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The interaction domain of the redox protein adrenodoxin is mandatory for binding of the electron acceptor CYP11A1, but is not required for binding of the electron donor adrenodoxin reductase ☆

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Abstract

Adrenodoxin (Adx) is a [2Fe–2S] ferredoxin involved in electron transfer reactions in the steroid hormone biosynthesis of mammals. In this study, we deleted the sequence coding for the complete interaction domain in the Adx cDNA. The expressed recombinant protein consists of the amino acids 1–60, followed by the residues 89–128, and represents only the core domain of Adx (Adx-cd) but still incorporates the [2Fe–2S] cluster. Adx-cd accepts electrons from its natural redox partner, adrenodoxin reductase (AdR), and forms an individual complex with this NADPH-dependent flavoprotein. In contrast, formation of a complex with the natural electron acceptor, CYP11A1, as well as electron transfer to this steroid hydroxylase is prevented. By an electrostatic and van der Waals energy minimization procedure, complexes between AdR and Adx-cd have been proposed which have binding areas different from the native complex. Electron transport remains possible, despite longer electron transfer pathways.

Keywords: Adrenodoxin; Electron transfer; Ferredoxin-NADP$^+$ reductase; Protein–protein interaction; Electrostatic potential; CYP11A1

In mammals, the adrenal cortex is the primary site of synthesis of a number of important steroid hormones. Also here, oxygenases play an important role, which were discovered by Osamu Hayaishi 50 years ago as enzymes that catalyse the oxidative cleavage of substrates by incorporation of atmospheric dioxygen into the substrate. Therefore this publication is dedicated to him and his pioneering discovery. The presence of different oxygenases (i.e., cytochrome P450s), localized in either the mitochondria or endoplasmic reticulum, work in concert for the sequential oxygenation of the steroid nucleus. The initial and rate-limiting step of this biosynthesis, the conversion of cholesterol to yield pregnenolone, the precursor of all steroid hormones, is performed by the mitochondrial cholesterol converting P450 system composed of the NADPH-dependent FAD-containing adrenodoxin reductase (AdR),[1] adrenodoxin (Adx), and the cytochrome P450$_{scc}$ (CYP11A1).

Bovine Adx, localized in the mitochondrial matrix, serves as an electron transfer protein in this P450 system and also directs electrons to the cytochrome CYP11B1 involved in producing the steroid hormones cortisol and aldosterone [1]. The mature soluble form of adrenodoxin consists of 128 amino acids and belongs to the group of ferredoxins carrying a [2Fe–2S] cluster. The Adx molecule is organized in two structural domains, a core domain containing the

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[1] *Abbreviations:* Adx, bovine adrenodoxin wild type; Adx-cd, deletion mutant of adrenodoxin lacking amino acids 60–88; AdR, adrenodoxin reductase; CYP11A1, cytochrome P450$_{scc}$; CD, circular dichroism; ET, electron transfer; SOE-PCR, site overlapping PCR; PCR, polymerase chain reaction; EDC, *N*-ethyl-*N*′-dimethylaminopropyl-carbodiimide; NHS, *N*-hydroxysuccinimide.

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iron–sulfur cluster, coordinated with four cysteines [2], and a hairpin domain [3]. The hairpin, also referred to as the recognition domain, is required for recognition and interaction of Adx with its redox partners. This recognition is mainly based on electrostatic interactions of negatively charged amino acids on the surface of Adx with positively charged amino acids of AdR or of cytochrome P450, respectively [4,5]. The different acidic residues of the recognition domain contribute unequally to the stabilization of the two complexes, but show a significant overlap for the binding site of AdR and CYP11A1 [6]. Amino acids 68–86 of Adx were shown to interact with both, AdR and CYP11A1 [7]. Aspartates 76 and 79 seem to be essential for interaction with AdR and CYP11A1, while aspartate 72 and glutamate 73 seem to interact only with CYP11A1 [8]. Tyrosine 82 has also been suggested to be close to the binding site of AdR and CYP11A1 [9]. Additional interaction sites localized in the core domain have been identified by the resolved crystal structure of a cross-linked 1:1 complex of Adx and AdR [5] as well as by site-directed mutagenesis studies. These interaction sites are localized in a second acidic patch around position Asp-39, the putative electron transfer region around the iron–sulfur cluster loop [10,11], and involve C-terminal residues of the ferredoxin [12].

Apart from the interacting residues of the redox partners, the overall organization of the functional electron transferring complex is still under investigation. Kido and Kimura [13] have reported that in the presence of cholesterol a stable 1:1:1 complex of Adx with its redox partners was formed. In contrast, Lambeth et al. [14] have proposed that Adx functions as a mobile electron shuttle transferring electrons from AdR to the cytochrome P450. According to a third model proposed by Hara and Takeshima [15], electron transfer occurs using two molecules of Adx transferring the electrons needed to the cytochrome P450. Additional evidence that Adx forms dimers has been obtained recently [16,17]. Nevertheless, how the reducing equivalent is transferred from AdR to CYP11A1 by Adx is still a matter of controversial discussion.

In this study, we describe a deletion mutant, Adx-cd, consisting of only the core domain, generated by SOE-PCR cloning. In order to investigate the specific role of the remaining interaction sites in the adrenodoxin core domain for its redox partners, the deletion mutant Adx-cd was characterized in comparison with full-length Adx by biophysical and biochemical methods. In particular, Adx-cd was analyzed in vitro for its ability to associate with AdR and with CYP11A1, for its function as electron acceptor for AdR, and for its function to support the P450-catalyzed cholesterol side-chain cleavage. The experimental results have been complemented by protein–protein docking calculations.

Materials and methods

Reagents and biochemicals. Taq DNA polymerase was from Biozym and restriction endonucleases were from GE Healthcare. Steroids were purchased from Sigma. All other reagents were of the highest purity grade commercially available.

Construction of the cDNA for adrenodoxin mutant. The cDNA of the deletion mutant Adx-cd was obtained in two subsequent reactions. In a first amplification step, two fragments with overlapping ends were produced by PCR. Fragment 1, obtained using primer pair 1 (Table 1), codes for amino acids 4–60 in wild-type adrenodoxin and continues with amino acids 89–94, which serve as the overlapping region. Fragment 2, which codes for amino acids 89–128 with an overlapping region from amino acid 55 to 60 in the N-terminal position, was produced using the primer pair 2. Together, these two fragments serve as the template for the following PCR using the forward primer from primer pair 1 and the reverse primer from primer pair 2 as a new primer pair. In this second amplification step, a fragment was obtained which codes for the mutant adrenodoxin protein. The fragment was cloned into the expression vector *pET3d* [18]. The accurate cDNA sequence was verified by sequencing.

Expression and purification. Escherichia coli strain BL21(DE3) was used for protein expression. Recombinant Adx and AdR were purified as described [2,19]. Protein concentration was calculated using $\varepsilon_{414} = 9.8$ $(\text{mM cm})^{-1}$ for Adx [20], and $\varepsilon_{450} = 11.3$ $(\text{mM cm})^{-1}$ for AdR [21]. CYP11A1 from bovine adrenal glands was isolated according to Akhrem et al. [22]. Adx-cd was purified using standard protocols for Adx isolation.

Spectroscopic methods. Absorption spectra in the UV/visible region were recorded at room temperature using a Shimadzu double-beam spectrophotometer UV2101PC.

CD spectra were recorded as described [23] using a Jasco 715 spectropolarimeter. Temperature-dependent measurements were carried out at a heating rate of 50 °C h^{-1} from 20 to 65 °C with a temperature increment of 0.2 °C, monitoring the decrease of the circular dichroism signal at 440 nm. T_m was calculated from CD scans with a nonlinear regression program using a two-state model [24,25].

EPR spectroscopy. Two hundred microliters of a 1 mM adrenodoxin solution was reduced with dithionite under anaerobic conditions in a glove box. After transferring the sample into an EPR tube, it was frozen in liquid nitrogen. EPR spectra were recorded with a Bruker spectrometer ER 420 with a microwave frequency of 9.5 GHz and a field modulation frequency of 100 kHz. An immersion Dewar flask was used for the measurements at liquid nitrogen temperature (77 K).

Redox potential measurements. Redox potentials of Adx and Adx-cd were determined using the dye photoreduction method with Safranin T as indicator and mediator as described [26].

Table 1
Oligonucleotides for SOE-PCR

	Oligonucleotide
Primer pair 1	
Forward	5′-gggg**CCATgg**gCAgCTCAgAAgATAAAATAACAgTC-3′
Reverse	5′-gATCTggCAgCCCAACCgTTCAAAgATgAggTgACA-3′
Primer pair 2	
Forward	5′-TgTCACCTCATCTTTgAAgCCTTgggCTgCCAgATC-3′
Reverse	5′-gggg**ggATCC**TTAAggTACTCgAACAgTCATATTg-3′

The sequences which code for the overlapping region are underlined and restriction sites for cloning are in bold letters. The polymerase chain reaction primers with appropriate cloning sites and overlapping sites were chemically synthesized by BioTez GmbH, Berlin.