THE PURIFICATION AND PROPERTIES OF NADPH-ADRENODOXIN REDUCTASE FROM BOVINE ADRENOCORTICAL MITOCHONDRIA

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1. Introduction

The steroid hydroxylating system of adrenocortical mitochondria has been resolved into three components: a flavoprotein (NADPH-adrenodoxin reductase), an iron–sulfur protein (adrenodoxin) and a cytochrome *P*-450 [1]. The purification of these components would be desirable in order to reconstruct this electron transfer system and to study the reaction mechanism. The improved procedure for the crystallization of adrenodoxin has recently been introduced by Suhara et al. [2].

NADPH-adrenodoxin reductase, which catalyzes the reduction of adrenodoxin by NADPH, has been isolated from adrenocortical mitochondria of beef [1], pig [3] and rat [4]. However, so far as we know, no detailed description concerning the molecular properties of the purified enzyme has been found.

We now wish to report the purification of NADPH-adrenodoxin reductase from bovine adrenocortical mitochondria and to describe some of the properties of the purified enzyme.

2. Materials and methods

Mitochondrial pellets used for the extraction of NADPH-adrenodoxin reductase were prepared from bovine adrenal cortex as previously described [2]. Crystalline adrenodoxin (A_{414}/A_{276}, 0.86) was prepared by the method described previously [2]. Crystalline cytochrome *c* was prepared from bovine heart by the method of Hagihara et al. [5]. The activity of the reductase was routinely assayed by measuring the rate of the adrenodoxin-dependent reduction of cytochrome *c* as described by Omura et al. [1]. The assay mixture (1 ml) contained 50 nmoles of NADPH (Boehringer und Sohne, GmbH), 50 μmoles of potassium phosphate (pH 7.4), 2 nmoles of adrenodoxin, 20 nmoles of cytochrome *c*, and an appropriate amount of the reductase. Polyacrylamide disc gel electrophoresis was carried out according to the method of Davis [6]. Electrophoresis in sodium dodecyl sulfate polyacrylamide gel was performed as described by Weber and Osborn [7]. Protein was determined by the biuret method with bovine serum albumin (Sigma) as standard.

3. Results and discussion

The NADPH-adrenodoxin reductase was purified according to a modification of the original method of Omura et al. [1]. All purification steps described below were performed at 4°. As the buffer system, potassium phosphate buffer (pH 7.4) was used.

Mitochondrial pellets were suspended in 100 mM of the buffer to approx. 30 mg protein/ml. Fifty milliliter aliquots of the suspension were sonicated at 0° for 10 min in a Quigley-Rochester sonic desmenbrator (20 KHZ). The sonicated samples were centrifuged at 37,000 *g* for 90 min. The yellow-brown supernatant fraction was immediately fractionated between 35 and 60% saturation of ammonium sulfate. The precipitate was dissolved in 10 mM buffer and dialyzed overnight against the same buffer. The dialyzate was applied to a DEAE-cellulose column (2.8 × 30 cm) equilibrated in 10 mM buffer. The reductase, yellow

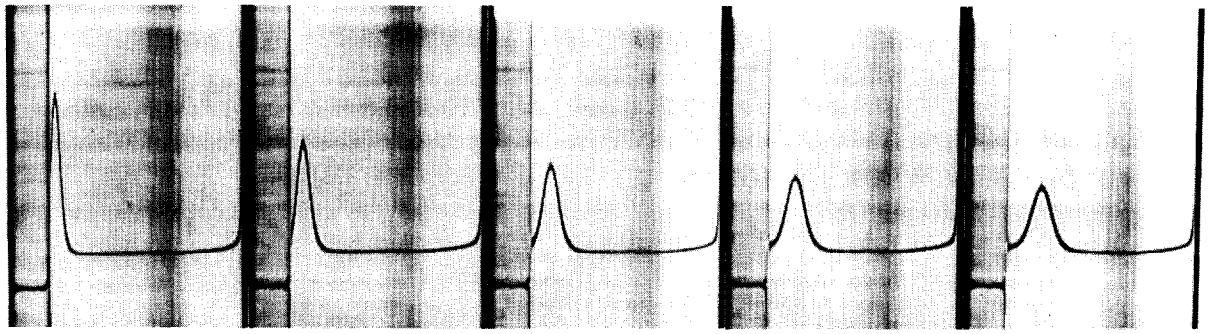

Fig. 1. Ultracentrifugation pattern of the reductase in 50 mM buffer. The concentration of protein was 7.5 mg per ml. Photographs were taken at 8-min intervals after attaining a roter speed of 59,780 rpm. Sedimentation was from left to right.

band, was washed onto the column with 10 mM buffer and finally eluted with 50 mM buffer. The eluate containing the reductase was fractionated between 35 and 55% saturation of ammonium sulfate. The precipitate was dissolved in 10 mM buffer and dialyzed overnight against the same buffer. The dialyzate was again applied to a DEAE-cellulose column (2.8 × 25 cm) equilibrated in 10 mM buffer and then eluted with a linear gradient between 10 mM and 50 mM buffer. The active fractions were precipitated between 40 and 55% saturation of ammonium sulfate and passed through a Sephadex G-100 column (2.8 × 60 cm) equilibrated in 10 mM buffer containing 100 mM KCl. The active fractions were combined, concentrated with ammonium sulfate (60% saturation) and dissolved in 50 mM buffer. After dialysis against 50 mM buffer, the preparation could be stored for several weeks at −80° without substantial loss of activity. The yield from 2 kg of bovine adrenal cortex was about 20 mg of the reductase.

The rate of the reduction of cytochrome *c* by the reductase was found to be dependent on the amount of adrenodoxin. When adrenodoxin was present in excess, the activity of the purified reductase was saturated and the specific activity was estimated to be 4 μmoles of cytochrome *c* reduced per min per mg protein under the conditions described in Methods.

When subjected to disc electrophoresis on acrylamide gel at pH 8.2, the reductase was resolved into one major and one minor band. When the reductase which had been treated with sodium dodecyl sulfate was subjected to electrophoresis at pH 7.0, only a single protein band appeared. Upon high speed sedimentation of the reductase in the analytical ultracentrifuge, a single symmetrical peak appeared (fig. 1) and $s_{20,w}$ was estimated to be 3.93 S(7.5 mg of protein/ml).

The reductase has a typical absorption spectrum of flavoprotein (fig. 2). The peaks were at 272, 378 and 450 nm and a shoulder appeared at 475 nm. Its absorbance ratio at 272 nm/450 nm in the oxidized form was 8.4.

The flavin was liberated from the protein by the acid treatment. A paper chromatography of the acid-liberated flavin with three different solvent systems [1-butanol–acetic acid–H_2O (4:1:5), pyridine–H_2O (2:1) and 5% $NaHPO_4$] revealed a yellow spot with the same mobility as authentic FAD. The fluorescence of the enzyme flavin at 523 nm was also

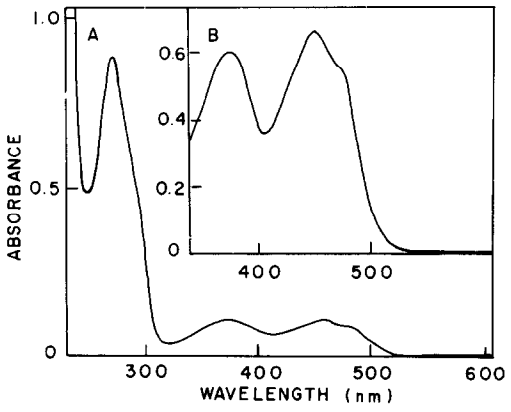

Fig. 2. The absorption spectrum of the reductase dissolved in 50 mM buffer. Concentrations of protein were 0.5 mg/ml (A) and 3 mg/ml (B), respectively.

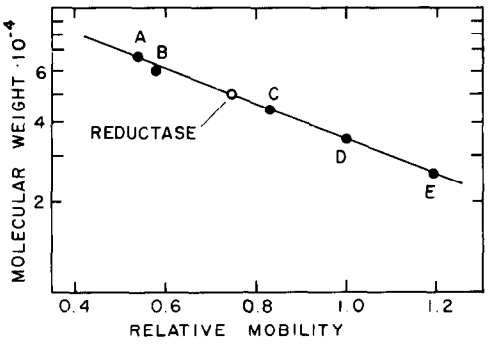

Fig. 3. Estimation of the molecular weight of the reductase by sodium dodecyl sulfate gel electrophoresis. Prior to electrophoresis, the reductase and standard proteins were treated with 1% sodium dodecyl sulfate and 1% mercaptoethanol. Mobilities are expressed relative to carboxypeptidase A. A) bovine serum albumin (mol. wt. 68,000); B) bovine liver catalase (mol. wt. of monomer, 60,000); C) ovalbumin (mol. wt. 43,000); D) carboxypeptidase A (mol. wt. 34,600); E) chymotrypsinogen (mol. wt. 25,700).

identical with that of authentic FAD, when activated by light having wavelength of 470, 375 and 275 nm. The enzyme flavin could fully activate the apo-salicylate hydroxylase from *Pseudomonas putida* which is specifically reactivated by FAD [8]. The apoenzyme prepared by the acid–ammonium sulfate [9] was also reactivated by FAD, but not FMN. Thus, analyses of the enzyme flavin reconfirmed that the prosthetic group of the reductase is FAD [3, 10].

The minimal molecular weight of the reductase was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and its value was estimated to be 50,000 (fig. 3). The molecular weight of the native enzyme in 50 mM buffer, as determined by gel filtration with a Sephadex G-100 column [11], was estimated to be 54,000, with cytochrome *c*, trypsin inhibitor, carboxypeptidase A, ovalbumin and bovine serum albumin as standard proteins. The minimal molecular weight based on the FAD content was estimated to be 55,400. These results suggest that the reductase is a single chain protein containing one FAD.

NADH-putidaredoxin reductase in the camphor methylene hydroxylase from *Pseudomonas putida* [12] and NADH-rubredoxin reductase in the ω-hydroxylation system of fatty acids and hydrocarbons from *Pseudomonas oleovorans* [13] have a molecular weight of 43,500 and 55,000, respectively. Both contain 1 molecule of FAD in a single chain protein. Such results suggest that NADPH-adrenodoxin reductase is very similar to these reductases in the molecular properties.

Acknowledgements

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