ISOLATION FROM BOVINE LIVER MITOCHONDRIA OF A SOLUBLE FERREDOXIN ACTIVE IN A RECONSTITUTED STEROID HYDROXYLATION REACTION

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SUMMARY. An iron-sulfur protein has been isolated from bovine liver mitochondria and purified 140-fold on DEAE-cellulose and Sephadex G-100. During the isolation the protein was detected by its NADPH-cytochrome c reductase activity in the presence of adrenal NADPH-ferredoxin reductase. The molecular weight of the protein (12,400), the optical spectrum (peaks at 414 nm and 455 nm which disappear upon reduction), and the EPR spectrum ($g_x = g_y = 1.935$ and $g_z = 2.02$) were typical for a ferredoxin. In the presence of soluble adrenal cytochrome P_{450}, ferredoxin reductase and NADPH, this protein could support the formation of pregnenolone from cholesterol. Under similar conditions, but in the presence of a cytochrome P_{450} solubilized from rat liver mitochondria, cholesterol was transformed into a more polar compound tentatively identified as 26-hydroxycholesterol.

Steroid hydroxylation reactions are known to take place in the mitochondria of steroidogenic tissues such as the adrenal cortex (1,2), the testis and ovary (3) and the placenta (4). Three redox active enzyme components take part in these reactions: a flavoprotein (or NADPH-ferredoxin reductase), a ferredoxin (an iron-sulfur protein), and cytochrome P_{450} (1,5).

Recently a steroid 26-hydroxylation reaction involved in bile acid formation, has been demonstrated in liver mitochondria (6,7). This reaction is inhibited by carbon monoxide (6,7) and thus is most likely cytochrome P_{450} dependent. The presence in liver mitochondria of a cytochrome P_{450} nor of a soluble ferredoxin has so far not been demonstrated.

The present communication describes the isolation of a soluble ferredoxin-type iron-sulfur protein from bovine liver mitochondria. We have demonstrated that this protein is active in a reconstituted steroid hydroxylation system composed of NADPH, a NADPH-ferredoxin reductase and either soluble adrenal cytochrome P_{450} or a cytochrome P_{450} solubilized from rat liver mitochondria.

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MATERIALS AND METHODS

The liver mitochondrial ferredoxin was prepared from 1.4 kg of bovine liver essentially as described for the isolation of a renal ferredoxin (8). The mitochondria were prepared by standard procedure in 0.25 M sucrose containing 15 mM Hepes[x] buffer, pH 7.4, and 0.5 mM EGTA. During the isolation procedure the iron-sulfur protein was detected by its NADPH-cytochrome c reductase activity in the presence of added adrenal ferredoxin reductase (8).

Bovine adrenal ferredoxin (adrenodoxin) was prepared essentially as described in the procedure of Orme-Johnson and Beinert (9). The ratio A_{414nm}/A_{280nm} of the preparation used was 0.81. NADPH-ferredoxin reductase was partially purified from bovine adrenal cortex mitochondria (10). After sonication the mitochondrial extract was subjected to ammonium sulfate fractionation (35 to 65% saturation) followed by 2 consecutive gel filtrations on Sephadex G-100 and G-50 respectively. The final preparation was dissolved in 0.3 M Mops buffer pH 7.4 containing 20% glycerol. The specific NADPH-cytochrome c reductase activity in the presence of excess adrenal ferredoxin was 800 nmol cytochrome c reduced x mg^{-1} x min^{-1}. Essentially no activity was detected in the absence of added ferredoxin. The concentration of the preparation was determined from the absorbance at 450 nm using a millimolar extinction coefficient of 11.3 (11).

Cytochrome P_{450} was prepared from bovine adrenal cortex mitochondria by a modification of the method of Mitani and Horie (12) (N.R. Orme-Johnson and R. White-Stevens, personal communication). The principal modification consists in introducing 2% butanol at the low cholate extraction step. Also 1 mM dithiothreitol was used in all buffers, and 25% glycerol was added to the buffer at the high cholate extraction step. The cholate was removed as described (8). The specific content of the preparation was determined from the CO difference spectrum of the reduced sample (13) and found to be 3.17 nmol/mg protein. Cytochrome P_{450} solubilized from rat liver mitochondria by the same procedure contained 0.15 nmol/mg protein. Protein was determined by the method of Lowry et al. (14). (For comments to this method see ref. 8.)

NADPH-cytochrome c reductase activity was essayed at 25°C essentially as described by Foster and Wilson (10). The reaction medium contained the following in 600 µl of 0.1 M Mops buffer pH 7.4: 0.6 µmol glucose-6-phosphate, 25 nmol KCN, 0.5 µmol cytochrome c, 12.5 nmol NAD 0.1 unit glucose-6-phosphate dehydrogenase. The reaction was started by the addition of 44 pmol of adrenal ferredoxin reductase to the sample cuvette. Under these conditions linear reaction rates were observed as well as proportionality between rate and amount of enzyme (ferredoxin) present up to an upper limit determined by the amount of ferredoxin reductase. The reduction of cytochrome c was followed at 550 nm on a Shimadzu MPL 50 recording spectrophotometer with full scal deflection corresponding to 0.2 absorbance units. A millimolar extinction coefficient of 20.5 for the reduced minus the oxidized form was used for calculation (15).

The optical spectra were recorded at room temperature on a Cary 118 spectrophotometer. Electron paramagnetic resonance (EPR) spectroscopy was performed on a Varian E-9 spectrometer equipped with an Oxford Instrument temperature control system.

x) Abbreviations:
Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid;
Mops, 3-(N-morpholino)-propanesulfonic acid;
Tris, 2-amino-2(hydroxymethyl)-1,3-propandiol.

The conversion of cholesterol to pregnenolone by soluble enzyme components was essayed in an incubation mixture that contained in 1 ml of 0.1 M Mops buffer, pH 7.4: 1 µmol glucose-6-phosphate, 0.5 unit glucose-6-phosphate dehydrogenase, 1 µmol $MgCl_2$, and 24,000 cpm of [4-^{14}C] cholesterol (specific activity 58 mCi/mmol) in 20 µl of aceton. (No unlabelled cholesterol was added. After gas chromatographic determination of the endogenous cholesterol of the cytochrome P_{450} preparation, the specific activity in the incubation mixture was found to be approximately 10,000 cpm/µg cholesterol). The amounts of enzymes added are given in Table 2. The reaction was started by the addition of 50 nmol of NADP and continued for 30 min at 30°C under oxygen. The reaction was stopped by the addition of 5 ml of chloroform:methanol (2:1). After extraction overnight a 2 phase system was established by the addition of 5 ml of chloroform and 2.5 ml H_2O. The chloroform phase was reduced to a small volume and pregnenolone formation was estimated on duplicate aliquotes of the lipid extract by radioactive essay after separation had been achieved by thin layer chromatography (2).

In reconstitution experiments with cytochrome P_{450} solubilized from rat liver mitochondria [1α, 2α, (n)-^{3}H] cholesterol with a specific activity of 43 Ci/mmol was used as a substrate. To each incubation flask 0.83 µCi of labelled cholesterol was added (no unlabelled substrate was added). The amounts of enzymes added are given in Table 3. The incubations and extractions were performed as above and the separation of labelled compounds were achieved by thin layer chromatography (16).

All chemicals and biochemicals were standard commercial high purity materials.

RESULTS

From the supernatant of a liver mitochondrial sonicate a ferredoxin-like activity could be detected by its ability to reconstitute a NADPH-cytochrome c reductase in the presence of adrenal ferredoxin reductase. The enzyme could be purified 140-fold with a yield of about 2% (Table 1) by a procedure similar to the one described for the isolation of a renal ferredoxin. The preparation was found to be rather labile and in sipte of precautions taken such as the inclusion of 20% glycerol and 0.5 mM dithiothreitol in the elution buffers a considerable amount of activity was lost during the isolation procedure.

The elution volume of the active fractions from a Sephadex G-100 column was exactly the same as for cytochrome c from horse heart (MW 12,384). This suggests a molecular weight of about 12,400 for the isolated protein which is similar to that of adrenodoxin and of the previously reported renal mitochondrial ferredoxin (8).

The absolute oxidized spectrum of the purified material exhibited a peak at 414 nm and a shoulder at 455 nm (Fig. 1). The peaks disappeared upon reduction with dithionite concomittant with a small

TABLE 1

PURIFICATION OF LIVER MITOCHONDRIAL FERREDOXIN

Purification step	Total protein (mg)	Total activity+ (units)	Specific activity (units x mg^{-1})	Purification	Yield (%)
Mitochondrial extract	18382	33413	1.82	1	100
First DEAE-cellulose	936	19892	21.2	12	60
Second DEAE-cellulose	221	14292	65	36	43
First Sephadex G-100	49.2	9167	186	103	27
Second Sephadex G-100	13.0	2454	189	104	7.3
Sephadex G-75++	2.43	638	262	144	1.9

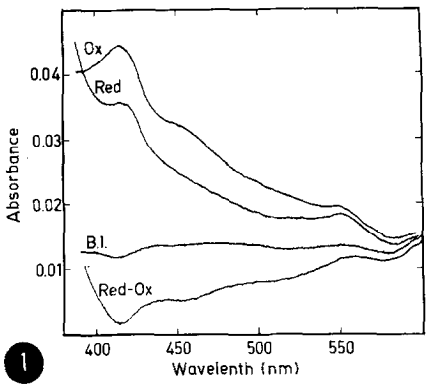
+ NADPH-cytochrome c reductase measured as described in MATERIALS AND METHODS. One activity unit is defined as the amount of enzyme catalyzing the reduction of 1 nmol of cytochrome c x min^{-1} under the given experimental conditions.

++ Total yield of iron-sulfur protein at this step estimated to be 1.5-1.9 nmol (see text).

but discernible increase at 550 nm. These features, most clearly seen in the reduced minus oxidized difference spectrum (Fig. 2) are all characteristic for both the adrenal and the testis ferredoxins (17). As can be seen in the Soret region the absolute reduced spectrum was complicated by a small amount of not identified heme.

An EPR spectrum of the isolated material reduced enzymatically by NADPH in the presence of adrenal ferredoxin reductase (Fig. 2) permitted the conclusion that the preparation contained a ferredoxin-like iron-sulfur protein. The g-values recorded were $g_x = g_y = 1.935$ and $g_z = 2.02$ i.e. identical to those reported for reduced adrenal and renal ferredoxins (8).

The spin concentration was determined by integrations of the EPR spectrum using a copper standard and found to be 1.6 μM for the preparation shown in Fig. 2. The concentration of the liver ferredoxin could also be estimated by comparing the enzymatic activity (NADPH-cytochrome c reduction in the presence of ferredoxin reductase) to that of known amounts of adrenal ferredoxin. This last method gave a value 76% of that obtained by the EPR method. From the yield of the purest fraction (see Table 1), the yield of protein and the molecular weight determination it might be inferred that the preparation



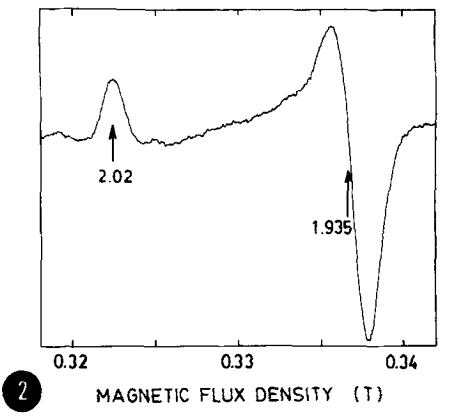


Fig. 1. Absorption spectra of liver ferredoxin. After recording the baseline (B.l.) the oxidized spectrum (Ox) of a sample diluted to 0.62 mg of protein per ml of buffer (0.1 M KCl, 0.05 M Tris, pH 7.4, 0.5 mM DTT, 20% glycerol) was recorded. The reduced spectrum (Red) was recorded after addition of 2.5 mM dithionite (dissolved in anaerobic 1 M Tris base) to the sample. The reduced minus oxidized (Red - Ox) spectrum was recorded after replacing the buffer in the reference cuvette by an oxidized sample also at a concentration of 0.62 mg protein per ml.

Fig. 2. Electron paramagnetic resonance spectrum of enzymatically reduced liver ferredoxin. To 250 µl of a sample of liver ferredoxin (3.5 mg protein per ml, side fraction from the gel filtration on Sephadex G-75) was added 0.2 µmol NADPH in 20 µl and 44 pmol ferredoxin reductase in 10 µl. The spectrum was recorded after 6 minutes at room temperature. The conditions for EPR spectroscopy were: microwave frequency, 9120 MHz, and power, 20 µW (higher power caused saturation); temperature, 25 K. The g-values are indicated in the figure.

is not highly pure. Also the ratio A_{414nm}/A_{280nm} was found to be 0.15 which is considerably lower than usually found for purified adrenal ferredoxin (see above). The specific NADPH-cytochrome c reductase activity in the presence of excess ferredoxin reductase was found to be 509 nmol cytochrome c reduced x mg^{-1} x min^{-1}. By disc gel electrophoresis on 7% polyacrylamid gel 3 fast moving bands were observed (not shown).

The isolated liver ferredoxin was found to be active in a reconstituted hydroxylation system composed of NADPH, soluble adrenal cytochrome P_{450}, adrenal ferredoxin reductase and labelled cholesterol. It is seen (Table 2) that liver ferredoxin can substitute for adrenal ferredoxin in this system and that the formation of pregnenolone depends on the amount of liver ferredoxin added.

TABLE 2

CONVERSION OF CHOLESTEROL TO PREGNENOLONE BY SOLUBLE ENZYME COMPONENTS

For incubation conditions see Materials and Methods

Components present in the incubation medium	Incubation number 1	'2	'3	'4	'5	'6
Adrenal cytochrome P_{450} (nmol)	0.92	0.92	0.92	0.92	0.92	0.92
Adrenal ferredoxin reductase (pmol)	-	66	66	66	66	66
Adrenal ferredoxin (nmol)	-	-	-	-	-	1.52
Liver ferredoxin (pmol)+	-	-	2.4	24	244	-
Pregnenolone formation (%)	10.1	13.6	18.2	50.8	83.3	87.1

+ Concentration determined on the basis of enzyme activity (NADPH-cytochrome c reductase) as described in the text.

DISCUSSION

The properties of the isolated protein are markedly similar to those of adrenal ferredoxin. These similarities are emphasized by their molecular weights, their optical and EPR spectra. The amount of labile sulfur relative to the iron content of the preparation was not possible to determine because of the small amount of protein available. Enzymatically the liver ferredoxin can substitute for adrenal ferredoxin in a reconstituted NADPH-cytochrome c reductase (in the presence of NADPH-ferredoxin reductase). In fact, this reaction enabled us to detect the protein during its isolation. Furthermore, this protein can substitute for adrenal ferredoxin in a reconstituted cytochrome P_{450} dependent hydroxylase. Thus, it must be concluded that the liver and adrenal ferredoxins are fundamentally similar both in structure and catalytic behaviour.

Until recently it was generally considered that soluble mitochondrial iron-sulfur proteins of this type were only present in typical steroidogenic tissues such as the adrenal glands, the gonads and the placenta. A liver "mitochondrial" fraction which supported 11β-hydroxylation in the presence of bovine adrenal "mitochondrial" particles has been reported, however, but no iron-sulfur protein was detected in that preparation (18). A new development in this area was the isolation from rachitic chick kidneys of a mitochondrial ferredoxin active in the 25-hydroxyvitamin D_3-1α-hydroxylase system (8)

TABLE 3

HYDROXYLATION OF CHOLESTEROL BY SOLUBLE CYTOCHROME P_{450} FROM RAT LIVER MITOCHONDRIA

For incubation conditions see Materials and Methods

Components present in the incubation medium	Incubation number 1	2	3	4	5
Liver mitochondrial cytochrome P_{450} (nmol)	0.162	0.162	0.162	0.162	0.162
Liver ferredoxin (pmol)+	-	-	122	122	-
Adrenal ferredoxin reductase (pmol)	-	66	-	66	66
Adrenal ferredoxin (nmol)	-	-	-	-	1.5
Product formation (%)++	0.1	0.3	1.4	7.6	9.0

+ See foot note Table 2.

++ Based on its chromatographic behaviour on TLC (16) the product has tentatively been identified as 26-hydroxycholesterol.

It is remarkable that the cytochrome P_{450} dependent hydroxylases of both the adrenal cortex and the kidney mitochondria are physiologically strictly regulated to the needs of the organism. It may be speculated if similar hydroxylation reactions also take place in liver mitochondria. The most likely candidate is the steroid 26-hydroxylase involved in bile acid formation (6,7). The inhibition of this hydroxylase by CO is maximally reversed by light at 450 nm (19) which strongly suggests the involvement of a cytochrome P_{450}. The soluble enzyme components of this hydroxylase have previously not been identified, however. Recently we have solubilized a cytochrome P_{450} from rat liver mitochondria (J.I.Pedersen, to be published). When incubated in the presence of a ferredoxin (either the adrenal or liver ferredoxin) adrenal ferredoxin reductase and NADPH, this cytochrome catalyzes the conversion of cholesterol to a more polar form, that we have tentatively identified as 26-hydroxycholesterol (Table 3). Work is now in progress towards the characterization of the physical and catalytic properties of this newly isolated heme protein.

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