Purification and biochemical characterization of hepatic ferredoxin (hepatoredoxin) from bovine liver mitochondria

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Received 24 September 1985

Hepatic ferredoxin (hepatoredoxin) was purified from bovine liver mitochondria. The monomeric molecular mass of the hepatoredoxin was larger than that of adrenocortical ferredoxin (adrenodoxin) from bovine adrenocortical mitochondria at 14 kDa. We studied the amino acid residues and NH_2-terminal sequence of this protein. The hepatoredoxin was organ-specific protein. The optical absorption spectrum of oxidized hepatoredoxin had two peaks, at 414 and 455 nm in the visible region. Hepatoredoxin formed an immunoprecipitin line against anti-adrenodoxin immunoglobulin in Ouchterlony double diffusion, and an immunochemical staining band in Western blotting.

Ferredoxin *Iron-sulfur protein (Bovine liver)* *Nonheme iron protein* *Mitochondrial* *Cytochrome P-450-linked monooxygenase* *Cytochrome P-450-oxygenase system*

1. INTRODUCTION

Ferredoxin in mammalian tissues is a component of the monooxygenase system linked with cytochrome P-450 in mitochondria [1–4]. NADPH-ferredoxin reductases of cattle are organ-specific and the hepatic ferredoxin from bovine hepatocytes has been tentatively named hepatoredoxin [5,6]. We purified hepatoredoxin from bovine liver mitochondria and characterized it biochemically. Here, we discuss the amino acid residues, molecular mass, immunochemical properties and amino-terminal sequence of this protein. Hepatoredoxin was compared with bovine adrenocortical ferredoxin (adrenodoxin).

2. MATERIALS AND METHODS

2.1. *Materials*

Livers of 3-year-old Holstein-Friesian cattle were obtained from a local slaughterhouse, chilled on ice and brought immediately to the laboratory. The livers were perfused with ice-cold 1.15 M KCl solution adjusted with 1 M Tris to pH 7.4 to remove blood through the portal vein. Fat, connective tissue and the main blood vessels were carefully removed. The liver was then cut into small pieces and homogenized in 5 vols ice-cold 0.25 M sucrose solution adjusted with 1 M Tris solution to pH 7.4. The mitochondrial fraction of the liver was prepared using the method of Hatefi and Lester [7].

2.2. *Enzyme assays*

The activity of hepatoredoxin was measured in the presence of NADPH-adrenodoxin reductase and cytochrome *c* as the activity of NADPH-cytochrome-*c* reductase, by the method of Hiwatashi et al. [8].

2.3. *Protein*

Protein was assayed by the method of Lowry et al. [9] or by the biuret reaction [10], using bovine serum albumin as the standard.

2.4. *Amino acid analysis*

The amino acid residues were analyzed as described elsewhere [8] on a Hitachi 835 amino acid analyzer.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

2.5. *Sequence of amino acid residues*

We determined the sequence of amino acid residues on an Applied Biosystems, model 470A, sequencer.

2.6. *Electrophoresis*

SDS-polyacrylamide slab gel electrophoresis was done by the method of Laemmli [11]. Bovine adrenodoxin (12.5 kDa), horse heart cytochrome *c* (12.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), and bovine serum albumin (67 kDa) were used as the standard proteins.

2.7. *Serology and immunochemistry*

Antibody to adrenodoxin was prepared as in [2].

The Ouchterlony double diffusion test was done on a slide glass covered with 1.0% (w/v) agar in 0.86% (w/v) NaCl solution containing 0.02% (w/v) NaN_3. The center well was filled with the antibody to adrenodoxin and the outer wells with bovine hepatoredoxin and adrenodoxin. The agar plate was placed in a dark box at 4°C for 2 days. Wells were 3 mm in diameter and the center to center distance between the antigen and the antibody wells was 5 mm.

2.8. *Western blotting*

The amount of hepatoredoxin was estimated immunochemically using various concentrations of bovine adrenodoxin as the standard, by the method of Towbin et al. [12].

2.9. *Reconstitution of 25- and 27-hydroxylations of steroids*

Reconstitutions of 25- and 27-hydroxylations of cholecalciferol and 5-cholestane-3,7,12-triol were done as described in [2] and according to Atsuta and Okuda [13], respectively.

2.10. *Chemicals*

The chemical reagents used were of the highest grade commercially available.

3. RESULTS

The concentration of hepatoredoxin in the liver was 0.09 ± 0.01 nmol/mg protein. That of adrenodoxin in adrenocortices was 2.65 ± 0.07 nmol/mg protein. There was 3–4% as much hepatoredoxin as adrenodoxin.

Purification of hepatoredoxin was done at 0–4°C. Mitochondria were suspended in 10 mM Na-phosphate buffer, pH 7.4. The solution (16 mg protein/ml × 7300 ml) was homogenized twice for 5 min using a Matsushita homogenizer (model MX-150S), and the solution was sonicated by a Waken sonicator (model W-225R) for a total of 10 min. The sonicated mitochondria were centrifuged at 65000 × *g* for 90 min in a refrigerated Hitachi 55p ultracentrifuge with a refrigerated barrel rotor. The brown supernatant was put on a DEAE-cellulose column (5 × 29 cm) equilibrated with 30 mM K-phosphate buffer, pH 7.4. The column was washed with 1 l of 30 mM K-phosphate buffer. Next, the active fraction of hepatoredoxin was eluted with 50 mM K-phosphate buffer, pH 7.4, containing 0.13 M KCl. The eluted solution was diluted with the same volume of 50 mM K-phosphate buffer, pH 7.4, and put on a DEAE-cellulose column (2.9 × 15 cm) equilibrated with 50 mM K-phosphate buffer, pH 7.4. The column was washed with 50 mM K-phosphate buffer, pH 7.4, containing 60 mM KCl and the sample eluted with 50 mM K-phosphate buffer, pH 7.4, containing 0.3 M KCl. The eluted solution was diluted with a 2.5-fold volume of 50 mM K-phosphate buffer, pH 7.4, and put on a DEAE-cellulose column (1.7 × 10 cm) equilibrated with 50 mM K-phosphate buffer, pH 7.4. The column was washed with 50 mM K-phosphate buffer, pH 7.4, containing 86 mM KCl. The sample was eluted with 50 mM K-phosphate buffer, pH 7.4, containing 0.4 M KCl. The eluted solution was dialyzed against 50 mM K-phosphate buffer, pH 7.4, and used as the partial purified hepatoredoxin.

The optical absorption spectrum of the partial purified hepatoredoxin is shown in fig.1. The solution contained hepatoredoxin with strong activity of NADPH-cytochrome-*c* reductase in the presence of NADPH-adrenodoxin reductase. The spectrum of oxidized hepatoredoxin had peaks at 414 and 455 nm in the visible region. The reduced form did not absorb light. The hepatoredoxin solution was studied by SDS-polyacrylamide gel electrophoresis. The main protein band was at 14 kDa and some contaminants were found on the slab gel. We found by Western blotting that the main protein band is identical with hepatoredoxin. This

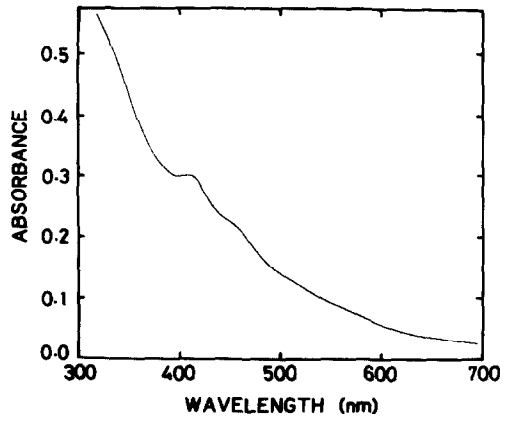


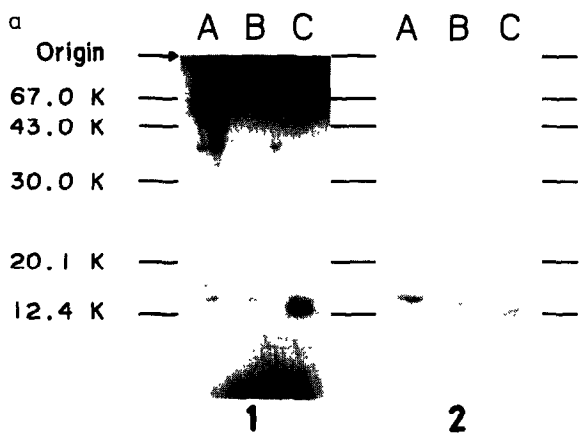
Fig.1. Absolute optical absorption spectrum of oxidized hepatoredoxin in the visible region. The cuvette contained 30 μM hepatoredoxin in 0.1 M K-phosphate buffer, pH 7.4, at 25°C.

band of hepatoredoxin was obtained by preparative electrophoretic fractionation. The solution of hepatoredoxin was lyophilized before study on the amino acid analyzer and sequencer. The lyophilized preparation was seen electrophoretically and immunochemically to be a single protein band and a single immunochemical staining band against nitrocellulose sheet. The results and densitometric scanning patterns are shown in fig.2. The monomeric molecular mass of hepatoredoxin was estimated electrophoretically to be 14 kDa (fig.3).

The amino acid composition and amino-terminal sequence of the hepatoredoxin are given in table 1. Larger amounts of Glx and Pro and small amounts of Asx, Thr, Ile, His, and Arg were present.

Ouchterlony double diffusion was done to compare adrenodoxin with hepatoredoxin. The results of the Ouchterlony double diffusion test are in fig.4. In the test, an immunochemical precipitin line against the antibody to adrenodoxin was formed. Spur formation between hepatoredoxin and adrenodoxin was not seen, although the amino acid residue composition and the NH_2-terminal sequence of hepatoredoxin differed from those of adrenodoxin.

Purified hepatoredoxin was useful for the reconstitution of 25- and 27-hydroxylations of steroids such as cholecalciferol, cholestanediol, cholestanetriol and dihydroxycholestenone (not shown).



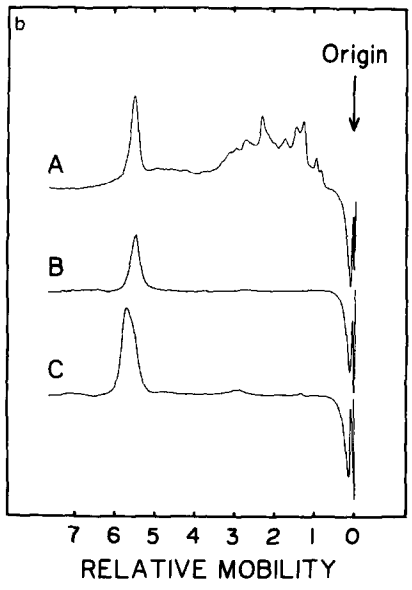


Fig.2. (a) 1, electrophoretic pattern of hepatoredoxin on SDS-polyacrylamide gel. The slab gel received 5 μg hepatoredoxin. Lanes: A, crude hepatoredoxin; B, purified hepatoredoxin; C, bovine adrenodoxin. 2, immunochemical staining nitrocellulose sheet of samples A, B and C. (b) Densitometric scanning patterns of the slab gel [(a), 1].

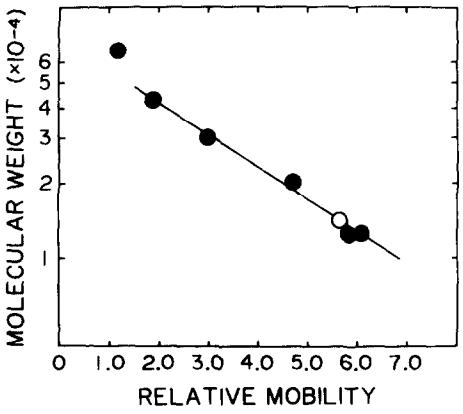


Fig.3. Estimation of the monomeric molecular mass of hepatoredoxin by SDS-polyacrylamide gel electrophoresis using 15% (w/v) acrylamide. The amount of the sample and marker proteins was 5 μg each. (○) Hepatoredoxin, (●) marker proteins.

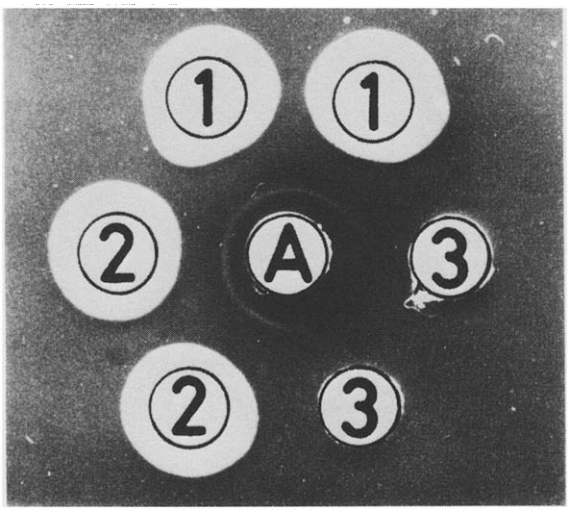


Fig.4. The Ouchterlony double-diffusion test. Wells: A, antibody to adrenodoxin; 1, hepatoredoxin; 2, adrenodoxin; 3, saline.

Table 1

Amino acid composition and amino-terminal sequence of hepatoredoxin

Composition	Residue/ molecule[a]	NH_2-terminal sequence	
		Cycle	Amino acid
Asx	13.9	1	X
Thr	6.3	2	Val
Ser	8.7	3	Arg
Glx	22.2	4	Lys
Pro	5.8	5	Phe
Gly	10.1	6	Thr
Ala	8.4	7	Glu
Val	8.3	8	Lys
Met	2.4	9	His
Ile	5.1	10	Glu
Leu	11.3	11	X
Tyr	4.2	12	Val
Phe	4.4	13	Thr
Lys	8.4	14	Thr
His	1.4	15	[Glu]
Trp	1.3	16	[Asp]
Arg	2.4	17	[Gly]
Cys	[5.0]		
M_r	13900		

[a] The amino acid composition of hepatoredoxin was determined by duplicate analysis and expressed based on an M_r of 14000

Adrenodoxin

^{1}Ser–Ser–Ser–Gln–^{5}Asp–Lys–Ile–Thr–Val–^{10}His–
^{11}Phe–Ile–Asn–Arg–^{15}Asp–Gly–Glu–

Hepatoredoxin

^{1}X–Val–Arg–Lys–^{5}Phe–Thr–Glu–Lys–His–^{10}Glu–
^{11}X–Val–Thr–Thr–^{15}Glu–Asp–Gly–

Fig.5. Amino acid sequences of NH_2-terminal portions of hepatoredoxin and adrenodoxin.

4. DISCUSSION

Hepatoredoxin and adrenodoxin had different chemical properties. The amino acid composition of the hepatoredoxin differed greatly from adrenodoxin, and resembled that of bovine renal ferredoxin (renoredoxin) [4,14]. The primary structures of hepatoredoxin and adrenodoxin differed completely. The NH_2-terminal sequences of these 2 iron-sulfur proteins are given in fig.5.

The monomeric molecular mass of hepatoredoxin found by electrophoresis was larger than that of adrenodoxin. This result was not due to an artifact produced with β-mercaptoethanol [15]. However, the immunochemical precipitin reaction of

hepatoredoxin in Ouchterlony double diffusion was similar to that of adrenodoxin. Hepatoredoxin formed an immunoprecipitin line against antibody to adrenodoxin; the line seemed to be caused by fuse formation.

The optical absorption spectrum of the hepatoredoxin had 2 peaks in the visible region, suggesting that hepatoredoxin is of 2Fe-2S type [16].

ACKNOWLEDGEMENT

We are grateful to Mr Akira Miyatake for determinations of amino acid composition, and NH_2-terminal sequence.

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