Isolation of Rat Liver Mitochondrial Ferredoxin and Its Reductase Active in the 5β-Cholestane-3α,7α,12α-triol 26-Hydroxylase*

Yohko Atsuta and Kyuichiro Okuda
From the Department of Biochemistry, Hiroshima University School of Dentistry, Hiroshima 734, Japan

A ferredoxin-like iron-sulfur protein and a flavoprotein, which reduce cytochrome c in the presence of liver ferredoxin or adrenodoxin, have been solubilized from rat liver mitochondria, partially purified, and 5β-cholestan-3α,7α,12α-triol 26-hydroxylase activity was reconstituted from these proteins together with the purified liver mitochondrial cytochrome P-450.

Liver ferredoxin was purified about 127-fold from rat liver mitochondria through DEAE-cellulose column chromatography and ammonium sulfate fractionation. The optical absorption spectrum of the purified preparation exhibited a peak at 403 nm with a shoulder around 452 nm in the oxidized form, both of which disappeared on reducing with sodium dithionite. The electron paramagnetic resonance spectrum showed signals at 1.94 and 2.03 g-values which are characteristic of ferredoxin. The molecular weight, determined by sodium dodecyl sulfate-gel electrophoresis, was 12,400. The enzymatically reduced liver ferredoxin has been shown to carry out the univalent reduction of oxygen. The superoxide radicals, so generated, were detected by their ability to cause the oxidation of epinephrine to adrenochrome.

A NADPH-cytochrome c reductase, active in the presence of adrenodoxin or liver ferredoxin, was eluted in the void volume of the DEAE-cellulose column for preparation of liver ferredoxin. The optical absorption spectrum of this fraction showed peaks at 442, 367, and 272 nm, which are characteristic of flavoprotein.

5β-Cholestane-3α,7α,12α-triol 26-hydroxylase activity could be reconstituted from these two proteins and the purified liver mitochondrial cytochrome P-450, prepared by the method reported previously.

5β-Cholestane-3α,7α,12α-triol is an intermediate in the conversion of cholesterol to cholic acid and subjected to 26-hydroxylation prior to cleavage of the side chain. The subcellular location of this 26-hydroxylase system has been studied in this laboratory and it was established that the system exists in the inner membrane-matrix region of liver mitochondria (1, 2). Furthermore, it was suggested that the enzyme system contains a member of the cytochrome P-450 family because of its sensitivity to carbon monoxide and phenylisocyanide, although the final conclusion has been withheld owing to an observation that the inhibition by carbon monoxide was not decreased by illumination with white light up to 21.5 klm/m². Conclusive evidence for the participation of cytochrome P-450 in 26-hydroxylation was, however, recently obtained by Okuda et al. (3), who took a photochemical action spectrum exhibiting a maximum at 450 nm. Meanwhile, we have partially purified a novel cytochrome P-450 from the inner membrane fraction of rat liver mitochondria and have shown that it was functional as terminal oxidase for 26-hydroxylation of 5β-cholestan-3α,7α,12α-triol when reconstituted with NADPH-adrenodoxin reductase and adrenodoxin, both purified from adrenocortical mitochondria, and NADPH (4).

These observations seem to suggest strongly that in liver mitochondria there exists an oxidase system involving cytochrome P-450 similar to that of steroidogenic tissues such as the adrenal cortex. An observation reported by Ohashi et al. (5) that bovine liver mitochondria contain NADPH-ferredoxin reductase and ferredoxin, which are immunologically identical with bovine adrenocortical adrenodoxin reductase and adrenodoxin, respectively, seems to provide additional support for the above conclusion. However, the purification from liver of a soluble ferredoxin or of a NADPH-ferredoxin reductase has not so far been reported. In this paper we will describe the partial purification of a ferredoxin-like iron-sulfur protein and a NADPH-ferredoxin reductase from rat liver mitochondria, which are functional for 26-hydroxylation when reconstituted with partially purified liver mitochondrial cytochrome P-450.

MATERIALS AND METHODS

Preparation of Mitochondrial Extract—Mitochondria (1.4 g of protein) were prepared from livers of male Wistar rats weighing 150 to 200 g according to the method described by Wilgram et al. (6). They were suspended in 10 mM potassium phosphate buffer, pH 7.4, to give a concentration of 25 mg of protein/ml and stored overnight in the cold. The suspension was sonicated with cooling in an ice bath for a total period of 3 min with 15-s intervals at 20 KHz and 60 watts output. The sonicated sample was centrifuged at 105,000 g for 60 min. The supernatant was carefully collected and the pellet was washed with phosphate buffer. The combined supernatant and washings were used as starting material for further purification.

Enzyme Assay—NADPH-cytochrome c reductase was assayed according to the modified method of Omura et al. (7) by following the increase in absorbance at 550 nm. The assay mixture contained, in a total volume of 1 ml: 50 μmol of potassium phosphate buffer (pH 7.4), 20 μmol of cytochrome c, 100 μmol of NADPH, 0.32 unit of adrenodoxin reductase (or an appropriate amount of liver ferredoxin reductase), and an appropriate amount of iron sulfur protein fraction (or 1.4 nmol of adrenodoxin). One millimolar KCN was added if necessary. The reaction was started by adding adrenodoxin (or liver ferredoxin) or adrenodoxin reductase (or liver ferredoxin reductase). The initial velocity of reduction of cytochrome c was expressed in terms of micromoles of cytochrome c reduced/min/ml using millimolar extinction coefficient difference of 21.0 (8).

NADPH-diaphorase activity was assayed spectrophotometrically by following the absorbance change at 690 nm at 25°C. The assay mixture contained in a total volume of 1 ml, 100 μmol of potassium phosphate buffer (pH 7.4), 40 nmol of dichlorophenolindophenol, 100 nmol of NADPH, and an appropriate amount of the flavoprotein fraction. The reaction was initiated by adding enzyme. The initial velocity of reduction of dichlorophenolindophenol was expressed in terms of micromoles of dichlorophenolindophenol reduced per min per ml using a millimolar extinction coefficient difference of 21.0 (9).

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The rate of accumulation of adrenochrome was measured according to the method described by Misra et al. (10). The reaction mixture contained 50 μmol of potassium phosphate buffer (pH 7.4), 100 nmol of EDTA, 50 nmol of NADPH, 200 nmol of epinephrine, 0.1 unit of NADPH-adrenodoxin reductase, and the appropriate amount of liver ferredoxin in a final volume of 1.0 ml. The reaction was initiated by adding NADPH and the formation of adrenochrome was followed by measuring the absorbance change at 480 nm. The rate of adrenochrome formation was expressed in terms of nanomoles of adrenochrome formed per min per ml, using a molar extinction coefficient of 4020.

**Isolation of 5β-Cholesterol-3α,7α,12α-triol 26-Hydroxylase System**—The standard reaction mixture contained 10.7 pmol of liver mitochondrial cytochrome P-450, 0.1 unit of bovine adrenodoxin reductase or 12.8 milliunit of liver ferredoxin reductase, 5 nmol of adrenodoxin or 148 pmol of liver ferredoxin, 1.2 μmol of MgCl₂, 20 μmol of potassium phosphate buffer (pH 7.0), 0.5 pmol of NADPH, and 30 nmol of [5β-14C]cholesterol-

As noticed in the figure two fractions revealing NADPH-cytochrome c reductase activity were eluted in the elution volume from 1300 to 1500 ml (peak I) and 1420 to 1700 ml (peak III), of which peak II activity was revealed by the addition of NADPH alone, whereas that of peak III was elicited by the addition of both NADPH and adrenodoxin reductase. The activity found in peak II seemed to be due to NADPH-cytochrome c reductase existing in microsomes or in the mitochondrial outer membrane, or both, according to Ohashi et al. (5) and Pedersen et al. (17), and therefore the fraction was not studied any further. However, the activity in peak III seemed to be due to liver ferredoxin, because NADPH-cytochrome c reductase activity could only be demonstrated in the presence of added adrenodoxin reductase. Fractions of peak III were therefore subjected to further purification. Peak III fractions were combined and were subjected to ammonium sulfate fractionation. Solid ammonium sulfate was added to give a final concentration of 60% saturation. The resulting solution was centrifuged at 13,000 × g for 20 min. The supernatant was dialyzed overnight against 6 liters of 10 mM buffer, then the dialysate was applied to a DEAE-cellulose column (1.7 × 12 cm) equilibrated with 10 mM buffer. After washing the column with 70 ml of 10 mM buffer containing 0.17 M KCl liver ferredoxin was eluted by a gradient from 0.17 to 0.5 M KCl. The fractions corresponding to liver ferredoxin were combined and dialyzed for 20 h against 2 liters of 10 mM buffer. Then the following procedures were applied to concentrate the enzyme solution. The dialysate was applied on a DEAE-cellulose column (1.4 × 5 cm) equilibrated with 10 mM buffer. A reddish yellow band formed on the top of the column was carefully removed and resuspended in a small amount of 10 mM buffer. The cellulose, containing adsorbed protein and was packed in a small pencil-size column, and the column was eluted with 10 mM buffer containing 0.5 M KCl. By these procedures the solution was concentrated 15- to 45-fold. The concentration of the liver ferredoxin was estimated by comparing the NADPH-cytochrome c reductase activity (in the presence of adrenodoxin reductase) with that of known amounts of adrenodoxin. Thus the specific concentration of liver ferredoxin in the final preparation was found to be 2.07 nmol/mg of protein, which implies about 127-fold purification from rat liver mitochondria. This preparation, however, still contained a slight adrenodoxin reductase independent cytochrome c reductase activity. Attempts to further purification have not so far been successful. The results at this purification are summarized in Table I. The ferredoxin preparation could be stored at least for 1 month at −70°C without substantial loss of activity.

**Spectral Properties of Partially Purified Liver Ferredoxin Reductase**—The absorption spectrum of the partially purified ferredoxin reductase in the oxidized state is shown in Fig. 2. As shown in the figure absorption maxima were observed at 272, 367, and 442 nm, respectively. Upon reduction with solid sodium dithionite the maximum at 442 nm disappeared with concomitant bleaching of the color of the solution. These
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FIG. 1. DEAE-cellulose column chromatography of the extract from rat liver mitochondria. The experimental conditions are given in the text. The left side of the inner scale applies to NADPH-cytochrome c reductase activity in peak I fraction (O), and the right side, to that in peaks II (0) and III (O). The enzyme activity of Peak I fraction was measured by adding both 1.4 nmol of adrenodoxin and 0.1 μmol of NADPH to the reaction medium containing 1 mM KCN and that of Peak II fraction, by adding 0.1 μmol of NADPH alone, and that of Peak III, by adding both 0.32 unit of adrenodoxin reductase and 0.1 μmol of NADPH (see the text). O, NADPH-diaphorase activity; ——, A_{410} nm; ——, A_{280} nm; --, KCl concentration.

![Graph](image)

TABLE I

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*The activity represents the velocity of reduction of cytochrome c upon addition of 0.32 unit of adrenodoxin reductase to the reaction medium containing 0.1 μmol of NADPH as described under “Experimental Procedures.” One activity unit is defined as the amount of enzyme catalyzing the reduction of 1 μmol of cytochrome c/min under the given experimental conditions.

**Total yield of iron-sulfur protein at this step estimated to be 0.56 nmol (see the text).

results together with the fact that the enzyme showed NADPH-cytochrome c reductase activity only in the presence of adrenodoxin strongly suggest that the enzyme is a flavoprotein acting as liver ferredoxin reductase.

Spectral Properties of Partially Purified Liver Ferredoxin—The absorption spectra in the oxidized and reduced forms of liver ferredoxin are shown in Fig. 3. It shows a maximum at 408 nm with a slight shoulder around 462 nm. These absorptions are similar to those of adrenodoxin and of renal mitochondrial ferredoxin (17). When the protein was reduced with sodium dithionite, these absorptions disappeared and a new maximum emerged at about 420 nm, which may be due to cytochrome b, contaminating the preparation.
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**FIG. 3.** Absorption spectra of purified peak III fraction. The spectra of the peak III fraction from the third DEAE-cellulose column were measured at a protein concentration of 2.8 mg/ml (specific activity, 1.9 unit/mg of protein). —, oxidized form; ——, dithionite-reduced form.

**FIG. 4.** Electron paramagnetic resonance spectrum of the purified peak III fraction. The sample was reduced enzymatically, i.e. to an aliquot (450 µl) of peak III fraction from the third DEAE-cellulose column were added 0.6 µmol of NADPH and 1.2 unit of adrenodoxin reductase, and the mixture was allowed to stand at room temperature. After 9 min, the spectrum was measured. The conditions for EPR spectrometry were as follows: modulation frequency, 100 KHz; modulation amplitude, 10 G; microwave power, 10 milliwatts; temperature, 77 K; time constant, 3 s. The numbers shown on the spectrum refer to the measured positions of the absorption maxima on the g-value scale.

The ratio of the absorbance at 408 nm to that at 280 nm of the purified sample was 0.23, which is somewhat lower than that of adrenodoxin.

**Electron Paramagnetic Resonance Spectrum of Liver Ferredoxin**—Fig. 4 shows the electron paramagnetic resonance spectrum of the liver ferredoxin, reduced with NADPH and adrenodoxin reductase. As shown in the figure signals were observed at g-values of 1.943 ± 0.002 and 2.028 ± 0.002, which are similar to those reported for the reduced renal ferredoxin (17).

**Disc Gel Electrophoresis of Liver Ferredoxin**—The partially purified liver ferredoxin showed one major band and a few small bands on sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis (Fig. 5). The electrophoresis performed on an ordinary disc gel gave a similar result. To determine which band corresponded to liver ferredoxin, the gel was sliced and extracted with 10 mM buffer containing 0.5 M KCl, and the extracts were incubated with adrenodoxin reductase, cytochrome c, and NADPH to detect NADPH-cytochrome c reductase. As a result only the major band was found to give rise to activity.

**FIG. 5.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified liver ferredoxin preparation. Electrophoresis was carried out under the conditions described under “Experimental Procedures.” The amount of protein applied was 3.5 µg (specific activity, 2.2 unit/mg of protein). The stained gel was subjected to gel scanning using a spectrophotometer equipped with a linear transport.

**FIG. 6.** Determination of the molecular weight of liver ferredoxin by sodium dodecyl sulfate-gel electrophoresis. Electrophoresis was carried out as described in “Experimental Procedures,” using an aliquot of the purified liver ferredoxin (3.5 µg of protein; specific activity, 2.2 unit/mg of protein). Protein standards used were: A, α-chymotrypsinogen (25,700); B, myoglobin (17,200); and C, cytochrome c (12,400). The mobility of the liver ferredoxin was the same as that of cytochrome c (O).
Molecular Weight Estimated by Calibration Polyacrylamide Gel Electrophoresis—The partially purified liver ferredoxin was subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gel calibrated with proteins of known molecular weight. The results are shown in Fig. 6. By this technique liver ferredoxin was found to have an apparent molecular weight of 12,400, which is similar to that of renal ferredoxin.

Generation of Superoxide Radical during Autooxidation of Liver Ferredoxin—Clostridial and spinach ferredoxin as well as adrenodoxin, reduced enzymatically, have been shown to carry out the univalent reduction of oxygen (10). It has been tested whether liver ferredoxin shows a similar reduction of oxygen or not, by detecting the superoxide by their ability to cause the oxidation of epinephrine to adrenochrome. Fig. 7 shows the dependence of the rate of adrenochrome formation on the concentration of liver ferredoxin. Increasing the concentration of liver ferredoxin resulted in a nearly proportional increase of adrenochrome formation.

Reconstitution of NADPH-Cytochrome c Reductase Activity—Reconstitution of NADPH-cytochrome c reductase activity has been performed by using liver ferredoxin reductase and liver ferredoxin preparations, both from liver mitochondria. When the concentration of liver ferredoxin was fixed and that of liver ferredoxin reductase was varied, the rate of cytochrome c reduction was proportional to the amount of the reductase added (Fig. 8). Conversely, when the concentration of the liver ferredoxin reductase was fixed and that of liver ferredoxin was varied, the rate of cytochrome c reduction increased with the increase of liver ferredoxin (Fig. 9).

Reconstitution of 5β-Cholestane-3α,7α,12α-triol 26-Hydroxylase—Reconstitution of the liver mitochondrial 26-hydroxylase activity was performed in three different ways. One was carried out using liver ferredoxin, adrenodoxin reductase, and liver mitochondrial cytochrome P-450; the second one was done using liver ferredoxin, liver ferredoxin reductase, and liver mitochondrial cytochrome P-450; and the third one was carried out using adrenodoxin, adrenodoxin reductase, and liver mitochondrial cytochrome P-450, which was the same as that described before (4). As shown in Table II the enzyme activity could be reconstituted effectively in these reconstituted systems. Elimination of any one of cytochrome P-450, non-heme iron protein (adrenodoxin or liver ferredoxin) or reductase (adrenodoxin reductase or liver ferredoxin reductase) resulted in complete or nearly complete loss of the activity. A slight activity observed in the absence of non-heme iron protein seems to be due to the direct electron flow from flavin to cytochrome P-450 or to the presence of a small amount of liver ferredoxin in the partially purified cytochrome P-450 preparation.

Fig. 10 shows the dependence of the 26-hydroxylase activity on the amount of added liver ferredoxin. From this figure the concentration of liver ferredoxin which gave rise to half-maximal velocity was calculated to be 0.24 μM. When this value is compared to that of adrenodoxin (3 to 6 μM), it is clear that liver ferredoxin has more than 10 times the affinity of adrenodoxin for liver mitochondrial cytochrome P-450.
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This non-heme iron protein could be used as the electron transporting carrier between flavin and cytochrome P-450 in the reconstituted system of liver mitochondrial 26-hydroxylase activity. It was therefore concluded that this non-heme iron sulfur protein is functional in the 26-hydroxylase system of the intermediates of cholesterol catabolism.

During the preparation of this manuscript, Pedersen et al. (18) have reported the isolation of a ferredoxin-like protein in bovine liver mitochondria, which is functional in the system reconstituted from adrenocortical mitochondrial cytochrome P-450 and adrenodoxin reductase. It may be surmised that our present rat liver ferredoxin preparation may be similar to that isolated by them. Nonetheless, there are some differences between both preparations. Thus our sample showed an absorption peak at 408 nm as compared to 414 nm (18), and there is a slight difference in electron paramagnetic resonance spectra between them.

The specific content of liver ferredoxin in liver mitochondria was calculated to be 16.3 pmol/mg of protein. This value is much higher than that reported by Pedersen et al. (18), but is lower than that described by Ohashi et al. (5).

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REFERENCES


Figure 10. Dependence of reconstituted 5β-cholestan-3α,7α,12α-triol 26-hydroxylase activity on the amount of liver ferredoxin added. The reconstitution was carried out as described under "Experimental Procedures" using 0.1 unit of bovine adrenodoxin reductase.
Isolation of rat liver mitochondrial ferredoxin and its reductase active in the 5beta-cholestan-3alpha, 7alpha, 12alpha-triol 26-hydroxylase.
Y Atsuta and K Okuda


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