Chaperone-assisted expression of authentic bovine adrenodoxin reductase in *Escherichia coli*

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Abstract Adrenodoxin reductase is an essential component of the mitochondrial monooxygenase systems that are involved in the synthesis of steroid hormones and related compounds. After removing by mutagenesis a secondary ribosome binding site and an mRNA loop formed between the gene and the vector, large amounts of the enzyme could be produced in *Escherichia coli* by coexpression with the HSP60-chaperone system. The purified protein was homogeneous enough for reproducible crystallization. The crystals diffracted X-rays isotropically beyond 1.7 Å resolution permitting a structure analysis.

Key words: Adrenodoxin reductase; Chaperone coexpression; Crystallization; mRNA loop; Secondary ribosome binding site

1. Introduction

Adrenodoxin reductase (adrenal ferredoxin-NADPH oxidoreductase, AdR, EC 1.18.1.2) is an FAD-containing enzyme that represents the first component in the mitochondrial P450 electron transfer systems [1,2]. AdR receives two electrons from NADPH and transfers them one at a time to adrenodoxin, which then transports them to the P450 cytochromes. All proteins of these P450 systems are located at the matrix side of the inner mitochondrial membrane.

AdR is encoded by one nuclear gene and is expressed in all human tissues that have been examined [3]. The AdR-adrenodoxin couple provides the electrons for at least six different mitochondrial P450 cytochromes that are expressed in a tissue-specific manner. The reactions catalyzed by these P450 systems include cholesterol side chain cleavage, steroid 11β- and 18-hydroxylations, sterol C27-hydroxylation and vitamin D3 1α- and 25-hydroxylations which represent crucial steps in the biosynthesis of steroid hormones, bile acids and active vitamin D derivatives.

To elucidate the enzyme mechanism in detail, bovine [4–6] and porcine AdR [7] have been crystallized. Further crystals were obtained from bovine AdR cross-linked with adrenodoxin [8], the structure of adrenodoxin is known [9]. All AdR crystals were apparently not adequate for detailed analysis. Therefore, we undertook to improve AdR production by expression in *Escherichia coli*. Unfortunately, *E. coli* shows a tendency, especially for eukaryotic proteins like AdR, to express the synthesized peptide in inclusion bodies [10]. The amount of correctly folded protein, however, can be increased by coexpression of chaperones [11,12]. Here, we report a high-yield expression system with subsequent purification that resulted in readily growing X-ray-grade crystals of authentic AdR.

2. Materials and methods

2.1. AdR isolation from bovine adrenal cortex

AdR was purified as described [13]. In brief, 1.0 kg adrenal cortex was obtained from about 100 adrenals. The mitochondria were isolated and sonicated. An AdR-enriched fraction was isolated by ammonium sulfate precipitation, dialyzed against 10 mM KH_2PO_4, 0.04 mM EDTA and loaded onto a DE52 ion exchange column (Whatman), from which it was then eluted with 50 mM KH_2PO_4, 0.04 mM EDTA and dialyzed against 10 mM KH_2PO_4. AdR was then loaded onto an adrenodoxin-Sepharose affinity column, washed with 40 mM KCl, 10 mM KH_2PO_4 and eluted with 240 mM KCl, 10 mM KH_2PO_4. All handling was at pH 7.35 and 4°C.

2.2. Mutagenesis and expression vectors

The cDNA of AdR was transferred from vector pBAR1607 [14] into vector pET22b (Novagen). Using PCR [15] with the N-terminal primer 5′-TTATCCATGGCAAGCACTCAAGAACAAACCCC-3′ and the C-terminal primer 5′-GTATCAAGCTTCTAGGCTCAGTGTCCCAGCAG-3′, the cDNA was amplified and restriction sites were introduced. For mutagenesis we applied the mega-primer method [16]. The primers for the destruction of the secondary ribosome binding site and for the removal the mRNA loop were 5′-TCGCCCGCGCAAACGGCTGATGGAACTG-3′ and 5′-CCTCTAGAAATATTTTTGTTTAAAATTAAGAAGG-3′, respectively. PCR was performed with *Pfu* polymerase (Stratagene). DNA was sequenced on a blotter (GATC-1500) using Thermo Sequenase (Amersham). For coexpression with chaperones we transformed [17] *E. coli* BL21(DE3) harboring the AdR-encoding plasmid pET22b3-AR242 (ampicillin resistance) with either the HSP60 or the HSP70 system containing expression vectors pREP4-groESL or pRDKJG, respectively (P. Caspers, Hoffmann-La Roche). Both vectors possess kanamycin resistance.

2.3. AdR expression and purification

E. coli BL21(DE3) harboring plasmids pET22b3-AR242 and pREP4-groESL were grown at 37°C in 8×250 ml LB medium with 100 μg/ml ampicillin and 25 μg/ml kanamycin. At an OD_{578} of 0.6 the temperature was lowered to 20°C, the cells were induced with 1 mM IPTG, further cultivated for about 15 h and harvested by centrifugation. All subsequent handling was at 4°C. The pellet was suspended in 30 ml 10 mM KH_2PO_4 pH 7.4, 1 mM EDTA (buffer P) with 1 mM PMSF and sonicated. The cell suspension was centrifuged (60 min at 47 000×*g*) and the cytosol was dialyzed overnight against buffer P at pH 8.0. The protein solution was diluted with buffer P to 300 ml and run through an ion exchange column (Source Q, Pharmacia), which

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Table 1
Crystal statistics and X-ray diffraction

Crystal form	A	A	A′	A″
Temperature (K)	300	300	100	100
Glycerol (%)	–	–	20	20
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$
Unit cell				
a (Å)	60.3[a]	85.5	60.8	57.8
b (Å)	63.1	63.0	62.5	62.0
c (Å)	81.2	220.4	78.4	83.0
β (°)	107.2	95.3	106.8	107.1
V_M (Å^3/Da)	2.9[b]	2.9[c]	2.8[b]	2.8[b]
Diffraction limit (Å)	2.6[d]	3.2	1.7[d]	2.3
Observations	76 890	69 695	268 079	58 709
Unique reflections	16 710	33 096	56 738	20 372
Completeness (%)	92	85	93	74
R_{sym} (%)	10.9	15.7	4.9	4.8

[a]Data were collected and processed ignoring the low intensity superstructure (Fig. 1).
[b]Assuming one AdR molecule in the asymmetric unit.
[c]Assuming four AdR molecules in the asymmetric unit.
[d]Data were collected at the beamline X11 at EMBL/DESY Hamburg.

was further washed with 200 ml buffer P. The flow-through was loaded onto a 2′,5′-ADP-Sepharose column (Pharmacia), washed, and eluted with a 0–400 mM NaCl gradient in buffer P.

2.4. Crystallization and X-ray diffraction

The protein was concentrated to 10 mg/ml (Centriprep, Millipore) and dialyzed against 50 mM sodium cacodylate pH 6.5, 100 mM calcium acetate (buffer C). For crystallization we applied vapor diffusion using the hanging drop method. The 10 μl droplets contained 4 mg/ml AdR in buffer C with 8% (w/v) PEG 8000. The reservoir consisted of buffer C with 12% (w/v) PEG 8000. For data collection at cryo temperature, the crystals were stepwise transferred into reservoir buffer with 20% glycerol.

X-ray diffraction data were collected on a multiwire detector (Siemens, model X-1000) attached to a rotating anode generator (Rigaku, model RU200B). The data were processed with program XDS [18]. Synchrotron data were collected at beamline X11 at EMBL/DESY-Hamburg using an image plate (MARresearch, model 30-cm) and processed with MOSFLM [19]. All data were scaled and reduced using programs SCALA and TRUNCATE [20].

3. Results and discussion

3.1. Isolation and crystallization of native AdR

At first AdR, a monomeric enzyme with an M_r of 51 079, was prepared from bovine adrenal cortex. This expensive and tedious procedure [13] yielded about 20 mg AdR per kg adrenal cortex obtained from more than 100 animals. Crystallization conditions were established by screening [21] and then refined. The resulting crystals were named form A, they diffracted to 2.6 Å resolution. A data set based on a unit cell with presumably one molecule per asymmetric unit was collected at room temperature and processed (Table 1). Subsequent analyses revealed a superstructure indicated by weak additional reflections. The relationship between the small, apparent unit cell and the four times larger proper unit cell is shown in Fig. 1.

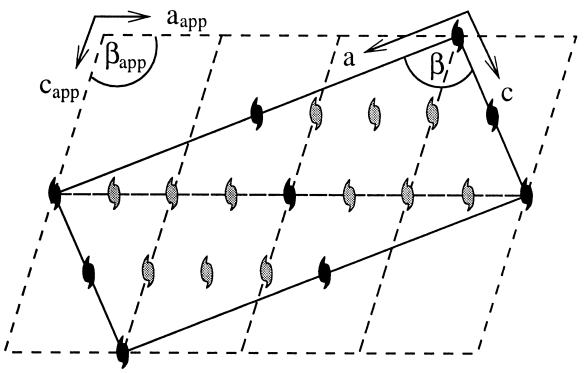

Fig. 1. Sketch of the relationship between the apparent small unit cell and the real unit cell with superstructure of crystal form A. The real cell is four times larger than the apparent cell.

3.2. AdR expression in E. coli

Because of crystal shortage, we changed to recombinant AdR, following Sagara et al. [14] who reported functional expression of AdR with a yield of 2.5 mg/l culture. Several attempts to reproduce the described procedure, however, resulted in AdR expression into inclusion bodies (Fig. 2, lanes 2 and 3). We therefore transferred the AdR cDNA from vector

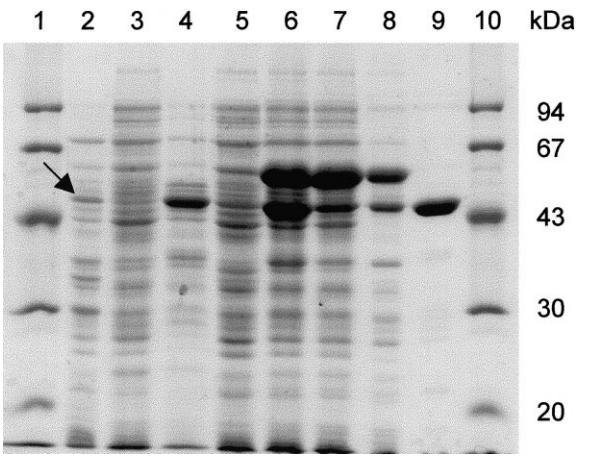

Fig. 2. AdR production steps analyzed by SDS-PAGE stained with Coomassie brilliant blue R250. Lanes 1 and 10, molecular mass markers; lanes 2 and 3, lysate and cytosolic fraction of induced expression system pBAR1607/JM109 [14] (arrow at presumed AdR band); lanes 4 and 5, lysate and cytosolic fraction of expression system pET22b3-AR242/BL21(DE3) after induction and addition of ethanol; lanes 6 and 7, lysate and cytosolic fraction of induced expression system pET22b3-AR242/pREP4-groESL/BL21(DE3); lane 8, after ion exchange chromatography; lane 9, purified AdR.

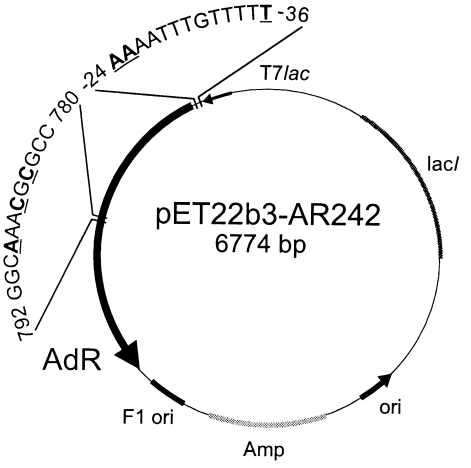

Fig. 3. Optimized expression vector pET22b3-AR242 encoding bovine AdR (mutations are shown as bold, underlined).

pBAR1607 into pET22b (Novagen) and examined the codon usage in the N-terminal region [22] and for the N-end rule [23], both of which were obeyed satisfactorily. We then checked for secondary *E. coli* ribosome binding sites in the AdR cDNA [24], found one and removed it by three silent point mutations (Fig. 3). A search for stable secondary mRNA structures near the Shine-Dalgarno sequence [25] revealed one putative loop formed between the cDNA of AdR and the vector with $\Delta G_{37} = -8.9$ kcal/mol. We destabilized this loop by three silent point mutations (Fig. 3). After these changes, the expression rate increased appreciably, but AdR was still expressed into inclusion bodies.

Since AdR is imported into mitochondria where it is folded with the help of chaperones, we subsequently enhanced the amount of chaperones in *E. coli* by the addition of ethanol to the medium after induction [26]. This increased the amount of soluble AdR (Fig. 2, lanes 4 and 5), though not to a sufficiently high level. Therefore we changed to direct chaperone expression by additional vectors [27]. We transformed either the HSP60 or the HSP70 system in a bacterial strain harboring the modified AdR-encoding plasmid pET22b3-AR242. This did not change the overall amount of AdR, but especially the HSP60 system increased the fraction of soluble, folded AdR (Fig. 2, lanes 6 and 7). AdR was isolated by ion exchange chromatography followed by an 2′,5′-ADP-Sepharose column (Fig. 2, lanes 8 and 9), yielding about 10 mg/l culture (4 mg/l for HSP-70). The protein bound to an adrenodoxin-Sepharose column (Section 2.1) indicating that it assumed its native conformation.

3.3. Crystals and X-ray data collection

The recombinant enzyme, crystallized under the same conditions as the native enzyme, yielding crystal form A with the same superstructure (Fig. 1). This is a further strong indication that the recombinant enzyme is authentic and in its native conformation. For X-ray diffraction measurements at a cryo temperature of 100 K, we transferred the crystals successively from the crystallization droplet into reservoir buffer containing 5, 10, 15 and 20% glycerol. During this procedure the crystals developed small cracks, but remained intact even after shock-freezing. X-ray analyses revealed that glycerol had changed the molecular packing scheme at 300 K as well as 100 K, giving rise to unit cell parameters similar to those of the apparent (small) unit cell of crystal from A. The superstructure had disappeared. This highly ordered crystal form was named A′ (Table 1). It could be produced directly by adding 5% glycerol to the usual crystallization set-ups, and it could be transferred to 20% glycerol without developing cracks. A data set was collected (Table 1). In one crystallization drop we obtained a further related crystal form A″ (Table 1). In conclusion, the high-yield expression system provided us with enough homogeneous material for determining the structure in order to understand the enzyme mechanism.

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