PURIFICATION AND CRYSTALLIZATION OF NADPH-ADRENODOXIN REDUCTASE FROM BOVINE ADRENOCORTICAL MITOCHONDRIA

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1. Introduction

Flavoprotein and iron sulfur protein as components of an electron transport system have been shown to form a complex under certain conditions [1,2]. Chu and Kimura [3] demonstrated that the complex formation between adrenodoxin reductase and adrenodoxin depends on the ionic strength of the environment.

We have succeeded in showing the complex formation of adrenodoxin reductase with immobilized adrenodoxin on Sepharose gel and achieving a high purification in one step. By the novel purification procedure including the affinity chromatography, the enzyme was crystallized as rhombic plates with a yield of 24% and 200-fold purification from the crude extract.

2. Materials and methods

Bovine adrenals were brought to the laboratory in the frozen state from a slaughterhouse. Crystalline adrenodoxin was prepared by the method of Suhara et al. [4]. NADPH-adrenodoxin reductase activity was assayed by measuring the rate of cytochrome *c* reduction in the presence of adrenodoxin. The assay mixture (1 ml) contained 100 nmol of NADPH, 10 nmol of oxidized cytochrome *c*, 20 nmol of adrenodoxin, 100 μmol of potassium phosphate, pH 7.4, and an appropriate amount of adrenodoxin reductase. One unit of enzymatic activity is defined as 1 μmol of cytochrome *c* reduced per min at 37°C. SDS-polyacrylamide gel electrophoresis was carried out as described by Weber and Osborn [5]. Protein was determined by the method of Lowry et al. [6].

Adrenodoxin reductase was extracted from bovine adrenocortical mitochondria according to the method of Suhara et al. [7], with some modifications.

Sepharose 6B was activated by Cyanogen Bromide (100 mg/ml settled gel) and washed, according to the method of Axén and Ernback [8]. The CNBr-activated gel (30 g) was suspended in 30 ml of 0.1 M sodium bicarbonate, pH 8.0, containing 40 mg of crystalline bovine adrenodoxin (absorbancy ratio at 414 nm/276 nm, 0.78) and gently stirred for 20 hr at 5°C in the dark. After filtration, the gel was resuspended in 30 ml of the same buffer containing 1.0 M glycine and stirred for 4 hr. The free coupling ligands, if any existed, on the Sepharose gel were saturated with glycine. This was confirmed by the fact that 99% of bovine serum albumin was recovered in a void volume through the adrenodoxin-Sepharose column.

3. Results and discussion

The adrenodoxin reductase was adsorbed on the adrenodoxin-Sepharose gel, but the binding capacity was dependent on the NaCl concentration (fig.1). The maximum capacity for the adsorption of the enzyme was obtained under the conditions of 0.07 M NaCl in 0.01 M phosphate buffer, pH 7.4, and the ionic strength being 0.13. The capacity was gradually decreased with increasing the concentration of NaCl, finally no binding was observed at 0.4 M NaCl in the same buffer.

The precipitate of 30–60% saturation of ammonium sulfate was completely dialyzed against 0.01 M phosphate buffer, pH 7.4, containing 0.07 M NaCl. The dialysate was applied to the adrenodoxin-Sepharose column and washed with 0.07 M NaCl in 0.01 M pho-

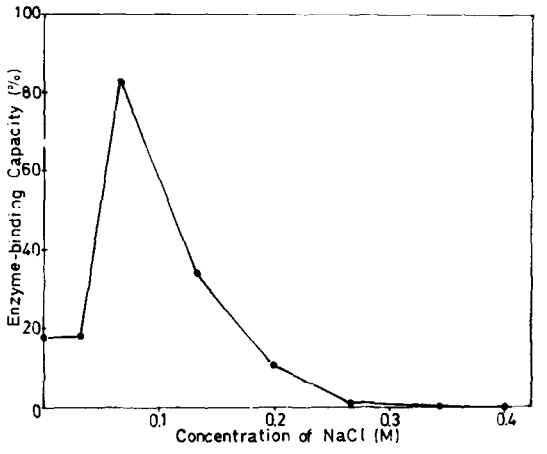

Fig.1. Effect of NaCl on the enzyme binding capacity of adrenodoxin-Sepharose gel. The reaction mixture was composed of 10.5 units of adrenodoxin reductase, 1.0 g of adrenodoxin Sepharose gel, and 5 ml of 0.01 M phosphate buffer, pH 7.4, containing the indicated concentration of NaCl. After gently shaking for 10 min at 5°C, the enzyme-bound gels were sedimented for 10 min at 3 000 *g* and washed with the same buffer. The reductase-binding capacity of each tube was expressed as the recovered activity in the supernatant after addition of 0.4 M NaCl in 0.01 M phosphate buffer, pH 7.4.

phate buffer until an absorbance at 280 nm was attained below 0.05. Fig.2 shows a typical elution pattern. Since the eluted fractions (No. 3–15), which colored dark brown, were rich in adrenodoxin, so these fractions were utilized for preparation of adrenodoxin. The adrenodoxin reductase was eluted by replacing the buffer to 0.4 M NaCl. The enzyme was thereby purified 30-fold in terms of specific activity, free of adrenodoxin, and the ratio of absorbance at 272 nm to 450 nm was shown to be 9.2.

The main fractions (No. 61–63) collected, precipitated by the 55% saturation of ammonium sulfate, dissolved in 0.2 M KCl in 0.01 M phosphate buffer, and then applied to a Sephadex G-100 column. In the void volume, some contaminants appeared (fig.3). The main fractions (No. 17–19) were collected and precipitated as described above. The purified amorphous precipitate was dissolved in a minimum amount of 0.01 M phosphate buffer, pH 7.4. Powdered ammonium sulfate was carefully added to the enzyme solution until about 40% saturation, and a small amount of precipitate was centrifuged off. Additional amount of ammonium sulfate was added most carefully, then the solution became turbid again. Since

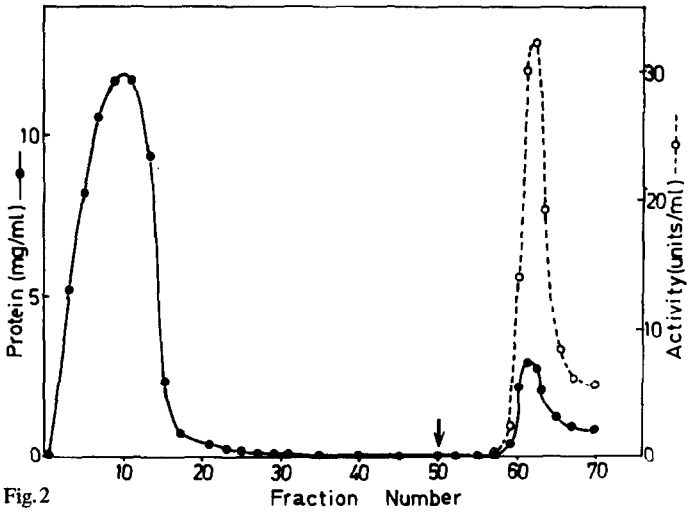

Fig.2

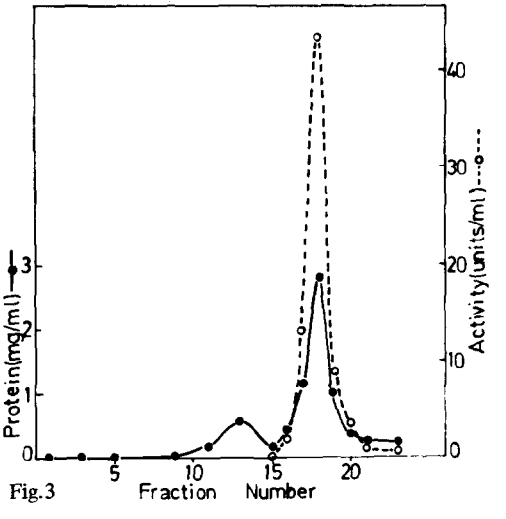

Fig.3

Fig.2. Chromatography of adrenodoxin reductase from bovine adrenocortical mitochondria on the adrenodoxin-Sepharose column. To the column (2 × 10 cm) equilibrated with 0.07 M NaCl in 0.01 M phosphate buffer, pH 7.4, 90 ml of the dialyzed enzyme (18 mg/ml) was applied, and the column was washed with 650 ml of the same buffer. Then the buffer was replaced by 0.4 M NaCl in 0.01 M phosphate buffer, pH 7.4, at the point indicated by the arrow. Fig.3. Further purification of the adrenodoxin reductase following affinity chromatography. The enzyme was precipitated by 55% saturation of ammonium sulfate and its precipitate was dissolved in 0.01 M phosphate buffer, pH 7.4, containing 0.2 M KCl, and then applied on a Sephadex G-100 column (1.5 × 40 cm) previously equilibrated with the same buffer.

Table 1
Purification of adrenodoxin reductase from bovine adrenocortical mitochondria

Steps	Total Volume (ml)	Total Protein (mg)	Specific Activity (units/mg)	Total Activity (units)	Yield (%)
1) Crude Extract	1000	16 000	0.11	1730	100
2) Dialysis	180	3450	0.31	1070	62
3) Affinity Chromatogr.	27	58	10.5	612	36
4) Sephadex G-100	13	33	13.2	436	28
5) Crystallization	1	18	22.3	404	24

at this stage the solubility of the enzyme was very sensitive to a temperature, the solution should be kept in an ise-packed jar. Most of the enzyme was crystallized within a week, and the supernatant solution was essentially colorless. A summary of the purification and crystallization steps is presented in table 1.

Fig.4 shows crystals of adrenodoxin reductase as rhombic plates, and some of them showed rosette-formation. The purity and properties of the crystalline reductase were examined. The enzyme was homogeneous on SDS-gel electrophoresis and showed a specific activity of about 22 units per mg protein. Fig.5 depicts the absorption spectrum of the crystalline reductase, which showed peaks at 272 nm 378 nm and 450 nm, and shoulders at 475 nm and 420 nm. These results are the same as those reported by Suhara et al. [7], but the absorbancy ratio at 272 nm/450 nm was 7.6. The flavin which was liberated from

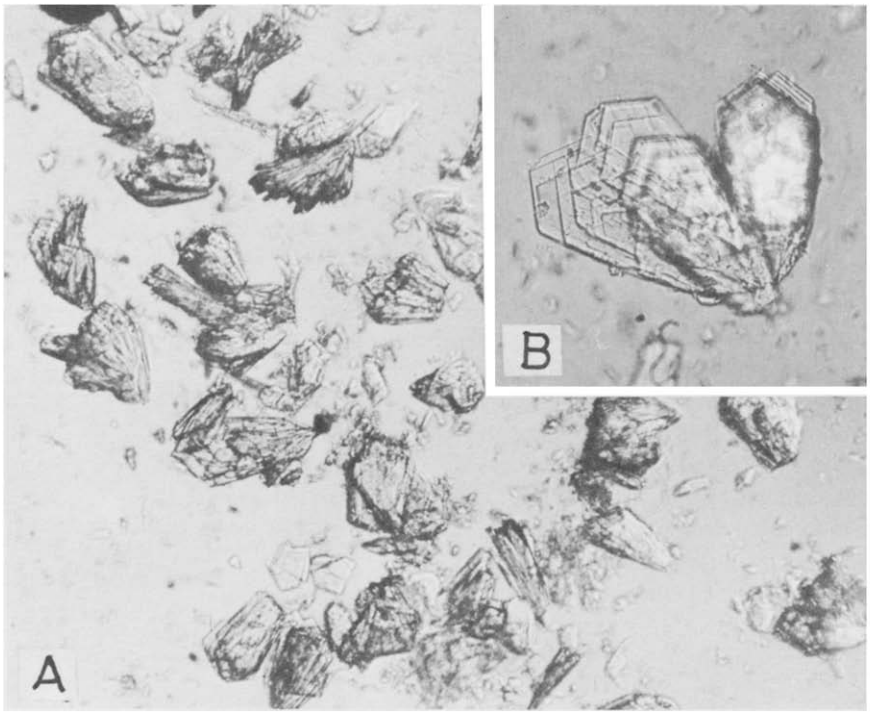

Fig.4. Crystals of bovine adrenodoxin reductase. Magnification: A, × 100; B, × 250.

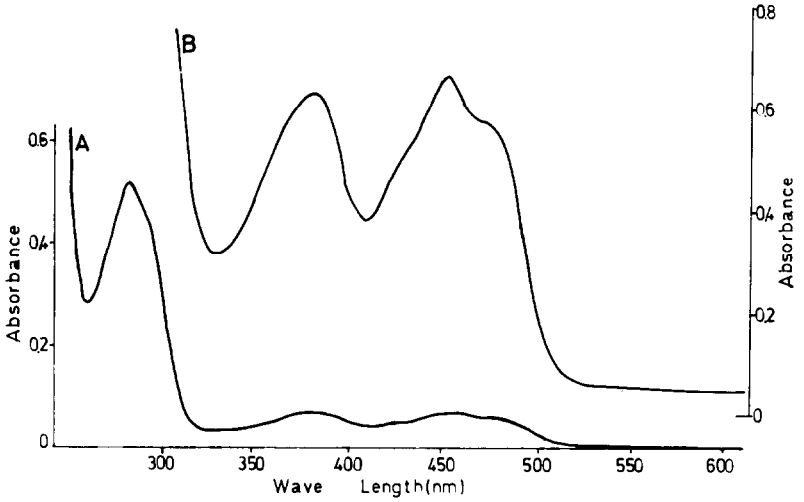

Fig.5. Absorption spectrum of crystalline adrenodoxin reductase. Enzyme concentrations: Curve A, 13 μM; Curve B, 60 μM; in 0.1 M phosphate buffer, pH 7.4.

the enzyme by the treatment of 80% methanol was coincident with the authentic FAD by the thin-layer chromatography. By using 11.3 of a millimolar extinction coefficient of FAD [9], the molecular weight of the enzyme was calculated as 54 300 per mol of FAD. This was in good agreement with the value of 54 000 which was obtained by SDS-gel electrophoresis.

In the same manner, the porcine adrenodoxin reductase was prepared and applied on this bovine adrenodoxin-Sepharose column under the same conditions described in fig.2. As bovine reductase, the porcine adrenodoxin reductase was tightly bound on the column and could be purified to homogeneity.

Furthermore, the spinach ferredoxin-Sepharose gel was also prepared and its binding characteristics of the adrenodoxin reductase was examined. The adrenodoxin reductase was bound on this gel at low ionic strength, and the binding capacity of this gel gradually decreased with increasing ionic strength. It is interesting that adrenodoxin reductase could be bound to immobilized ferredoxin under certain conditions. The reductase may generally be bound to iron sulfur proteins, and may be purified by appropriate affinity chromatography.

Acknowledgements

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References

[1] Foust, G. P., Mayhew, S. G. and Massey, V. (1969) J. Biol. Chem. 244, 964–970.
[2] Nelson, N. and Neumann, J. (1969) J. Biol. Chem. 244, 1932–1936.
[3] Chu, J. W. and Kimura, T. (1973) J. Biol. Chem. 248, 5183–5187.
[4] Suhara, K., Takemori, S. and Katagiri, M. (1972) Biochim. Biophys. Acta 263, 272–278.
[5] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[6] Lowry, O. H., Rosebrough, N. J., Faar, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[7] Suhara, K., Ikeda, Y., Takemori, S.and Katagari, M. (1972) FEBS Lett. 28, 45–47.
[8] Axén, R. and Ernback, S. (1971) Eur. J. Biochem. 18, 351–360.
[9] Koziol, J. (1971) Methods Enzymol. 18-B, pp. 253–285, Academic Press, New York.