Heterogeneity of adrenocortical ferredoxin

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Bovine adrenocortical ferredoxin (adreno-ferredoxin) was purified from adrenocortical mitochondria by an improved method that included hydrophobic chromatography on Toyopearl gels. The purified ferredoxin was electrophoretically homogeneous. It was further separated into five fractions by hydrophobic chromatography on a TSK-gel phenyl-5PW column with a high-pressure liquid chromatography system. The properties of the three main fractions were examined. The fractions had identical absorption spectra and almost the same activity in an NADPH-cytochrome *c* reducing system. Their amino-terminal sequences all corresponded to the reported sequence, but the carboxyl-terminal residues were glycine or serine, not alanine as reported. These results indicate that these adreno-ferredoxins had additional amino acid residues at the carboxyl end. It seems that adreno-ferredoxin extracted from mitochondria undergoes proteolytic attack during purification to become heterogeneous.

Ferredoxin *Adrenocortex* *Hydrophobic chromatography* *Iron-sulfur protein* *Heterogeneity*

1. INTRODUCTION

Adrenocortical ferredoxin (adreno-ferredoxin) is a protein of a low molecular mass with an iron-sulfur cluster of 2Fe-2S type and participates in electron transport from NADPH to cytochrome P-450 in the metabolism of steroid hormones in adrenocortical mitochondria. The ferredoxin was first noticed by Ichikawa and Yamano [1,2] as unusual signals at $g_m = 1.94$ and $g_s = 2.01$ during electron paramagnetic resonance (EPR) observation of bovine adrenocortical mitochondria. The protein containing iron with this EPR property was isolated from pig adrenocortical mitochondria by Kimura and Suzuki [3,4] and tentatively named adrenodoxin. Bovine adreno-ferredoxin has been highly purified to a homogeneous protein with a molecular mass of 12 kDa, but some investigators have reported that bovine adrenocortical mitochondria have two different ferredoxins [5,6]. Sakihama et al. [7,8] have developed a chromatographic method for separating two spinach ferredoxins that had not been separated before in native form with a TSK-gel phenyl-5PW column by hydrophobic high-pressure liquid chromatography (HPLC). Here we used hydrophobic chromatography to find whether bovine adreno-ferredoxin also contains more than one component.

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2. MATERIALS AND METHODS

2.1. *Purification of bovine adreno-ferredoxin*

Bovine adrenal glands were obtained from a local slaughterhouse and adrenocortical mitochondria were prepared from them by a method reported elsewhere [9]. The purification of adreno-ferredoxin from the mitochondria was improved by the introduction of hydrophobic column chromatography with Toyopearl HW-65C and butyl-Toyopearl 650M according to the purification method for plant ferredoxins [10]. The method included the following purification steps: i, extraction by sonication from the mitochondria;

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ii, batch-wise adsorption with DEAE-cellulose; iii, removal of impure proteins by ammonium sulfate precipitation at 60% saturation; and iv, hydrophobic chromatography with Toyopearl HW-65C and butyl-Toyopearl 650M columns. Details will be reported elsewhere.

2.2. *Separation of the purified adreno-ferredoxin by HPLC with a phenyl-5PW column*

Analytical hydrophobic chromatography of the purified adreno-ferredoxin was done on a TSK-gel phenyl-5PW column (0.75 × 7.5 cm) with a Toyo Soda HLC-CCPM HPLC system following the method of Sakihama et al. [7]. For preparation, the ferredoxin was separated on a large phenyl-5PW column (2.2 × 15 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 1.6 M ammonium sulfate with the same buffer containing 1.45 M ammonium sulfate. Phenyl-5PW columns were obtained from the Toyo Soda Manufacturing Co.

2.3. *Assay of enzymatic activity*

The enzymatic activity of adreno-ferredoxin was assayed in an NADPH-cytochrome *c* reducing system [9].

2.4. *Amino acid analysis*

Amino acid analysis was done on an automated amino acid analyzer (Hitachi model 835) using the method of Spackman et al. [11]. The amino acid sequence was obtained with an Applied Biosystems sequencer (model 470A) according to Laursen [12]. The carboxyl-terminal amino acid was identified by hydrazinolysis as in [13,14].

3. RESULTS AND DISCUSSION

Bovine adreno-ferredoxin was purified more easily by the improved method of hydrophobic chromatography with Toyopearl gels than by the conventional method. The highly purified preparation migrated as a single band on SDS-polyacrylamide gel electrophoresis and had an absorbance ratio, A_{414}/A_{280}, of 0.9, the highest purity reported so far [15]. Its molecular mass was estimated to be 14 kDa by SDS-polyacrylamide gel electrophoresis and 13 kDa by gel filtration HPLC on a TSK-gel G3000SW column. These observations confirmed that the highly purified preparation was homogeneous in monomeric form. However, the preparation was further separated into five fractions by hydrophobic HPLC with a phenyl-5PW column (fig.1). The five fractions of adreno-ferredoxin already existed in the crude preparation obtained after the batch-wise adsorption step in this purification (fig.2). Fig.2 also shows that the elution profile of the five fractions changed during incubation at room temperature (20°C), suggesting that adreno-ferredoxin was attacked by proteolytic enzymes during purification. To investigate properties of the individual fractions, we rapidly purified adreno-ferredoxin and immediately separated the fractions using a preparative phenyl-5PW column. Fractions III, IV and V were obtained in sufficient amounts, but fractions I and II were not. We therefore studied only the three main fractions extensively. They had identical absorption spectra; that of fraction V is shown in fig.3. No large difference in electron transport activity in an NADPH-cytochrome *c* reducing system [9] was found between them. The fractions migrated as a single band on SDS-polyacrylamide gel electrophoresis, where their molecular mass was estimated to be 14 kDa. This value was slightly larger than that reported elsewhere (12–13 kDa; [16,17]). Tanaka et al. [18]

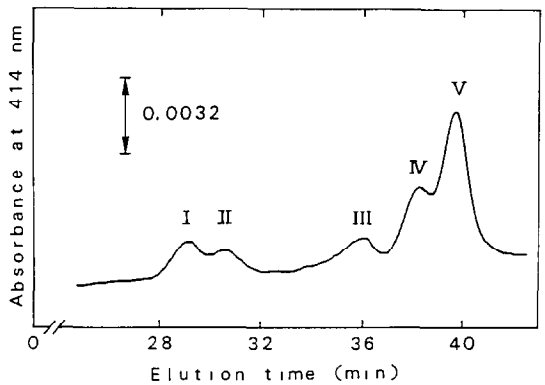

Fig.1. Separation of highly purified bovine adreno-ferredoxin by hydrophobic HPLC. 10 μg of adreno-ferredoxin was loaded on a TSK-gel phenyl-5PW column (0.75 × 7.5 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 2 M ammonium sulfate and eluted with a 40-min linear gradient of ammonium sulfate (2–1 M) at a flow rate of 1 ml/min. Adreno-ferredoxin was detected by measurement of absorbance at 414 nm.

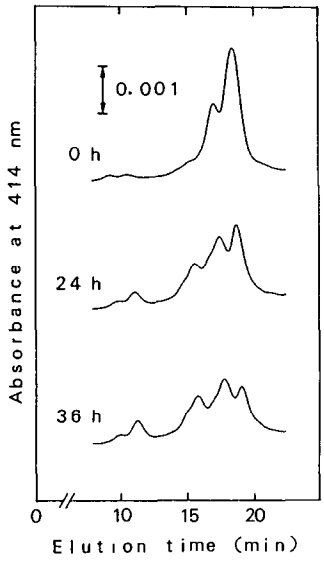

Fig.2. Detection of adreno-ferredoxin fractions in a crude preparation by hydrophobic HPLC. The crude adreno-ferredoxin preparation that was freshly prepared was incubated at 20°C for the indicated number of hours and analyzed by hydrophobic HPLC as in fig.1, except that a 20-min linear gradient of ammonium sulfate (1.5–1 M) was used.

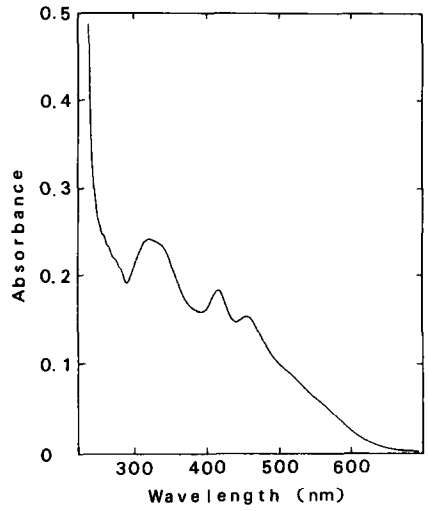

Fig.3. The optical absorption spectrum of adreno-ferredoxin fraction V in the oxidized form. The spectrum was recorded in 20 mM Tris-HCl buffer, pH 7.4, with a Shimadzu spectrophotometer (model UV-240).

reported that bovine adreno-ferredoxin has 114 amino acid residues including 18 Asx, 7 Ser, 11 Glx, 8 Gly, 3 Met, 8 Ile, 6 Lys and 5 Arg, with a molecular mass of 12.5 kDa. According to Okamura et al. [19] it contains 128 amino acid residues including 20 Asx, 10 Ser, 13 Glx, 9 Gly, 5 Met, 10 Ile, 6 Lys, and 5 Arg, with a molecular mass of 14 kDa. This group deduced the primary structure of bovine adreno-ferredoxin by nucleotide sequencing of the protein gene and found an additional peptide at the carboxyl-end, which was composed of 14 amino acid residues, 2 Asx, 3 Ser, 2 Glx, 1 Gly, 2 Met, 2 Ile, 1 Lys, and 1 Arg (fig.4). The amino acid compositions of our adreno-ferredoxins are shown in table 1. They have 20 Asx, 7–10 Ser, 13 Glx, 10 Gly, 4–5 Met, 8–9 Ile, 5 Lys, and 5 Arg in 123–126 residues on the basis of a molecular mass of 14 kDa. Their amino-terminal sequences were identified up to 18 cycles. They all had the same sequence of NH_2-Ser-Ser-Ser-Glu-Asp-Lys-Ile-Thr-Val-His-Phe-Ile-Asn-Arg-Asp-Gly-Glu-Thr-, which was the same as that reported by Okamura et al. [19]. The carboxyl-terminal amino acids of fractions III and IV were found to be glycine and serine, respectively, rather

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                                  114                 120                 128
Okamura et al.  ---Asp-Ala-Arg-Glu-Ser-Ile-Asp-Met-Gly-Met-Asn-Ser-Ser-Lys-Ile-Glu-COOH

Tanaka et al.   ---Asp-Ala-COOH

Fraction III    ---Asp-Ala-Arg-Glu-Ser-Ile-Asp-Met-Gly-COOH

Fraction IV     ---Asp-Ala-Arg-Glu-Ser-Ile-Asp-Met-Gly-Met-Asn-Ser-COOH

Fraction V a)   ---Asp-Ala-Arg-Glu-Ser-Ile-Asp-Met-Gly-Met-Asn-Ser-Ser-COOH

a) Main component of Fraction V.
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Fig.4. Proposed carboxyl-terminal sequences of bovine adreno-ferredoxin fractions.

Table 1

Amino acid compositions of bovine adreno-ferredoxin fractions

Amino acids	Highly purified prep.	Fractions					Tanaka et al.[a]	Okamura et al.[b]
		III	IV		V			
Asx	20.47	20.72	21.19	(21.0)	20.65	(21.0)	18	20
Thr	8.95	9.59	9.37	(10.2)	9.52	(10.0)	10	10
Ser	6.63	7.74	7.13	(10.0)	8.53	(9.8)	7	10
Glx	13.91	13.55	13.05	(13.1)	13.29	(13.2)	11	13
Pro	1.38	0.88	0.65	(1.2)	0.90	(2.6)	1	1
Gly	10.13	9.94	9.98	(10.2)	10.34	(10.2)	8	9
Ala	7.28	7.52	7.53	(7.8)	7.49	(7.3)	7	7
Val	7.00	6.82	6.87	(6.2)	6.71	(5.8)	7	7
Met	4.80	4.57	5.08	(5.2)	4.84	(5.0)	3	5
Ile	8.54	8.71	8.80	(8.6)	8.70	(8.2)	8	10
Leu	12.73	12.67	12.74	(12.8)	12.38	(12.2)	12	12
Tyr	1.09	1.01	1.00	(0.9)	0.99	(0.8)	1	1
Phe	4.07	4.40	4.38	(4.0)	4.33	(3.2)	4	4
Lys	5.69	5.28	5.25	(5.2)	5.10	(4.9)	5	6
His	3.09	3.12	3.62	(3.0)	3.16	(2.8)	3	3
Trp	0.0	0.0	0.0	(n.d.)	0.0	(n.d.)	0	0
Arg	6.10	5.85	5.82	(5.4)	5.67	(5.4)	4	5
Cys	n.d.	n.d.	n.d.	(n.d.)	n.d.	(n.d.)	5	5
Total	(125)	(124)	(123)	(126)	(125)	(126)	(114)	(128)

[a] By protein sequencing [18]
[b] By nucleotide sequencing [19]

Calculated after 24 h hydrolysis based on a molecular mass of 14 kDa without an iron-sulfur cluster. The value in parentheses was determined by extrapolation of amino acid analysis data after 12, 24 and 48 h hydrolysis

than the alanine reported by Tanaka et al. [18]. The carboxyl-terminal amino acid of fraction V was mainly serine and glycine was also found as contaminant.

Our highly purified adreno-ferredoxin preparation was a mixture of fractions with different carboxyl-terminal ends. The probable carboxyl-terminal structure of the three fractions is given in fig.4. The structures were longer than that found by Tanaka's group with Ala-114 and shorter than that found by Okamura's with Glu-128. Okamura et al. [19] proposed that the elongated peptide might be a precursor segment to be processed during the importation of pro-adreno-ferredoxin into adrenocortical mitochondria. However, our adreno-ferredoxin retained a part of the precursor segment. The main component of fraction V is the longest and only three amino acid residues were lacking from that reported by Okamura's group. Since fraction V in the crude preparation decreased during incubation while fractions III and IV increased (fig.2), fractions III and IV were probably derived from the main component of fraction V by proteolytic attack during purification. We have no evidence that fraction V is the intact structure of adreno-ferredoxin that is present in adrenocortical mitochondria.

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