CRYSTALLIZATION AND PROPERTIES OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-ADRENODOXIN REDUCTASE OF PIG ADRENOCORTICAL MITOCHONDRIA

Atsuo HIWATASHI, Yoshiyuki ICHIKAWA and Toshio YAMANO
Department of Biochemistry, Osaka University Medical School, 33 Joan-cho, Kita-ku, Osaka, Japan

Received 8 August 1977

1. Introduction

NADPH-adrenodoxin reductase is a component of an adrenodoxin-linked cytochrome *P*-450 mixed-function oxidase system of adrenocortical mitochondria, and many steroids are hydroxylated by this mixed-function oxidase system [1]. We have reported previously on crystalline NADPH-adrenodoxin reductase from bovine adrenocortical mitochondria [2,3]. This communication describes the crystallization of pig NADPH-adrenodoxin reductase from pig adrenocortical mitochondria by two methods and the properties of the NADPH-adrenodoxin reductase.

2. Materials and methods

Crystalline NADPH-adrenodoxin reductase was purified from pig adrenocortical mitochondria by affinity chromatography using 2′5′-ADP–Sepharose 4B and by a method described previously [2]. Pig adrenodoxin was crystallized by Ichikawa's method [1].

Molecular weight of the reductase was estimated by three methods:

(i) SDS–polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn [4] using 7.5% acrylamide.

(ii) Gel filtration was carried out at 4°C using Sephadex G-100 by Andrew's method [5].

(iii) Analytical ultracentrifugation was carried out by the method of Edelstein and Schachman [6].

Amino acid analysis was performed by the method of Moor and Stein [7] in the absence and presence of 2% thioglycolic acid [8]. The tryptophan and tyrosine contents were also estimated by the method of Beaven and Holiday [9]. The number of sulfhydryl groups was determined by Ellman's method [10] in the absence and presence of 8 M guanidine–HCl.

Total content of sugars was determined by the method of Masamune and Sakamoto [11]. Content of each sugar composition was analyzed by Sinohara's method [12]. Succinate dehydrogenase was assayed by King's method [13]. Glucose 6-phosphatase was assayed by Swanson's method [14]. The 2′5′-ADP–Sepharose 4B and 5′-AMP–Sepharose 4B were synthesized by the method of Brodelius et al. [15]. Glycine–Sepharose 4B was synthesized from 1,6-diaminohexane, glycine and Sepharose 4B according to Cuatrecasas's method [16]. The enzymatic activities of NADPH-adrenodoxin reductase were measured as reported previously [2]. Molar extinction coefficients of NADH and NADPH used were 6.30×10^3 $M^{-1}cm^{-1}$. This value was obtained by McComb et al. [17] and Ziegenhorn et al. [18]. Protein content was determined by the method of Lowry et al. [19] and the biuret reaction [20]. Flavin was determined by paper chromatography with three different solvent systems (1-butanol/acetic acid/H_2O(4:1:5); pyridine/

Abbreviations: 2′5′-ADP–Sepharose 4B, Sepharose 4B-bound N^6-(6-amino-hexyl)-adenosine 2′5′-bisphosphate; 5′-AMP–Sepharose 4B, Sepharose 4B-bound N^6-(6-aminohexyl)-adenosine 5′-monophosphate; glycine–Sepharose 4B, Sepharose 4B-bound (6-aminohexyl)-glycine; SDS, sodium dodecyl sulfate

H_2O(2:1); 5% $NaHPO_4$) [21] and by activation of the apo-enzyme of D-amino acid oxidase, which was prepared by the method of Negelein and Brömel [22]. All reagents were of the best grade available from commercial sources.

3. Results and discussion

Fresh pig adrenal glands were obtained from a local slaughter house and stored in a desiccator at −20°C for a week. Fat and connective tissues of pig adrenal glands were removed from about 1.5 kg whole adrenals with scissors. Unless otherwise indicated, all purification procedures were carried out at below 4°C. The whole adrenal glands were homogenized with 4 vol. ice-cold 0.25 M sucrose solution (adjusted to pH 7.4 with 0.1 M Tris solution) in a Matsushita Model MX-140S homogenizer at a speed of 10 000 rev/min. The homogenate was passed through gauze to remove fat and cell debris. The mitochondrial fraction was prepared from the homogenate of adrenal glands by the method of Hatefi and Lester [23]. The mitochondrial fraction showed the activities of succinate dehydrogenase (205.4 ± 29.9 nmol/min/mg protein) and glucose 6-phosphatase (20.8 ± 8.1 nmol P_i released/min/mg protein). The mitochondrial pellet was suspended in 10 mM Na-phosphate buffer, pH 7.4, at a protein concentration of about 20 mg/ml and homogenized twice for 4 min periods in the homogenizer. The broken mitochondrial fraction was centrifuged at 65 000 × *g* for 60 min in a refrigerated Hitachi 55P ultracentrifuge with a refrigerated barrel rotor. The yellow–brown supernatant fluid was fractionated between 35% and 60% saturation of ammonium sulfate, and the precipitate obtained was dissolved in 75 ml 10 mM K-phosphate buffer, pH 7.4, then dialyzed against the same buffer (10 liters) for 20 h using a magnetic stirrer with three changes of the outer medium. The dialyzed solution was centrifuged at 9000 × *g* for 60 min, then the supernatant was applied to a DEAE-cellulose column (3 × 35 cm) pre-equilibrated with 10 mM K-phosphate buffer, pH 7.4. The column was washed with 2 liters 10 mM K-phosphate buffer, pH 7.4. This removed a red pigment. Then the yellow band of NADPH-adrenodoxin reductase was eluted with 50 mM K-phosphate buffer, pH 7.4, while the brown pigment, adrenodoxin, remained at the top of the column. The eluted NADPH-adrenodoxin reductase (55 ml) was fractionated between 35% and 55% saturation of ammonium sulfate. The precipitate was dissolved in 10 mM K-phosphate buffer, pH 7.4, and dialyzed to remove the ammonium sulfate against 500 vol. (5 liters) of the same buffer for 10 h. Crystalline NADPH-adrenodoxin reductase was obtained from the crude solution of adrenodoxin reductase by the following two methods.

Method 1

The dialyzed adrenodoxin reductase was applied to 2′5′-ADP–Sepharose 4B affinity column (2 × 7 cm) preequilibrated with 10 mM K-phosphate buffer, pH 7.4. The column was washed with 5 column vol. 50 mM K-phosphate buffer, pH 7.4, then the adrenodoxin reductase was specifically eluted with 0.5 mM $NADP^+$ in the buffer. The adrenodoxin reductase was fractionated between 40% and 55% saturation of ammonium sulfate. The precipitate was dissolved in 10 mM K-phosphate buffer, pH 7.4. Next, the solution was passed through a Sephadex G-100 column (1 × 30 cm) preequilibrated with 10 mM K-phosphate buffer, pH 7.4, containing 0.1 M KCl. The eluted main fractions with an absorption ratio of 272 nm/450 nm of 8.0–8.8, were combined and finely powdered ammonium sulfate was added to a 60% saturation. The precipitate was dissolved gradually in 50 mM K-phosphate buffer, pH 7.4, until the solution became slight turbid. Crystallization of NADPH-adrenodoxin reductase (11 mg protein in 0.6 ml) was allowed to proceed at 0°C for a few days.

Method 2

The dialyzed sample solution was treated according to the method of Hiwatashi et al. [2]. The NADPH-adrenodoxin reductase was crystallized by addition of finely powdered ammonium sulfate, until the solution became slightly turbid. The adrenodoxin reductase (9 mg protein in 0.5 ml) crystallized out from the sample solution within a few days at 0°C.

The 2′5′-ADP–Sepharose 4B was particularly better for purification of NADPH-adrenodoxin reductase than 5′-AMP–Sepharose and glycine–

Sepharose affinity chromatographies. Table 1 shows a typical purification scheme for pig NADPH-adrenodoxin reductase. The adrenodoxin reductase obtained after 2′5′-ADP–Sepharose affinity chromatography showed a slightly higher specific activity than DEAE-cellulose chromatography. Affinity chromatography using 2′5′-ADP–Sepharose(Method 1) can be used repeatedly to purify pig NADPH-adrenodoxin reductase, after the column is washed with 1 liter 0.5 M NaCl in 50 mM K-phosphate buffer, at pH 7.4, then 1 liter 10 mM K-phosphate buffer, at pH 7.4. However, the DEAE-cellulose column (Method 2) must be newly prepared for the purification each time. Figure 1 shows crystals of pig NADPH-adrenodoxin reductase. They were rhombic plates. In SDS–polyacrylamide gel electrophoresis, the adrenodoxin reductase was a single band of protein. It was electrophoretically homogeneous and highly pure, as shown in fig.2. Figure 3 shows that the optical absorption spectra of pig NADPH-adrenodoxin reductase had absorption peaks at 272 nm, 377 nm and 450 nm and shoulders at 280 nm, 366 nm, 423 nm and 475 nm in the oxidized form at 25°C. This absorption spectrum is that of a typical flavoprotein. The ratio of the optical density of the peaks at 272 nm and 450 nm was 8.2, and the ratio of those at 450 nm and 377 nm was 1.12.

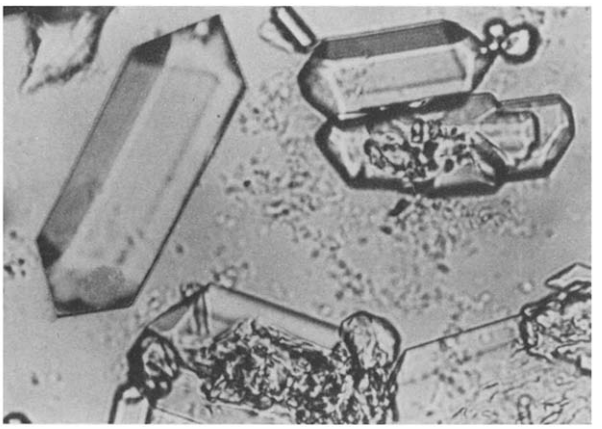

Fig.1. Microphotograph of crystals of pig NADPH-adrenodoxin reductase. These crystals were obtained by Method 1 in this text. The same crystals of pig NADPH-adrenodoxin reductase were also obtained by Method 2. Magnification: × 180.

Table 1
Purification of NADPH-adrenodoxin reductase of pig adrenocortical mitochondria

	Total volume (ml)	Total protein (mg)	Specific activity (μmol/min/mg protein)	Purification (fold)	Total activity (μmol/min)	Yield (%)
Homogenate	5000	89 500	0.03	1	2685	100
Mitochondria	1000	20 150	0.09	3	1814	67.6
Crude extract	875	2280	0.43	14	980	36.5
Ammonium sulfate (35–60%)	75	503	1.15	38	578	21.5
DEAE-cellulose (I)	55	39	8.84	295	345	12.8
Method 1						
2′5′-ADP–Sepharose	16	17	18.63	621	317	11.8
Sephadex G-100	8	13	22.54	751	293	10.9
Crystallization	0.6	11	23.05	768	254	9.5
Method 2						
DEAE-cellulose (II)	18	18	16.23	541	292	10.9
Sephadex G-100	9	11	21.24	708	234	8.7
Crystallization	0.5	9	22.80	760	205	7.6

These activities were monitored with NADPH-ferricyanide reductase activity

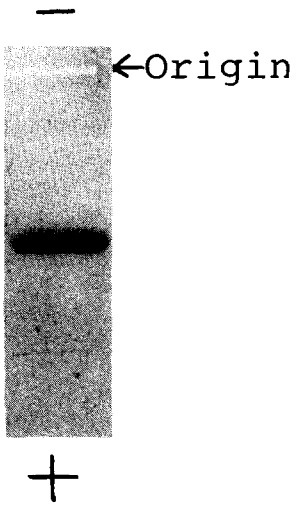

Fig.2. Electrophotograph of pig NADPH-adrenodoxin reductase on SDS–polyacrylamide gel. Five μg protein of the reductase obtained by Method 1 was used.

Table 2 shows the amino acid residue, sugar and flavin contents of NADPH-adrenodoxin reductase of pig and bovine adrenocortical mitochondria. Pig NADPH-adrenodoxin reductase, unlike bovine adrenodoxin reductase was free of sugar. Although the amounts of arginine, asparagine and aspartate residues of bovine adrenodoxin reductase were higher than those of the pig adrenodoxin reductase, the contents of the other amino acid residues were similar. It is not clear whether the arginine, asparagine and aspartate residues are related to the binding of sugar. The flavin of pig adrenodoxin reductase was released by the treatment with acidic ammonium sulfate [22], the prosthetic group of pig adrenodoxin reductase was confirmed to be a single yellow spot with the same mobility as authentic FAD by paper chromatography, and the flavin could activate the apo-enzyme of D-amino acid oxidase. These facts demonstrated that the prosthetic group of pig adrenodoxin reductase was FAD, like that of bovine adrenodoxin reductase [21]. Table 3 summarizes the molecular weight of pig NADPH-adrenodoxin reductase estimated by the three methods. It was estimated to be 49 000 ± 3000.

The activities of NADPH-ferricyanide reductase and adrenodoxin-linked NADPH-cytochrome *c* reductase of pig NADPH-adrenodoxin reductase were 23.1 and 9.51 μmol/min/mg protein at pH 7.4 and 25°C, respectively.

Acknowledgment

We thank Professor H. Sinohara, Department of Biochemistry, Kinki University Medical School, Osaka, Japan, for help in determination of the sugar composition of the reductase.

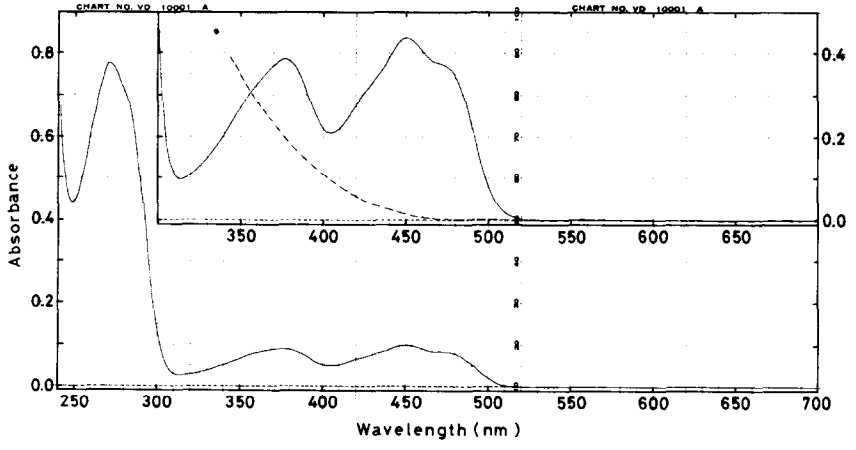

Fig.3. Optical absorption spectra of pig NADPH-adrenodoxin reductase. The cuvette contained 0.5 mg protein/ml (2.1 mg protein/ml for magnified spectra in the visible region) in 0.1 M K-phosphate buffer, pH 7.4 (25°C). (——) Oxidized form; (– – – –) dithionite-reduced form; (- - - - -) base line.

Table 2
Amino acid residue, sugar and flavin contents of pig and bovine NADPH-adrenodoxin reductases

	Pig	Bovine
Amino acid[a]		
Aspartate + Asparagine	29.4	37.7
Threonine	22.4	22.9
Serine	30.3	23.5
Glutamate + Glutamine	42.4	48.9
Proline	35.3	33.8
Glycine	42.1	40.9
Alanine	34.4	35.8
Cysteine	5.3[d]	6.1[d]
Valine	29.0	27.1
Methionine	4.0	6.0
Isoleucine	13.8	14.0
Leucine	36.9	41.1
Tyrosine	6.8	8.3
Phenylalanine	12.4	14.1
Histidine	8.2	9.3
Lysine	18.4	19.0
Tryptophan	13.3[d] (9.2)[e]	14.1[d] (10.8)[e]
Arginine	23.7	29.9
Sugar[b]		
Ribose	0.0	0.0
Rhamnose	0.0	0.0
Mannose	0.0	13.5
Fucose	0.0	1.6
Galactose	0.0	3.5
Xylose	0.0	0.0
Glucose	0.0	1.4
Glucosamine	0.0	6.2
Galactosamine	0.0	0.0
Mannosamine	0.0	0.0
Sialic acid	0.0	1.3
Flavin[c]		
FAD	1.0	1.0
FMN	0.0	0.0

[a] Values are amino acid residues/mol NADPH-adrenodoxin reductase
[b] Values are mol sugar/mol adrenodoxin reductase
[c] Values are mol flavin/mol adrenodoxin reductase
[d] Values were obtained spectrophotometrically
[e] Values in parentheses were obtained in the presence of 2% thioglicolic acid

Table 3
Molecular weight of pig NADPH-adrenodoxin reductase

Method	Mol. wt
Analytical ultra-centrifugation	45 000[a]
SDS–polyacrylamide gel electrophoresis	53 000
Sephadex G-100 gel filtration	50 000

[a] Partial specific volume 0.73

References

[1] Ichikawa, Y. (1971) in: Mitochondria (Hagihara, B. ed) pp. 266–291, Asakura Publishing Co., Tokyo.
[2] Hiwatashi, A., Ichikawa, Y., Maruya, N., Yamano, T. and Aki, K. (1976) Biochemistry 15, 3082–3090.
[3] Hiwatashi, A., Ichikawa, Y., Yamano, T. and Maruya, N. (1976) Biochemistry 15, 3091–3097.
[4] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[5] Andrew, P. (1964) Biochem. J. 91, 222–233.
[6] Edelstein, S. J. and Schachman, H. K. (1967) J. Biol. Chem. 242, 306–311.
[7] Moor, S. and Stein, W. H. (1963) Meth. Enzymol. 6, 819–831.
[8] Matsubara, H. and Sasaki, R. M. (1969) Biochem. Biophys. Res. Commun. 35, 175–181.
[9] Beaven, G. H. and Holiday, E. R. (1952) Adv. Protein Chem. 7, 319–386.
[10] Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[11] Masamune, H. and Sakamoto, M. (1956) Tohoku J. Exp. Med. 63, 345–355.
[12] Sinohara, H. (1977) Insect Biochem. 7, 3–4.
[13] King, T. E. (1967) Meth. Enzymol. 10, 322–331.
[14] Swanson, M. A. (1967) Meth. Enzymol. 2, 541–543.
[15] Brodelius, P., Larsson, P.-O. and Mosbach, K. (1974) Eur. J. Biochem. 47, 81–89.
[16] Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059–3065.
[17] McComb, R. B., Bond, L. W., Burnett, R. W., Keech, R. C. and Bowers, G. N., jr (1976) Clin. Chem. 22, 141–150.
[18] Ziegenhorn, J., Seen, M. and Bücher, Th. (1976) Clin. Chem. 22, 151–160.
[19] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[20] Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) J. Biol. Chem. 177, 751–760.
[21] Suhara, K., Ikeda, Y., Takemori, S. and Katagiri, M. (1972) FEBS Lett. 28, 45–47.
[22] Negelein, E. and Brömel, H. (1936) Biochem. Z. 300, 225–239.
[23] Hatefi, Y. and Lester, R. L. (1958) Biochim. Biophys. Acta 27, 83–88.