Different Effects of Carboxy-Terminal Deletion in the Adrenodoxin Molecule on Cytochrome c and Acetylated Cytochrome c Reductions

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In immunoblotting analysis using a rabbit antibody to bovine adrenodoxin, the total proteins of the bovine adrenal cortex gave two bands, suggesting the presence of two forms of adrenodoxin in vivo: full-length and carboxy-terminal deleted adrenodoxins. To examine the effect of the carboxy-terminal deletion of adrenodoxin on its activity, cDNAs for Arg(115)stop mutant adrenodoxin and for Asp(113)stop mutant adrenodoxin were constructed. The wild type [Ad (2-128)] and carboxy-terminal deleted [Ad (2-114) and Ad (2-112)] recombinant adrenodoxins expressed in Escherichia coli were purified to give a single band on SDS-PAGE. They showed an A414/A276 value of 0.92. In an NADPH-cytochrome c reduction assay, the Km values for cytochrome c in the reconstituted system with Ad (2-128), Ad (2-114) and Ad (2-112) were 39, 235 and 618 nm, respectively. The Vmax values were 638, 700 and 898 mol/min/mol flavin, respectively. In an NADPH-acetylated cytochrome c reduction assay, the maximum activity of Ad (2-128) was obtained at 50 mM NaCl, while the maximum activities of Ad (2-114) and Ad (2-112) were obtained at 100 mM NaCl; the latter values were 4 times higher than that of Ad (2-128). In the presence of 100 mM NaCl, the Km values for acetylated cytochrome c in the system reconstituted with Ad (2-128), Ad (2-114) and Ad (2-112) were 320, 33 and 22 µM, respectively. The Vmax values were 352, 305 and 382 mol/min/mol flavin, respectively. These results indicate that the effects of the carboxy-terminal deletion of adrenodoxin on NADPH–cytochrome c and acetylated cytochrome c reductions are different: the carboxy-terminal region (residues 113–128) of adrenodoxin largely contributes to the binding with cytochrome c but disturbs the binding with acetylated cytochrome c.

Key words: adrenodoxin; carboxy-terminal deletion; electron transfer; acetylated cytochrome c; adrenodoxin reductase; cytochrome P-450

Adrenodoxin is a [2Fe-2S]-type ferredoxin located in the mitochondrial matrix of steroidogenic tissues, and it functions as the common electron transporter from NADPH–adrenodoxin reductase to mitochondrial cytochromes P-450. The primary structure of bovine adrenodoxin, determined by amino acid sequencing, has 114 amino acid residues. However, the amino acid sequence of mature bovine adrenodoxin, as deduced from the cDNA sequence, consists of 128 amino acid residues. This result indicated the presence of 14 amino acid residue extensions at the carboxy-terminal end. The adrenodoxin purified by a conventional method from bovine adrenal glands showed two bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The presence of the adrenodoxin molecule with the carboxy-terminal extension in vivo was confirmed by immunological detection. Adrenodoxin with an elongated carboxy-terminal end has been purified from the bovine adrenal gland, using HPLC at pH 9.0. Only the carboxy-terminal Glu128 was deleted in the purified adrenodoxin.

When the carboxy-terminal region (residues 116–128) of adrenodoxin was deleted by limited proteolysis with trypsin, the trypsin-treated adrenodoxin showed enhanced activity regarding electron transport to cytochromes P-450 and to cytochrome c. The increase in steroid hydroxylation activity in the reconstituted system with carboxy-terminal deleted adrenodoxin was also observed by using adrenodoxins purified from a heterologous expression system with the cDNAs constructed by site-directed mutagenesis and PCR. However, the mechanism of the increase in activity accompanied by the carboxy-terminal deletion of adrenodoxin remains to be clarified.

In this study, to explore the above mechanism, we applied a new electron acceptor, acetylated cytochrome c, which is suggested to be a good model electron acceptor for cytochrome P-450. We observed strikingly different effects of the carboxy-terminal deletion of adrenodoxin on NADPH–cytochrome c and acetylated cytochrome c reductions.

MATERIALS AND METHODS

Materials Reagents and biochemicals were obtained from the following sources: restriction endonucleases and modifying enzymes, Takara Shuzo Co., Ltd.; NADPH, Oriental Yeast Co., Ltd.; cytochrome c (horse heart) and superoxide dismutase (bovine liver), Sigma Chemical Co.; Sephadex G-75 Superfine, Pharmacia P-L. Biochemicals, Inc.; DE52, Whatman Paper, Ltd.; protein molecular mass standards, Gibco BRL. All other reagents used were of a guaranteed grade.

Analytical Methods The concentration of adrenodoxin was determined using an extinction coefficient of 9.8 mM−1·cm−1 at 414 nm. The protein concentration was determined by the method of Lowry et al., using bovine serum albumin as a standard. The purity of the adrenodoxin was analyzed by SDS-PAGE, according
to the method of Laemmli.\textsuperscript{14} Immunoblotting analysis using rabbit antibody to bovine adrenodoxin was carried out according to the method of Towbin \textit{et al.}\textsuperscript{15} The amino-terminal amino acid sequence of the purified adrenodoxins was determined by Edman degradation using a gas phase protein sequencer (Applied Biosystems, model 473A).

### Construction of Mutant Adrenodoxin cDNA by Site-Directed Mutagenesis

The HindIII–EcoRI fragment containing the cDNA for the mature form of bovine adrenodoxin from pBAL15\textsuperscript{16} was ligated to the HindIII–EcoRI site of M13mp19 RF DNA. The cDNAs for mutant adrenodoxins which lack a carboxy-terminal region were constructed by the method of Kunkel \textit{et al.}\textsuperscript{17} The sequences of synthetic oligonucleotide primers for site-directed mutagenesis were 5′-TGCCGTGTCCTGATCAAGAGAGTCCA-3′ (26-mer, for Asp113stop) and 5′-GTCGATGGGCTAGAGTTTGCAT-3′ (21-mer, for Arg115stop). The underlined sequences indicate the stop codon introduced into the cDNA for mutant adrenodoxin. Letters in italics indicate the position of mismatches. The entire coding region of the mutant adrenodoxin cDNA was sequenced to confirm the proposed construction by the dyeoxy chain termination method. General DNA techniques used were according to Sambrook \textit{et al.}\textsuperscript{18}

### Expression and Purification of Recombinant Bovine Adrenodoxin

The HindIII–EcoRI fragment containing mutant adrenodoxin cDNA was cloned into the HindIII–EcoRI site of pKK223-3. The plasmid was used for the transformation of \textit{Escherichia coli} D1210. Culture of the \textit{E. coli} cells harboring the plasmid for wild type or mutant adrenodoxin, as well as the induction for adrenodoxin production, were as described elsewhere.\textsuperscript{16}

All purification procedures were carried out at 0–4 °C. The cells harvested from 4 liters of culture by centrifugation at 5000 × g for 5 min were washed once with 10 mM K-phosphate (pH 7.4). The cells were resuspended into 5 volumes of the phosphate buffer, then sonicated with a Branson Cell Disruptor 200 in ice-cold 10 mM K-phosphate (pH 7.4) using 120 s pulses at 1 min intervals and at 40% of the maximum power. The suspension was centrifuged at 5000 × g for 5 min, after which the supernatant was centrifuged at 100000 × g for 60 min. The recombinant adrenodoxin was purified from the resultant supernatant, according to the method of Kimura \textit{et al.}\textsuperscript{19} but with some modifications. The supernatant was applied onto a DE52 column (2.7 × 20 cm) equilibrated with 10 mM K-phosphate (pH 7.4). The column was washed with 10 mM K-phosphate (pH 7.4) containing 170 mM KCl until A\textsubscript{280} of the eluate decreased to lower than 0.05. The white layer of DE52 above the brown band containing adrenodoxin was removed. The remaining adrenodoxin was eluted with 4 volumes of 10 mM K-phosphate (pH 7.4) containing 300 mM KCl. The fractions which had an A\textsubscript{444}/A\textsubscript{276} ratio higher than 0.4 were combined and diluted 3 times with 10 mM K-phosphate (pH 7.4). The diluted adrenodoxin-rich fraction was applied onto a second DE52 column (2.0 × 20 cm) equilibrated with 10 mM K-phosphate (pH 7.4). After washing and removal of the upper white DE52 layer, adrenodoxin was eluted with 10 mM K-phosphate (pH 7.4) containing 300 mM KCl, as described above. The fractions which had an A\textsubscript{444}/A\textsubscript{276} ratio higher than 0.8 were combined and diluted 3 times with 10 mM K-phosphate (pH 7.4). After being concentrated using a small DE52 column (1.0 × 1.0 cm) and 10 mM K-phosphate (pH 7.4) containing 500 mM KCl, adrenodoxin was filtered through a Sephadex G-75 Superfine column (3.0 × 100 cm) equilibrated with 10 mM K-phosphate (pH 7.4) containing 100 mM KCl.

### Enzyme Assays

NADPH-cytochrome c reduction activity was spectrophotometrically measured at 550 nm in 2 ml of 5 mM HEPES buffer (pH 7.5) containing 0.05 mM adrenodoxin reductase, 0.05 mM adrenodoxin, 20 mM cytochrome c and 100 mM NADPH at 25 °C.\textsuperscript{1,2} The concentration of cytochrome c was determined using the reduced minus oxidized extinction coefficient of 19.5 mM⁻¹ cm⁻¹ at 550 nm.\textsuperscript{20} NADPH-acetylated cytochrome c reduction activity was measured at 550 nm in 2 ml of 5 mM HEPES buffer (pH 7.5) containing 0.1 mM adrenodoxin reductase, 0.1 mM adrenodoxin, 20 µg/ml superoxide dismutase, 30 µM acetylated cytochrome c and 100 µM NADPH at 25 °C.\textsuperscript{21} Acetylated cytochrome c was prepared by the method of Azi \textit{et al.}\textsuperscript{22} Cholesterol side-chain cleavage activity was measured in 0.5 ml of 30 mM K-phosphate (pH 7.2) containing 0.8 mM adrenodoxin reductase, 8 µM adrenodoxin, 0.4 µM P-450 11A1, 200 µM cholesterol, 0.1% (w/v) Emulgen 911 and 500 µM NADPH at 37 °C.\textsuperscript{23} The adrenodoxin reductase and cytochrome P-450 11A1 used were authentic enzymes purified from the bovine adrenal cortex.\textsuperscript{1,2,21}

### RESULTS

#### Presence of Two Forms of Adrenodoxin in Vivo

The bovine adrenal cortex was prepared from a freshly excised adrenal gland, which was immediately frozen with liquid nitrogen, then homogenized in the presence of protease inhibitors (10 µg/ml each of leupeptin, pepstatin and aprotinin). In the immunoblotting analysis using a rabbit antibody to bovine adrenodoxin, the total proteins of the bovine adrenal cortex gave two bands (Fig. 1). When the homogenate was incubated in the absence of protease inhibitors at 37 °C for 30 min, the intensity of both bands decreased. Only a slight change was observed in the ratio of the bands (Fig. 1). This result was confirmed with five individual adrenal glands. Thus, two forms of adrenodoxin apparently exist in vivo. These phenomena are consistent with the findings that the authentic adrenodoxin purified from the bovine adrenal gland showed two bands on SDS-PAGE.\textsuperscript{5,10} It is suspected that the upper and lower bands correspond to the full-length adrenodoxin and the carboxy-terminal deleted adrenodoxin, respectively.

#### Purification of Recombinant Adrenodoxins

To examine the effect of the carboxy-terminal deletion of adrenodoxin on its activity, we constructed cDNAs for Arg115stop and Asp113stop mutant adrenodoxin and for Arg115stop and Arg113stop mutant adrenodoxin. The former was designed to delete the carboxy-terminal 14 amino acid residues. The primary structure of its product was expected to be identical with that originally reported by Tanaka \textit{et al.}\textsuperscript{23} except for the amino-terminal Met. The latter was designed to delete the carboxy-
terminal 16 amino acid residues. These mutant adrenodoxins and the wild type adrenodoxin, Ad(Met)\(^1\), which corresponds to the mature form of bovine adrenodoxin,\(^1\) were purified from the lysate of transformed E. coli cells by two steps of DE52 column chromatography and one step of Sephadex G-75 gel filtration. As shown in Fig. 2, the wild type and the two mutant adrenodoxins with carboxy-terminal deletion each gave a single band on SDS-PAGE. The apparent sizes of the mutant adrenodoxins were smaller than that of the wild type, as expected from the cDNA sequence. The purified adrenodoxins showed a typical absorption spectrum of adrenodoxin, with peaks at 455, 414 and 320 nm (data not shown). The ratio of \(A_{444}/A_{276}\) was 0.92. The amino-terminal amino acid sequences of the purified wild type, the Arg115stop mutant and the Asp113stop mutant adrenodoxins were the same, Ser–Ser–Glu–Asp. This result indicates that the amino-terminal Met of the expressed adrenodoxins in E. coli had been excised. The amino acid composition of the purified wild type adrenodoxin was close to that calculated from the cDNA sequence when the excision of amino-terminal Met was considered (data not shown). Therefore, the purified wild type, Arg115stop mutant and Asp113stop mutant adrenodoxins probably consist of 2-128, 2-114 and 2-112 residues, respectively. These recombinant adrenodoxins are designated in this report as Ad(2-128), Ad(2-114) and Ad(2-112).

We also constructed a cDNA for Arg106stop mutant adrenodoxin. The cell lysate of E. coli expressing the mutant adrenodoxin showed a significant amount of adrenodoxin reductase-dependent NADPH-cytochrome c reducing activity. However, the cell lysate from a 37°C culture showed lower activity than that from a 28°C culture. We were not able to purify the active protein of Arg106stop mutant adrenodoxin, probably owing to the instability of this mutant protein (data not shown). This result is apparently inconsistent with the findings of Uhllmann et al.,\(^1\) who observed that the mutant 4–107, which lacks a unique proline residue at position 108, showed no EPR spectrum. They considered that Pro108 plays an essential role in the formation of the iron-sulfur cluster. However, both results suggest that the carboxy-terminal region, around 106–108, contributes to the stability of adrenodoxin.

**Interaction with Cytochrome c**

The complex formations of adrenodoxin with adrenodoxin reductase and with cytochrome c are mainly ionic and are affected by salts. We examined the effects of ionic strength on NADPH-cytochrome c reduction catalyzed by carboxy-terminal deleted adrenodoxins by using a system reconstituted with adrenodoxin reductase. The effect of ionic strength on the activity by Ad(2-128) was the same as that on the activity by bovine adrenodoxin.\(^1\) The reduction rate was highest at a low ionic strength, 5 mM HEPES buffer (pH 7.5) without NaCl, from which the rate gradually decreased when the salt concentration increased (Fig. 3). In the presence of 0–100 mM NaCl, Ad(2-114) and Ad(2-112) showed somewhat higher activities than that seen for Ad(2-128). This means that cytochrome c reduction by these mutant adrenodoxins is less sensitive to ionic strength than that by the wild type adrenodoxin.

The \(K_m\) values for cytochrome c in the reconstituted NADPH-cytochrome c reduction system with Ad(2-128),
Ad(2-114) and Ad(2-112) were 39, 235 and 618 nM, respectively. The $V_{\text{max}}$ values were 638, 700 and 898 mol/min/mol flavin, respectively. The $K_m$ values with Ad(2-114) and Ad(2-112) were markedly higher than that with Ad(2-128), while the $V_{\text{max}}$ values were not so largely different. These results clearly indicate that the carboxy-terminal deletion of adrenodoxins causes a decrease in the affinity of the adrenodoxins to cytochrome c.

**Interaction with Acetylated Cytochrome c** At the beginning of this study, we examined cholesterol side-chain cleavage activity using a system reconstituted with adrenodoxin, adrenodoxin reductase and cytochrome P-450 11A1. The mutant adrenodoxins with carboxy-terminal deletion showed apparently higher activity than that of the wild type in the electron transfer reaction. Ad(2-128), Ad(2-114) and Ad(2-112) gave values of 6.8, 10.9 and 9.5 mol pregnenolone produced/min/mol cytochrome P-450 11A1, respectively, when the molar ratio of adrenodoxin to cytochrome P-450 11A1 was 20. This result is essentially consistent with both the results concerning adrenodoxin with carboxy-terminal deletion by trypsin digestion$^9$ and those related to adrenodoxin with carboxy-terminal deletion constructed by PCR.$^{10}$

Acetylated cytochrome c functions as an electron acceptor from adrenodoxin, and was proposed to be a good model electron acceptor for cytochrome P-450 11A1.$^{11}$ In order to obtain information about the contribution of the carboxy-terminal region of adrenodoxin to its electron transfer to the hydrophobic electron acceptor, we examined the acetylated cytochrome c reduction by the carboxy-terminal deleted adrenodoxins using a system reconstituted with adrenodoxin reductase under various ionic strengths. The maximum activity of Ad(2-128) was 65 mol/min/mol flavin at 50 mM NaCl, but the activity of Ad(2-114) and Ad(2-112) was observed at a higher concentration of NaCl, 100 mM, and was 4 times higher than that of Ad(2-128) (Fig. 4).

In the presence of 100 mM NaCl, the $K_m$ values for acetylated cytochrome c in the reconstituted system with Ad(2-128), Ad(2-114) and Ad(2-112) were 220, 33 and 22 μM, respectively. The $V_{\text{max}}$ values were 352, 305 and 382 mol/min/mol flavin, respectively. The $K_m$ values with Ad(2-114) and Ad(2-112) were 7—10 times lower than that with Ad(2-128), while the $V_{\text{max}}$ values were not so largely different. These findings clearly indicate that the carboxy-terminal deletion of adrenodoxin causes an increase in the affinity of adrenodoxins to acetylated cytochrome c, in contrast to that to cytochrome c.

**DISCUSSION**

The electron transfer from the flavin of adrenodoxin reductase to the non-heme iron of adrenodoxin is considered to be the rate-limiting step in the cytochrome c reduction reaction.$^{26}$ In our previous study, we compared the catalytic properties of a chemically cross-linked complex between adrenodoxin reductase and adrenodoxin with those of the equimolar mixture of these enzymes (native complex). NADPH-cytochrome c reduction activity of the cross-linked complex was comparable (117%) to that of the native complex.$^{12}$ This result suggests that the adrenodoxin molecule has no need to dissociate from the complex during the electron transfer from NADPH to cytochrome c, and that the binding site of adrenodoxin to adrenodoxin reductase does not overlap with that to cytochrome c.

Coughlan and Vickery$^{27}$ reported that the site-directed mutagenesis of Asp76 and Asp79 of human ferredoxin, which correspond to Asp76 and Asp79 of bovine adrenodoxin, respectively, resulted in a dramatic decrease in affinity of the ferredoxin for adrenodoxin reductase. This result suggests that these two acidic residues are the binding sites of adrenodoxin to adrenodoxin reductase.

In the present study, the deletion of the carboxy-terminal region led to a decrease in affinity of the adrenodoxins to cytochrome c. It would thus appear that the carboxy-terminal 113—128 region of adrenodoxin largely
contributes to the complex formation with cytochrome c, but not to the binding with adrenodoxin reductase. Ad(2-114) lost three acidic amino acid residues (Glu116, Asp119 and Glu128) and two basic amino acid residues (Arg115 and Lys126) from Ad(2-128). Ad(2-112) lost one additional acidic amino acid residue (Asp113) from Ad(2-114) (Fig. 5). The lesser sensitivity to ionic strength of the adrenodoxins with carboxyl-terminal deletion suggests that these charged residues of adrenodoxin contribute to the complex formation of adrenodoxin with cytochrome c. The difference in affinity to cytochrome c might reflect the distinctive effect of the salt on cytochrome c reduction activity.

We previously observed that acetylated cytochrome c reduction by the cross-linked complex between adrenodoxin reductase and adrenodoxin was slow (0.4%) compared to that by the native complex. On the other hand, cytochrome c reduction by the cross-linked complex was comparable to that seen with the native complex. These results suggest that the binding site of adrenodoxin to acetylated cytochrome c does not completely overlap with that to cytochrome c, even if they are in close proximity.

In the present study, the deletion of the carboxy-terminal region led to a marked increase in affinity of the adrenodoxins to acetylated cytochrome c, in contrast to that to cytochrome c. Since the force of interaction between adrenodoxin and acetylated cytochrome c must be hydrophobic rather than ionic, this dramatic effect may be related to the removal of charged amino acid residues in the carboxy-terminal region of adrenodoxin. The removal of charged amino acid residues may lead to the enhanced activity of adrenodoxin in acetylated cytochrome c reduction through enhancement of the hydrophobic interaction. This notion is consistent with the finding that carboxy-terminal deleted adrenodoxins showed enhanced activity in the cholesterol side-chain cleavage reaction.

Another possible explanation for the effect of carboxy-terminal deletion is that the carboxy-terminal region of adrenodoxin interferes with binding to acetylated cytochrome c, by sterical hindrance. According to a recent report by Uhlmann et al., the CD spectrum of adrenodoxin mutants 4-108 and 4-114 was similar to that of the wild type 4-128, suggesting that carboxy-terminal deletion does not cause a large change in the tertiary structure of adrenodoxin. When compared with the ferredoxin of Spirulina platensis and the putidaredoxin of Pseudomonas putida, adrenodoxin has excess carboxy-terminal residues, 113–128. This region corresponds exactly to the carboxy-terminal extension noted with cDNA sequencing (Fig. 5). Although the tertiary structure of adrenodoxin is unknown, this hydrophilic carboxy-terminal region of adrenodoxin might be present in tail form. If this is the case, the binding site of adrenodoxin to acetylated cytochrome c would be in the proximity of residue 112 in steric distance.

We directly address to the presence of adrenodoxin molecules with carboxy-terminal deletion in bovine adrenocortical cells. The authentic bovine adrenodoxin originally reported by Tanaka et al. 21 consisted of residues 1-114. The corresponding carboxy-terminal deleted adrenodoxin, Ad(2-114), was active in electron transfers to cytochrome P-450 11A1, cytochrome c and acetylated cytochrome c. Furthermore, the carboxy-terminal deleted adrenodoxin was more active in its electron transfer to the hydrophobic electron acceptors than the full-length adrenodoxin. Uhlmann et al. 21 reported that the first step of electron transfer to cytochrome P-450 11B1, but not to cytochrome P-450 11A1, was accelerated by adrenodoxin mutants 4-108 and 4-114.

When the carboxy-terminal region was deleted to position 106, the adrenodoxin became unstable. Thus, the proximate carboxy-terminal region may also contribute to a stable conformation of adrenodoxin. The possibility that the carboxy-terminal deleted adrenodoxin is an intermediate form during the degradation of adrenodoxin cannot be ruled out. However, another possibility, that the carboxy-terminal deleted adrenodoxin has a positive and specific function in vivo, can also be considered. By using a new electron acceptor, acetylated cytochrome c, the significant influence of the carboxy-terminal region of
adrenodoxin on its electron transfer was further clarified. Processing of the carboxy-terminal region of adrenodoxin in the mitochondria may be physiologically important and might affect the cell type-specific production of steroid hormones.

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REFERENCES