Direct Expression of Adrenodoxin Reductase in Escherichia coli and the Functional Characterization

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A plasmid for direct expression in Escherichia coli of the mature form bovine adrenodoxin reductase was constructed from the full-size cDNA for the enzyme [Y. Sagara, Y. Takata, T. Miyata, T. Haru, and T. Horiuchi, J. Biochem. (Tokyo), 102, 1333 (1987)] and an expression vector pCWori+. The recombinant adrenodoxin reductase was purified from the transformed E. coli cell lysates using adrenodoxin-Sepharose affinity chromatography [T. Sugiyama and T. Yamano, FEBS Lett., 52, 145 (1975)] with a yield of 2.5 mg/l of culture. The purified recombinant enzyme showed a single band on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and migration was identical with that of the authentic enzyme purified from bovine adrenal cortex mitochondria. The recombinant enzyme had Ser at its amino-terminus and the sequence in the amino terminal 9 residues was identical with that of the authentic bovine enzyme. The absorption spectrum of the recombinant enzyme showed peaks at 270, 376, and 450 nm and shoulders at 425 and 475 nm. Flavin content of the recombinant enzyme was 0.8 mol FAD/mol. The apparent Kₘ value for bovine adrenodoxin in NADPH-cytochrome c reductase activity using a reconstitution system was 16 μM, a value comparable with that of the authentic bovine enzyme (17 μM). The cholesterol side chain cleavage activity with a reconstitution system was about 75% of that obtained when the authentic enzyme was used.

Keywords adrenodoxin reductase; cDNA; expression; E. coli; adrenodoxin; cytochrome P-450cc

Introduction

NADPH: Adrenodoxin oxidoreductase [adrenodoxin reductase; EC 1.18.1.2] (AdR) is a component of the mitochondrial steroid hydroxylating system in the adrenal cortex. AdR is synthesized as a large-sized precursor by cytoplasmic free polysomes and is processed to the mature form after transfer to the mitochondria. The mature AdR has an FAD and supplies reducing equivalents from NADPH to three forms of cytochrome P-450 (P-450 SCC, P-450 17p, and P-450 adR) via adrenodoxin (Ad). In liver and kidney mitochondria, the corresponding electron-transport system donates electrons to other forms of cytochrome P-450, which contribute to the production of bile acids and to the activation of vitamin D₃. The ferredoxin reductases in those tissues are called hepatoredoxin reductase and renoaredoxin reductase, respectively. The primary structure of AdR has been determined by nucleotide sequencing of the cDNA cloned from bovine adrenal cortex and from human testis, and the molecular weight of the mature bovine AdR was calculated to be 50,396. Purification and crystallization of bovine AdR have been reported, while X-ray analysis of the tertiary structure of AdR remains to be accomplished. The mechanism of electron transport from AdR to cytochrome P-450 via Ad has been investigated primarily using kinetic and chemical modification techniques. Expression of components of the system in bacteria using cDNAs, and construction of site-directed mutants are important approaches to investigate the mechanism of the electron transport in detail. Coghlan and Vickery reported the expression of human placental ferredoxin, which corresponds to Ad in adrenal cortex mitochondria, in E. coli and on site-directed mutagenesis of the ferredoxin. They first obtained ferredoxin as a fusion protein, then purified it using specific proteolysis. A similar strategy was used for expression of human NADH-cytochrome b₅ reductase, a component of the microsomal type electron transport system, and its mutants. In the case of NADPH-cytochrome P-450 reductase, the enzyme and mutants have been expressed as fusion proteins for the secretion system of E. coli. Akiyoshi-Shibata et al. investigated the transport of bovine AdR and Ad into mitochondria in yeast using an expression system constructed with the cDNA and an alcohol dehydrogenase promoter. They and Vickery have just reported on the expression of human ferredoxin reductase in E. coli, although yields of active enzyme were relatively low (0.1 mg/l of culture). We attempted to construct direct expression systems for components of mitochondrial P-450 electron transport system, by which the enzymes are not produced as a fused protein and thus can be directly purified from the bacteria. We have reported the direct expression of functional bovine AdR in E. coli, and in this report we describe direct expression of functional bovine AdR in E. coli, and its purification.

Materials and Methods

Plasmid Construction

Construction of the expression plasmid for the mature form of bovine AdR was carried out as follows: the full-size cDNA for bovine AdR, pBAR16, was digested with EcoRI and then with exonuclease Bal 31 to shorten the 3'-untranslated region. After the filling reaction with the large fragment of DNA polymerase I, the linearized DNA was digested with HindIII. The cDNA fragment was then cloned to pHSG399, a chloramphenicol resistant vector, and digested with Smal and HindIII. The nucleotide sequence of the remaining 3'-untranslated region was confirmed by the dyeoxy chain terminator method using a denatured plasmid templateand the Sequenase kit version 2.0 (United States Biochemical). The clone, pBAR1601, with 10 bases of 3'-untranslated region was thus selected. cDNA for the new amino terminal region covering from the NdeI site (CATATTG) and coding from the initiation codon to Gln108 which is 38 bp downstream from the internal Dral site was prepared by polymerase chain reaction. The template used was pBAR16; it had been linearized by EcoRI digestion. The primers used were 5'-GGGAATTCCATATG-
GCACCTCAAGAACAACCCCGATCTGTTGTG-3' for the amino terminal side and 5'-CTGATGGTCTCCTGGCCCTA-3' for the carboxy terminal side. The primer for the amino terminal side was designed to add the initiator Met just prior to the amino terminal Ser of bovine mature AdR, to change the resulting second codon TTC (Ser) to AGC (Ser), and to enhance the A-T richness of nucleotides which may increase expression of the AdR in E. coli, as originally devised for the expression of bovine cytochrome P-450m in E. coli.44 The product of polymerase chain reaction was blunt-ended by the large fragment of DNA polymerase I and subcloned to the Smal site of pUC19.45 The nucleotide sequence was confirmed as described above. The HinclI/DraII fragment (318 bp, the HinclI site in the multi-cloning site of pUC19 was utilized) encoding the amino terminal region of AdR was ligated to the HinclI/DraII fragment of pBAR1601. The latter encodes the remaining portion of AdR and pHSG399. The resultant plasmid, pBAR1603, was digested with EcoRI, blunt-ended by the large fragment of DNA polymerase I, then digested with NdeI. The NdeI/blunt-end fragment encoding the entire AdR was subcloned to the NdeI/blunt-end site of expression vector, pCWori +,46 which had been digested with XhoI, blunt-ended by the large fragment of DNA polymerase I, then digested with NdeI. The final construct was designated pBAR1607. General techniques for plasmid construction were as described by Sambrook et al.47

Expression and Purification of Recombinant AdR Overnight cultures of E. coli JM109 harboring pBAR1607 in LB medium containing 50 μg/ml ampicillin were seeded (1%) into 750 ml of Terrific Broth containing 50 μg/ml kanamycin and incubated at 37 °C until the absorbance at 600 nm reached about 0.5. Expression of AdR was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside. The incubation was continued for 24 h at 30 °C with gentle shaking. The E. coli cells were harvested by centrifugation at 5200 × g for 5 min at 4 °C and washed once in ice-cold 10 mM potassium phosphate (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA). The cells were resuspended in 40 ml of 10 mM potassium phosphate (pH 7.4) containing 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride, sonicated with Artok Sonic dismembrator model 150 (Artok Systems) in ice using 30 s pulses with 30 s intervals at maximum power, then centrifuged at 105,000 × g for 60 min. Authentic AdR and recombinant AdR were purified from the supernatant by the method of Sugiya and Yamano,21 using adrenodoxin-Sepharose affinity chromatography with some modification. Adrenodoxin was purified from bovine adrenal cortex as described by Suhara et al.31

Amino Acid Sequence Amino terminal amino acid sequences of purified AdRs were determined by Edman degradation using a gas phase protein sequencer (Applied Biosystems, model 472A).

Enzyme Assays NADPH-cytochrome c reductase activity was assayed by following the increase in absorbance at 550 nm using a spectrophotometer (IBMer, model 9420). The reaction mixtures contained 100 μM NADPH, 10–30 nM AdR, 5–30 mM Ad, and 20 μM cytochrome c in 1 ml of 50 mM potassium phosphate (pH 7.4). Cholesterol side chain cleavage activity was assayed as described elsewhere,20 except that 0.1 μM AdR and 0.2 or 0.3 μM P-450sec were used and that recombinant P-450sec and Ad [AdMet1] purified from E. coli8727 were used.

Analytical Methods Protein concentrations were determined using the BCA protein assay reagent (Pierce) and bovine serum albumin as the standard. Flavin content of AdR was determined by spectrophotometric methods.33,34 (Amino-Boomain, cat.14-8202). FAD and FMN used as standards were kindly provided by Dr. J. A. Peterson (Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas, U.S.A.). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out by the method of Laemmli39 using 10% acrylamide gels.

Results and Discussion

Expression and Purification of Recombinant AdR To obtain the mature form of bovine AdR, we constructed a direct expression system in which the enzyme can be directly purified from the bacterial cell lysate. In our pilot tests, the level of expression of recombinant AdR was higher in E. coli JM109 than in D1210 or in XL1-Blue,49,56,57 and the expression in JM109 at 30 °C was higher than that at 37 °C (data not shown). Thus, the recombinant AdR was expressed at 30 °C in JM109 and was purified from the cell lysate using adrenodoxin-Sepharose affinity chromatography.21 The purified AdR showed a single band on SDS-PAGE, with the same migration as that of the authentic AdR purified from bovine adrenal cortex mitochondria (Fig. 1). Yield of the recombinant AdR from the E. coli culture was 2.5 mg/l, a value 20-fold higher than that of human ferredoxin reductase expressed in E. coli.42

Amino Terminal Sequence of Recombinant AdR As shown in Table I, the recombinant AdR had Ser at its amino-terminus and the sequence of the amino terminal 9 residues was identical with that of the authentic bovine AdR. This result indicates that the amino terminal Met is removed from recombinant AdR in E. coli.

Spectral Properties Figure 2 shows the absorption spectrum of the recombinant AdR in the visible wavelength range. The spectrum has peaks at 376 and 450 nm and shoulders at 475 and 425 nm. The absorption spectrum also showed a peak at 270 nm and the absorbance ratio at 270 nm/450 nm in the oxidized form was 7.8 (data not shown). Sugiya and Yamano20 reported that AdR purified from bovine adrenal cortex showed an absorption spectrum having peaks at 272, 378, and 450 nm and shoulders at 475 and 420 nm and that the absorbance ratio at 272 nm/450 nm was 7.6. Thus, properties of purified recombinant AdR are comparable to those of the authentic bovine AdR.

Flavin Analysis Flavin contents of recombinant AdR and the authentic bovine AdR were assayed spectrophotometrically. As shown in Table II, recombinant AdR

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**Table I. Amino Acid Sequence of the Amino Terminal Region of Recombinant and Authentic AdR**

<table>
<thead>
<tr>
<th>Deduced from cDNA</th>
<th>MSTQEQTPQI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant AdR</td>
<td>STQEQTPQI</td>
</tr>
<tr>
<td>Bovine AdR</td>
<td>STQEQTPQI</td>
</tr>
</tbody>
</table>

Numbers above the sequence indicate residue number from the amino-terminus.
had 0.8 mol FAD/mol AdR but no FMN. The authentic bovine AdR had 1.0 mol FAD/mol AdR, as reported. These results suggest that recombinant AdR expressed in E. coli has a flavin content equal to that of its bovine counterpart.

**Enzymatic Activities of the Recombinant AdR** NADPH-cytochrome c reductase activity was measured using a reconstitution system with recombinant AdR and Ad purified from bovine adrenal cortex. Apparent $K_m$ values for bovine Ad were 16 nM for recombinant AdR and 17 nM for authentic bovine AdR (Fig. 3, average of three experiments), thereby indicating that the recombinant AdR has an affinity for Ad similar to that of the authentic bovine AdR. We also measured the cholesterol side-chain cleavage activity of the recombinant AdR, using a reconstitution system with recombinant Ad$^3$ and recombinant P-450$_{bec}^{5,2}$ The side-chain cleavage activity of recombinant AdR was about 75% (average of two experiments) of that obtained with bovine AdR (Table III). This result suggests that the recombinant AdR can transfer electrons to Ad, with efficiency comparable to that of authentic AdR.

We reported the direct expression of functional bovine Ad$^{3,1}$ and P-450$_{bec}^{4,5,2}$ in E. coli. In the present work, we have succeeded in the direct expression of functional bovine AdR in E. coli, the recombinant enzyme properties being essentially identical with those of the native enzyme. Using these recombinant enzymes, we are now proceeding to construct an electron transport system for P-450$_{bec}$ and the production of pregnenolone in E. coli cells. AdR and Ad function as a common electron transport system for P-450$_{bec}$ and P-450$_{11p}$. This E. coli system may aid in elucidating the molecular mechanisms of mitochondrial P-450 electron transport.

**Acknowledgments** Dr. T. Hara (Nakamura Gakuen College) provided critical comments and M. Ohara provided helpful editorial comments.

**References and Notes**

1) This study was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas of "Molecular Biology of Cytochrome P-450." From the Ministry of Education, Science and Culture of Japan (T.H.) and USPHS Grant GM37942 and Grant I-624 from The Robert A. Welch Foundation (M.R.W.).

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6) Abbreviations used are: AdR, NADPH-adrenodoxin reductase; Ad, adrenodoxin; P-450$_{bec}$, cytochrome P-450 catalyzing cholesterol side chain cleavage reaction; P-450$_{11p}$, cytochrome P-450 catalyzing steroid $11\beta$-hydroxylation; P-450$_{11a}$, cytochrome P-450 catalyzing aldosterone synthesis; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.