Overproduction in *Escherichia coli* and Characterization of the Precise Mature Form of Rat Adrenodoxin

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The mature form of rat adrenodoxin (Ad) was purified from a heterologous direct expression system in *Escherichia coli* with a yield of 56 mg/l culture. The purified Ad showed a A_{414}/A_{280} ratio of 0.91 and the sequence of 10 amino terminal residues was identical with that of authentic rat Ad. By Time of Flight/Mass spectrometry, the molecular mass of purified Ad was identical to that calculated from the cDNA sequence and the carboxy terminal residue was estimated to be Ser, which was also as expected from the cDNA. These results indicate that the purified recombinant Ad is a precise mature form. In measurements of NADPH-cytochrome *c* reductase activity reconstituted with bovine adrenodoxin reductase (AdR), the apparent K_m value for rat Ad was 46.9 ± 2.5 nM, indicating a somewhat lower affinity for rat Ad to bovine AdR than for bovine Ad. On the other hand, the spectral K_d value for rat Ad to bovine cytochrome P-450_{scc} was 0.46 ± 0.05 μ M, a value which was almost identical with that of the bovine counterpart.

Key words adrenodoxin; adrenodoxin reductase; cDNA; cytochrome P-450_{scc}; *Escherichia coli*; rat

Adrenodoxin (Ad) is a [2Fe-2S]-type ferredoxin, located in the mitochondrial matrix of steroidogenic tissues, and functions as the common electron transporter from NADPH-adrenodoxin reductase (AdR) to mitochondrial cytochromes P-450 which catalyze steroid hormone biosynthesis.¹⁾ Ad is synthesized as a large precursor by cytoplasmic polysomes and is transferred into the mitochondria by an energy-dependent mechanism, after which it is processed to a mature form by a metalloprotease.^{2–6)} Ad has been purified mainly from bovine adrenal gland⁷⁾ and crystallization and X-ray analysis of bovine Ad have been investigated.^{7–9)} After cDNA clonings of Ad were performed,^{10–13)} heterologous expression systems, mainly for bovine and human Ads, have been used for investigating mechanisms of electron transfer reaction from AdR to cytochromes P-450 via Ad.^{14–17)} However, the rat is one of the most suitable animals to examine regulation of the mitochondrial cytochrome P-450 system using whole animal tissues. In our previous study, a small amount of mature Ad was purified from 2300 rat adrenal glands and several enzymic properties of the protein were revealed.¹⁸⁾ Full-length cDNA cloning of rat Ad showed the entire amino acid sequence of the precursor and that the mature form consists of 124 amino acid residues.¹⁹⁾ In the present study, we developed a heterologous direct expression system to obtain large amounts of the precise mature form of rat Ad, and report herein overproduction in *Escherichia coli*, simple purification and further characterization of the protein.

MATERIALS AND METHODS

Materials NADPH, cytochrome *c* (horse heart), myoglobin (horse heart) and sinapinic acid were purchased from Sigma-Aldrich Co. HiLoad 16/10 Q Sepharose FF, HiTrap Q and HiLoad 16/60 Superdex 75 pg were from Amersham Pharmacia Biotech Ltd. DE52 was from Whatman Paper Ltd. All other reagents were of a guaranteed grade.

Plasmid Construction The cDNA fragment encoding the mature peptide region of rat Ad was prepared by polymerase chain reaction (PCR). pRA38, which contains a full-

size cDNA for rat Ad,¹⁹⁾ was linearized by *Xho*I digestion and used for the template. The primers used were 5'-GCGC-CATATGAGCAGCTCAGAAGATAAGGTA-3' for the amino terminal side and 5'-TGAAGCTTAGCTATTCTTGCTCAT-GTCAAC-3' for the carboxy terminal side. The *Nde*I site and *Hind*III site overlap the initiation codon and the stop codon, respectively. The primer for the amino terminal side was designed to add the initiator Met just prior to the amino terminal Ser of rat mature Ad and to change the resulting second codon AGT (Ser) to AGC (Ser) to increase the expression of Ad in *E. coli*.^{20,21)} After confirmation of the nucleotide sequence of the PCR product, the *Nde*I/*Hind*III fragment encoding rat mature Ad was ligated with the expression vector pCWori+.²¹⁾ The final construct was designated pRA3813.

Expression and Purification of Recombinant Rat Ad Overnight culture of *E. coli* D1210 harboring pRA3813 in LB broth containing 50 μ g/ml ampicillin was 1 % seeded into 500 ml of Terrific Broth containing 50 μ g/ml ampicillin and was incubated at 37 °C to OD₆₀₀=0.5. Expression of Ad was induced by adding 1 mM isopropyl β -D-thiogalactopyranoside. The incubation was continued overnight at 37 °C with gentle shaking. Thereafter, all the purification procedures were carried out at 0–4 °C. The *E. coli* cells were harvested by centrifugation at 5200 $\times g$ for 5 min and washed once with 10 mM potassium phosphate buffer (pH 7.5) containing 150 mM NaCl. The cells were resuspended in 19 vol. of 10 mM potassium phosphate buffer (pH 7.5), sonicated using 50% pulses for 10 min, then centrifuged at 105000 $\times g$ for 60 min. The supernatant was applied to a DE52 column (2.5 $\times$ 10 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.5). After washing with 4 column vol. of 10 mM potassium phosphate buffer (pH 7.5) containing 150 mM NaCl, Ad was eluted with 500 mM NaCl in 10 mM potassium phosphate buffer (pH 7.5). The Ad-rich fractions were combined and diluted 4 times with 10 mM potassium phosphate buffer (pH 7.5), then applied to a HiLoad 16/10 Q Sepharose FF column. After washing with 6 column vol. of 10 mM potassium phosphate buffer (pH 7.5) containing 150 mM

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Fig. 1. Mass Spectra of the Purified Recombinant Rat Ad (A) and That Pre-treated with Carboxypeptidase Y (B)

The small signal with about 200 mass larger than Ad probably indicates the sinapinic acid conjugate.

NaCl, Ad was eluted with a linear gradient of 150–800 mM NaCl (total 13.5 column vol.) in 10 mM potassium phosphate buffer (pH 7.5). The peak fractions, eluted at about 350 mM NaCl, were combined and concentrated on a HiTrap Q column using 1 M NaCl in 10 mM potassium phosphate buffer (pH 7.5). The Ad was then subjected to gel filtration on a HiLoad 16/60 Superdex 75 pg column equilibrated with 10 mM potassium phosphate buffer (pH 7.5). The peak fractions were combined and re-chromatographed on a HiLoad 16/10 Q Sepharose FF column with a linear gradient of 200–600 mM NaCl (total 9 column vol.) in 10 mM potassium phosphate buffer (pH 7.5).

Enzyme Assay NADPH-cytochrome *c* reduction activity was spectrophotometrically measured at 550 nm in 1 ml of 10 mM potassium phosphate buffer (pH 7.5) containing 20 nM AdR, 10–100 nM Ad, 20 μ M cytochrome *c* and 100 μ M NADPH at 25 °C.²²⁾ Ad-induced spectral change of cy-

tochrome P-450scc was measured as described by Kido and Kimura²³⁾ using 0.5 μ M cytochrome P-450scc and 0.5–2.0 μ M Ad in 20 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol, 0.1% Tween 20 and 40 μ M cholesterol. The AdR used was a recombinant enzyme—which corresponds to the mature form of bovine AdR—and was purified from *E. coli* harboring pBAR1607.²⁴⁾ The bovine Ad used was also a recombinant protein, Ad(2–128)—which corresponds to the mature form of bovine Ad—and was purified from *E. coli* harboring pBA1159.^{15,17)} The P-450scc used was an authentic enzyme purified from bovine adrenal cortex.²⁵⁾

Others General techniques for plasmid construction were as described by Sambrook *et al.*²⁶⁾ Nucleotide sequencing was carried out on a denatured plasmid template using a 370A DNA Sequencer (PE Applied Biosystems). Concentration of the Ad was determined using the millimolar extinc-

tion coefficient of 9.8 cm^{-1} at 414 nm .¹⁸⁾ Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out by the method of Laemmli²⁷⁾ using 15% acrylamide gels. The amino terminal amino acid sequence of purified rat Ad was determined using a 491 cLC Protein Sequencer (PE Applied Biosystems). The molecular mass of the purified rat Ad was measured by Matrix-Assisted Laser Desorption Ionization/Time of Flight/Mass Spectrometry (PerSeptive Biosystems, Voyager RP) using sinapinic acid and myoglobin as a matrix and a standard, respectively. The carboxy terminal residue of the Ad was estimated by mass spectrometry using a Sequazyme C-Peptide Sequencing Kit provided by PerSeptive Biosystems.

RESULTS AND DISCUSSION

There are many reports in which recombinant proteins with some modification at the amino and/or carboxy terminal region are used for analysis of enzymic properties in place of the authentic protein; for example, addition of several residues derived from a multi-cloning site of the vector at the amino terminus and the His-Tag at the carboxy terminus. In the case of Ad, however, modifications at both the amino terminal region and the carboxy terminal region affected the enzymic properties.^{15–17)} Therefore, we constructed an expression system to produce a precise mature Ad.

The mature form of rat Ad was overexpressed in *E. coli* and purified from the cell lysate by anion-exchange chromatographies and gel filtration. The purified Ad showed a single band on SDS-PAGE (data not shown) and the yield was 28 mg from the cell lysate of a 500 ml culture, *i.e.* 56 mg/l culture, in a typical case. The A_{414}/A_{280} ratio was 0.91 and the absorption spectrum was indistinguishable from that of authentic protein purified from rat adrenal gland (data not shown). The amino acid sequence of the 10 amino terminal residues was Ser-Ser-Ser-Glu-Asp-Lys-Val-Thr-Val-His. This sequence is identical with that of residues 2–11 of the heterologous expression product defined by the cDNA sequence,¹⁹⁾ indicating that the initiator Met just prior to Ser—which corresponds to the amino terminal residue of the mature Ad—had been excised in *E. coli* as expected.²⁸⁾ Consequently, the amino terminal amino acid sequence of the purified recombinant Ad was identical with that of authentic protein.¹⁸⁾ The molecular mass of the purified Ad was estimated to be 13584 by the Time of Flight/Mass spectrometry (Fig. 1A). This value is very close to that calculated from the cDNA sequence, 13588. When the Ad was treated with carboxypeptidase Y, the mass spectrum showed a new signal whose mass value was 87 lower than that of the major one (Fig. 1B). Therefore the carboxy terminal residue was estimated to be Ser, which was also as expected from the cDNA sequence. These results indicate that the purified recombinant rat Ad consists of the precise amino acid sequence of mature Ad, Ad(1–124).

Using the purified recombinant proteins, kinetic parameters of rat Ad were compared with those of the bovine counterpart. NADPH-cytochrome *c* reductase activity reconstituted with bovine AdR showed K_m values for the rat and the bovine Ads of 46.9 ± 2.5 and $36.9 \pm 2.1 \text{ nM}$, respectively (Fig. 2). This result suggests that affinity of rat Ad to bovine AdR is somewhat lower than that of bovine Ad, since the electron

Fig. 2. NADPH-Cytochrome *c* Reductase Activity Reconstituted Using the Rat and the Bovine Ads

The assay conditions are described in Materials and Methods.

Fig. 3. Spectrophotometric Titrations of Cytochrome P-450scc with the Rat and the Bovine Ads

$A_{390-420}$ values obtained from the titration experiments were plotted as $[\text{Ad}]_{\text{free}}/\Delta A$ versus $[\text{Ad}]_{\text{free}}$. Concentrations of free Ad were calculated using the following equation. $[\text{Ad}]_{\text{free}} = [\text{Ad}]_{\text{total}} - (\Delta A/\Delta A_{\text{max}})[\text{cytochrome P-450}]$

transfer from the flavin of AdR to the non-heme iron of Ad is considered to be the rate-limiting step in the cytochrome *c* reduction reaction.²⁹⁾ In our previous study, we observed that the K_m value for rat Ad ($0.44 \mu\text{M}$) in the cholesterol side-chain cleavage activity reconstituted with bovine AdR and bovine cytochrome P-450scc was significantly smaller than that for bovine Ad ($1.22 \mu\text{M}$).¹⁸⁾ From analysis of the Ad-induced high spin shift of cytochrome P-450scc in the present study, however, the spectral K_d values of these Ads to bovine cytochrome P-450scc were almost identical; 0.46 ± 0.05 and $0.42 \pm 0.02 \mu\text{M}$ for the rat and the bovine Ad, respectively (Fig. 3). These results suggest that the affinities of these Ads

in the oxidized state to the cytochrome P-450_{scc} are almost at the same level while the reduced form of rat Ad shows a higher affinity to cytochrome P-450_{scc} than the bovine counterpart. Conformational change accompanied by the change of redox state has been proposed for bovine Ad from an NMR study.³⁰⁾ The conformational change of rat Ad might be different from that of bovine Ad. Further study on electron transport *via* Ad in the rat mitochondrion is on going.

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