

Direct expression in *Escherichia coli* and characterization of bovine adrenodoxins with modified amino-terminal regions

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Received 10 December 1991; revised version received 5 February 1992

Four forms of bovine adrenodoxin with modified amino-termini obtained by direct expression of cDNAs in *Escherichia coli* are Ad(Met¹), Ad(Met¹⁻¹), Ad(Met¹⁻²), and Ad(Met⁶). The shoulder numbers represent the site of translation initiator Met at the amino-termini. The adrenodoxins, except for Ad(Met¹), were purified from the cell lysate and the ratios of A₄₁₄-to-A₂₈₀ of the purified proteins were over 0.92. NADPH-cytochrome *c* reductase activities of the three forms of adrenodoxin in the presence of adrenodoxin reductase were the same as that of purified bovine adrenocortical adrenodoxin. However, as cytochrome *P*-450_{SCC} reduction catalyzed by Ad(Met⁶) was about 60% of that by Ad(Met¹), the contribution of the amino-terminal region for the electron transfer or binding to cytochrome *P*-450_{SCC} would need to be considered.

Adrenodoxin; Adrenodoxin Reductase; cDNA; Cytochrome *c*; Cytochrome *P*-450_{SCC}; Expression of protein

1. INTRODUCTION

Adrenodoxin (Ad) is a component of the mitochondrial steroid hydroxylating system in the adrenal cortex, testis, ovary and placenta [1]. Ad is synthesized as a large precursor by cytoplasmic polysomes and is transferred to the mitochondria by an energy-dependent mechanism, after which it is processed to a mature form by a metalloprotease [2–6]. The mature Ad has a [2Fe-2S] center and acts as the common electron transporter from NADPH-adrenodoxin reductase (AdR) to cytochromes *P*-450_{SCC} and *P*-450_{11β}. The primary structure of bovine Ad has been determined by amino acid sequencing [7] and cDNA nucleotide sequencing [8,9]. Crystallization of bovine Ad was reported [10], and the X-ray analysis of the tertiary structure of Ad remains to be done. The structure–activity relationships of Ad and the mechanisms of electron transfer reactions from AdR to *P*-450_{SCC} via Ad have been investigated mainly using kinetic and chemical modification techniques [11–18]. Using cDNA, Coghlan and Vickery [19] noted the expression of human placental ferredoxin in *E. coli*,

which corresponds to Ad in adrenal cortex mitochondria [20,21]. The first obtained the ferredoxin as a fusion protein, then purified it using specific proteolysis.

We report here the direct and high level expression of active bovine Ad, not the fusion protein, in *E. coli*, and purification and catalytic properties of 3 forms of Ad modified in the amino-terminal region.

2. MATERIALS AND METHODS

The full-size cDNA for bovine Ad was digested with exonuclease Bal31 to obtain various lengths of deletion of the 5'-region as follows: the *Pst*I fragment of the cDNA which covers the entire sequence of bovine Ad [9] was subcloned to the *Pst*I site of pUC9. The *Eco*RI-*Xba*II fragment of the plasmid which includes the Ad cDNA was cloned to the *Eco*RI-*Sma*I site of the expression vector pKK223-3. With *Xba*II digestion, the 3'-untranslated region of the Ad cDNA was cleaved off 10 bases downstream from the stop codon. After a 5.5 kb *Eco*RI fragment of DNA derived from R plasmid was inserted into the *Eco*RI site of the clone, the plasmid was digested with *Sma*I and then with exonuclease Bal31. The *Sma*I site is derived from pUC9. The truncated cDNAs were flash-ended and ligated to the *Eco*RI site of the vector pKK223-3 using the *Eco*RI linker, CATGAATTCATG, which was designed to include an initiation codon. During this step, excess *Eco*RI linker and remaining R plasmid DNA were removed by *Eco*RI digestion. The final constructs were used for transformation of *E. coli* D1210. The clones producing Ad were selected by immunological methods from the truncated cDNA library [22]. For assay of the enzyme activity, the induction for Ad production was carried out as follows: overnight culture of the *E. coli* in L-Ap (L-broth containing 50 µg/ml ampicillin) was 2% seeded to PYPG-Ap (1% polypepton, 0.5% yeast extract, 0.1 M potassium phosphate buffer (pH 7.4), 0.5% glycerol, and 50 µg/ml ampicillin) and incubated at 37°C until the absorbance at 600 nm reached 0.5, then 4 vols. of PYPG-Ap and 1 mM IPTG (final concentration) were added to the culture and incubation was continued for a further 4 h at 37°C. The *E. coli* cells were har-

Abbreviations: Ad, adrenodoxin; AdR, NADPH-adrenodoxin reductase; M-Ad, mature form of bovine adrenocortical adrenodoxin; *P*-450_{SCC}, cytochrome *P*-450 catalyzing cholesterol side chain cleavage reaction; *P*-450_{11β}, cytochrome *P*-450 catalyzing steroid 11β-hydroxylation; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

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vested by centrifugation at 5,000×g for 5 min. and washed with 10 mM potassium phosphate buffer (pH 7.4). The cells suspended in 0.4 culture vols. of the same buffer were sonicated 6 times for 30 s, at 30 s intervals, using a Branson cell disruptor model 200 at level 6 in an ice bath. The cell lysate obtained by centrifugation at 100,000×g for 60 min was used for the enzyme assays. General DNA cloning and Western blotting techniques were carried out as described by Sambrook et al. [22]. Ad was detected using peroxidase-labeled antibody and 4-chloro-1-naphtol as the coloring substrate. The amount of Ad was estimated from densitometric tracing of the blotting sheet with Shimadzu TLC Scanner CS-930 at 560 nm under the reflection mode, cutting out the peak-area from the recording paper, weighing the piece, and comparing with the standards for which various amounts of pure M-Ad were applied on the same SDS-PAGE. A laser-type densitometer was not suitable for this experiment since color intensity of the Ad band was greatly reduced by the laser beam.

Purification of 3 modified Ad's from the *E. coli* cell lysate was carried out by the method of Kimura et al. [23] with some modifications. The ratios of A_{214} -to- A_{276} of the purified Ad's were over 0.92, a value greater than the reported value of Ad purified from bovine adrenal cortex, 0.86 [10].

NADPH-cytochrome *c* and $P-450_{\text{SCC}}$ reduction activities were measured as described elsewhere [17,18], except that the reaction mixtures for cytochrome *c* reduction activity of the cell lysate contained 0.7 μ M AdR and 14 nM bovine Ad or a corresponding amount of the cell lysate. Reagents and enzymes were purchased from the following sources: IPTG, Sigma; 7-DEAZA Sequencing kit, exonuclease Bal31, and restriction endonucleases, Takara Shuzo Co. All other reagents were of a guaranteed grade and were purchased from commercial suppliers.

3. RESULTS AND DISCUSSION

As part of a study on the structure-activity relationship of Ad, we constructed a cDNA library for expression of different forms of bovine Ad modified at the amino-terminal region. We isolated 4 clones from the cDNA library. As shown in Fig. 1, the Ad derived from pBA1159, Ad(Met¹) corresponds to the mature form in which the amino-terminal Ser is replaced by the initiator Met. pBA1156 gives a short form of Ad, Ad(Met⁶), which lacks the amino-terminal 6 residues of

| | | | | |
|------------------------------------|---|--------------------------------|------|---|
| pKK223-3 (vector) | AGGAAACAGAAATTC | CCGGGGATCCGTCGACCTGCAGCCAAGCTT | S.D. | Eco RI |
| pBA1156 Ad(Met ⁶) | AGGAAACAGAAATTCATGATAACAGTCCACTTTATAAACCGTGAT | | S.D. | Met Ile Thr Val His Phe Ile Asn Arg Asp 7 8 9 10 11 12 13 14 15 |
| pBA1158 Ad(Met ⁻¹²) | AGGAAACAGAAATTCATGATGCAATTCATGAGCGTATCGGGGCCA | | S.D. | Met His Glu Phe Met Ser Val Ser Gly Arg -7 -6 -5 -4 -3 |
| pBA1159 Ad(Met ¹) | AGGAAACAGAAATTCATGAGCTCAGAAGATAAAATAACAGTCCAC | | S.D. | Met Ser Ser Glu Asp Lys Ile Thr Val His 2 3 4 5 6 7 8 9 10 |
| pBA1161 Ad(Met ⁻¹) | AGGAAACAGAAATTCATGAGCTCAGAAGATAAAATAACAGTCCAC | | S.D. | Met Ser Ser Ser Glu Asp Lys Ile Thr Val His 1 2 3 4 5 6 7 8 9 10 |

Fig. 1. Nucleotide sequence of the amino-terminal regions of the isolated Ad clones. Numbers under the amino acid sequence indicate the residue numbers of the initial amino-terminus, the first serine of the mature Ad as 1. AGGA is possible Shine-Dalgarno (S.D.) sequence and GAATTC is an *Eco*RI site. Bold characters indicate the nucleotides derived from the *Eco*RI-ATG linker(s). As for initiation codon, TTG, present in the pBA1161, see text.

M-Ad and has the initiator Met. pBA1158 gives a 12 residue-long form of Ad, Ad(Met⁻¹²), which has 5 linker-derived residues and 7 carboxyl-terminal residues of the extension peptides in front of the amino-terminus of M-Ad. pBA1161 gives a 1 residue-long form of Ad, Ad(Met⁻¹), which has an extra residue of Met at the amino-terminus of M-Ad. Table I shows levels of expression of 4 clones and their NADPH-cytochrome *c* reductase activities. The four modified Ad contents determined by Western blotting analysis were 6.8–24.4 mg/l culture, in Experiment 1. All of the modified Ad's were active in NADPH-cytochrome *c* reduction in the presence of AdR. However, the specific activities dif-

Table I
Cytochrome *c* reduction activities and contents of Ad's in the cell lysate

| Source | Ad | | Cyt. c red. | | | |
|-----------------------------------|-------------------|-------------------|-------------|--------|----------------|--------|
| | μ g/ml | | mU/ml | | mU/ μ g Ad | |
| | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
| pKK223-3 [vector] | N.D. ^a | N.D. ^a | 12.8 | 10.4 | - | - |
| pBA1156 [Ad(Met ⁶)] | 61 ^b | 40 | 598 | 492 | 9.8 | 12 |
| pBA1158 [Ad(Met ⁻¹²)] | 39 ^b | 41 | 463 | 550 | 11 | 13 |
| pBA1159 [Ad(Met ¹)] | 58 ^b | 73 | 963 | 1260 | 17 | 17 |
| pBA1161 [Ad(Met ⁻¹)] | 17 ^b | 16 | 284 | 275 | 17 | 17 |
| Bovine Ad [M-Ad] | | | | | 28 | 28 |

The enzyme activities of the cell lysate were measured as described in section 2. The content of Ad was estimated from densitometric tracing of the Western blotting sheet.

^a Not detected.

^b The contents of Ad's (mg per liter of the culture) for experiment 1 were calculated to be 24.4, 15.6, 23.2 and 6.8 mg/l of the culture, from the top to the bottom, respectively. The coefficient of correlation between weights of the peak-area and M-Ad's (40, 80, 120, 160 ng) which were applied on the same SDS-PAGE as the standard was 0.997.

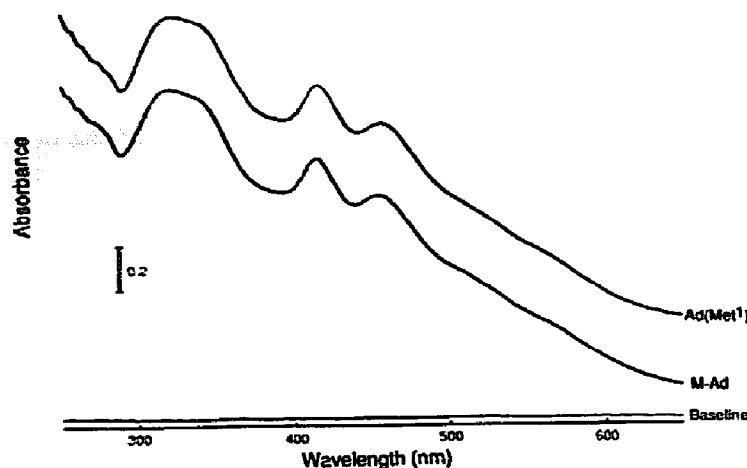


Fig. 2. Absorption spectrum of purified Ad(Met¹) in comparison with that of M-Ad. The absorption spectra of purified Ad's in 10 mM potassium phosphate buffer (pH 7.4) were measured using a Hitachi 228A spectrophotometer. The concentration of Ad's is 120 μ M, respectively.

ferred, thereby suggesting the presence of apo-Ad in the culture.

We attempted to purify the four modified Ad's from the extract of the *E. coli* cell lysate, but owing to the low level of the expression of Ad(Met¹), we were not successful. The major reason of the low expression is that the initiation codon of this form is TTG, as shown in Fig. 1. We purified 3 forms, Ad(Met¹), Ad(Met⁶) and Ad(Met¹²), from the sonicated extract of the cell lysate by 2 steps of DE-52 chromatography and Sephadex G-75 gel filtration. As ratios of A_{414}/A_{276} of all purified samples were over 0.92, the iron-sulfur centers of the modified Ad's remain more intact than that of M-Ad, because the highest value reported is 0.86 [10]. We observed a higher value of A_{414}/A_{276} than 0.900 using purified M-Ad from bovine adrenal cortex and other investigators obtained the same results [24]. However, this observation has not yet been taken into account because it is difficult to identify any significance between 0.86 and 0.90. The present study clearly shows the higher ratios of purified Ad's from *E. coli* extract. In our experience, the highest value of this ratio was 0.946 from Ad(Met¹). Fig. 2 shows the absorption spectra of Ad(Met¹) in comparison with that of M-Ad. The absorption maxima were observed at 455, 414 and 320 nm. The oxidized spectrum of Ad(Met¹) was indistinguishable from that of M-Ad, except that the value of absorption maximum at 414 nm was exactly the same as that of the absorbance observed as a trough at 290 nm; the former was significantly lower than the latter in the case of M-Ad. Fig. 3 shows SDS-PAGE patterns of purified Ad(Met¹) and M-Ad. M-Ad purified from the bovine adrenal cortex showed 2 bands on SDS-PAGE [4]. This was also observed on Western blots even when using a freshly excised adrenal gland (data not shown). Ad(Met¹) showed the same migration with the upper band of M-Ad. Ad(Met⁶) and Ad(Met¹²) also

showed a comparable migration with the sizes expected from their nucleotide sequences (data not shown). Tanaka et al. [7] reported a 114 residue sequence as an entire amino acid sequence of bovine Ad (M-Ad). However, 14 residues of the carboxyl-terminal extension were expected from the nucleotide sequence of the

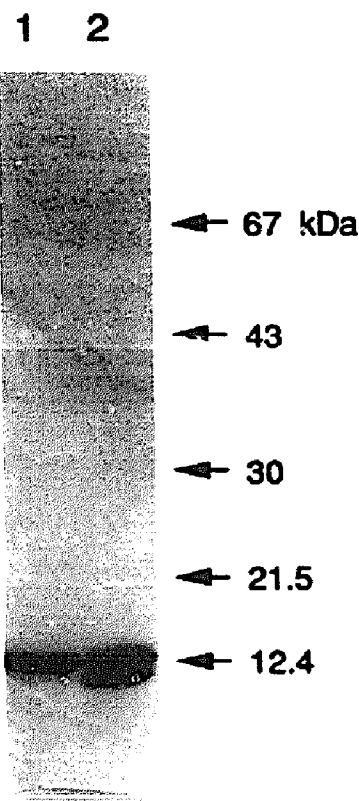


Fig. 3. SDS-PAGE patterns of purified Ad's modified in the amino-terminal region. 5 μ g of Ad's were applied on a gradient gel (8-16%). Lane 1, Ad(Met¹); lane 2, M-Ad.

Table II
Cytochrome *c* and *P*-450_{SCC} reduction activities of purified Ad's modified in the amino-terminal region

| Source | Cyt. <i>c</i> red. | | Cyt. <i>P</i> -450 _{SCC} red. (min ⁻¹) |
|-----------------------------------|--|-----------------------------------|---|
| | <i>V</i> _{max} (min ⁻¹) | <i>K</i> _m for Ad (nM) | |
| pBA1156 [Ad(Met ⁶)] | 266 ± 20 ^b | 31 ± 6 ^b | 7.3 ^c |
| pBA1158 [Ad(Met ⁻¹²)] | 279 ± 23 ^b | 34 ± 8 ^b | 9.2 ^c |
| pBA1159 [Ad(Met ¹)] | 242 ± 19 ^b | 29 ± 6 ^b | 13.2 ^c |
| Bovine Ad [M-Ad] | 279 ± 18 ^b | 40 ± 7 ^b | 13.2 ^c |

The enzyme activities were measured as described in section 2.

^a Flavin turnover numbers.

^b The numbers represent the mean ± S.E. (*n*=4).

^c Errors were within 10%.

cDNA, and were actually detected in M-Ad, using an immunological method [8,25]. Our M-Ad [4] gave a single peak on each cycle of the Edman degradation and the amino acid sequence was identical with the reported amino-terminal sequence (data not shown). We detected Lys-126 by peptide maps of lysylendopeptidase-digested Ad(Met¹) and M-Ad [26]. Our results suggest that the lower band of M-Ad is formed by processing of the carboxyl-terminal region in vivo, and that the carboxyl-terminal region of 3 modified Ad's produced in *E. coli* is not cleaved.

We next measured NADPH-cytochrome *c* and *P*-450_{SCC} reduction activities using 3 modified Ad's in comparison with M-Ad. As shown in Table II, the cytochrome *c* reduction catalyzed by Ad's and AdR was the same as flavin turnover numbers, and the *K*_m values for 3 modified forms of Ad were also the same as that of M-Ad. Thus, the amino-terminal region of Ad does not seem to contribute to the binding of AdR or cytochrome *c*, or electron transfer from AdR to cytochrome *c* via Ad. We measured NADPH-*P*-450_{SCC} reduction using 3 modified Ad's, under anaerobic conditions [18]. The reduction rates by Ad(Met⁶) and Ad(Met⁻¹²) were 7.25 and 9.23 min⁻¹, respectively, whereas those by Ad(Met¹) and M-Ad were 13.2 min⁻¹. Thus, while the amino-terminal region of Ad contributes to electron transfer of binding to *P*-450_{SCC}, the possibility that the small conformational change of Ad(Met⁶) affects electron transfer reactions would need to be ruled out. Very recently, Coghlan and Vickery reported that Asp-76 and Asp-79 were essential for interactions of ferredoxin both with AdR and with *P*-450_{SCC} using site-directed mutagenesis of human ferredoxin [27]. Miura et al. reported that modification of a His-56 residue in Ad with diethyl pyrocarbonate resulted in an increase of *K*_m in cytochrome *c* reduction by 2-fold and of *K*_d for *P*-450_{SCC} by 5-fold, indicating that His-56 is responsible for the reduction of binding affinities of Ad for redox partners [28]. These results suggest that several discontinuous surface domains of Ad may contribute to the binding to *P*-450_{SCC}. At all events,

determination of the tertiary structure of Ad is necessary to clarify the electron transport mechanism from AdR to *P*-450_{SCC} via Ad.

Acknowledgments: We are grateful to Prof. Michael R. Waterman, The University of Texas Southwestern Medical Center at Dallas for his valuable advice and critical reading of the manuscript. M. Ohara provided editorial comments. 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.

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