme, only one absorption maximum in the visible region was found, at 416 nm, and a low, broad absorbance at the position corresponding to an $\alpha$ band and $\beta$ band appeared. The oxidized enzyme was readily prepared by either standing the enzyme solution in a refrigerator for a few days at 4°C, or oxidizing the enzyme with potassium ferricyanide. The oxidized enzyme had a shoulder at 360 nm, which was converted to a broad but a clear peak at 335 nm upon reduction by the addition of spermidine. These observations strongly indicate that the intramolecular heme component is associated with spermidine oxidation. This is similar to that observed with a quinoprotein glucose dehydrogenase, in which an exogenously added heme b was available as an electron acceptor in glucose oxidation. With flavoproteins, no appreciable absorption maxima are seen in the visible region with the reduced enzyme. These spectral changes suggest the presence of another cofactor that is different from flavins. To identify the molecular species of heme concerned, a pyridine hemochrome was taken as shown in Fig. 3. It was apparent that the $\alpha$ band and $\beta$ band occurred at 556 nm and 523 nm, respectively, indicating the existence of a typical heme b. About 0.9 mol of heme b was found per mole of the enzyme. The presence of heme b in spermidine dehydrogenase was indicated in the enzyme from Serratia marcescens, although the enzyme was obtained from the particulate fraction of the organism.

Cofactor analysis
In addition to heme b, further cofactor analysis was done to see whether the enzyme contains flavin or PQQ. Tabor and Kellogg pointed out the presence of FAD in the enzyme from S. marcescens and partial resolution of the cofactor. With the enzyme from C. freundii the same treatment was done as with the amine dehydrogenase from Pseudomonas sp. Neither flavin nor PQQ was detected in the supernatant

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**Fig. 2.** Absorption Spectra of Spermidine Dehydrogenase.
The absorption spectrum of the oxidized enzyme (solid line) was taken at 1.0 mg per ml in 10 mM potassium phosphate buffer, pH 7.2. The reduced enzyme (broken line) was prepared by adding 2 μl (2 μmol) of spermidine trihydrochloride to the oxidized enzyme (1 ml). After the enzyme was immediately mixed with the substrate, the absorption spectrum of the reduced enzyme was taken.

**Fig. 3.** Absorption Spectrum of the Pyridine Hemochromogen Derivative of Heme in Spermidine Dehydrogenase.
To the crystalline enzyme solution (250 μg), pyridine was added to 20% (v/v). NaOH was added to the mixture to 0.2 N and mixed well. After addition of a small amount of solid sodium hydrosulfite, the absorption spectrum was taken.
after treating the enzyme with 90% methanol. This indicated the possibility of the absence of noncovalently bound FAD and PQQ. The precipitate from the 90% methanol treatment was hydrolyzed in 6 M HCl at 110°C for 48 hr. After neutralization with alkali, the solution was diluted 1,000 times with distilled water to decrease the ionic strength, and then adsorbed into a column of DEAE-Sephadex A-25 (1 x 10 cm) that had been equilibrated with 1 M potassium phosphate buffer, pH 7.0. The column was washed with the buffer containing 0.7 M KCl that elutes flavocompounds from the column. No reaction of flavins was detected with such fractions. The column was then treated with the buffer containing 2 M KCl to elute it at one time. A sample of the eluted fractions was used in the enzyme assay with quinoprotein apo-glucose dehydrogenase as previously mentioned to monitor the appearance of PQQ or its adduct.15 The pooled fractions were passed through a Sep-Pak cartridge to adsorb the cofactor and elution was done with 90% methanol, which is used for the elution of the authentic PQQ. After evaporation of methanol under reduced pressure in a centrifugal concentrator, the residue was dissolved in a small volume of water. A positive response to apo-glucose dehydrogenase was clearly observed with such fractions. This means the presence of PQQ or a PQQ adduct in the chromatophore fraction. The absorption spectrum and fluorescence spectra of the fraction (Fig. 4) were similar to those from other quinoproteins17 and quite different from those of flavin. Although the PQQ content, measured directly, was nearly 0.1 mol PQQ per mole of the enzyme, it is reasonable to conclude that PQQ is involved covalently in the enzyme. Since PQQ is so reactive and is readily converted to its adduct with various compounds and becomes inert,18 the correction of the PQQ measurement is essential to get a probable estimation, because PQQ must be converted to its adduct during acid hydrolysis and subsequent handleings. At this moment, however, there is no absolute method for PQQ measurement with a quinoprotein in which PQQ is involved covalently. To make a stoichiometric interaction with heme b during spermidine oxidation, the PQQ content in spermidine dehydrogenase must be deduced to be nearly one mole per mole of the enzyme. If the enzyme is localized in the periplasmic space or slightly attached to the outer surface of the cytoplasmic membrane facing to the periplasmic space, the possibility of the occurrence of flavin as coenzyme is far less than PQQ, judging from the information accumulated so far.17

As seen with well known examples of membrane-bound flavoproteins such as NADH dehydrogenase19,20 or D-lactate dehydrogenase,21–23 flavoprotein dehydrogenase is specifically localized at the inner surface of the cytoplasmic membrane.

References