The steroid hydroxylating system of adrenal cortex mitochondria consists of the membrane-attached NADPH-dependent adrenodoxin reductase (AR), the soluble one-electron transport protein adrenodoxin (Adx), and a membrane-integrated cytochrome P450 of the CYP11 family. In the 2.3-Å resolution crystal structure of the Adx:AR complex, 580 Å² of partly polar surface are buried. Main interaction sites are centered around diimide; r.m.s.d., root mean square deviation.

In mitochondria of the adrenal cortex, the cytochrome P450 enzymes of the CYP11 family catalyze the side chain cleavage of cholesterol to form pregnenolone (P450sc, CYP11A1) and are involved in the formation of cortisol (P45011β, CYP11B1) and aldosterone (P450aldo, CYP11B2). The enzymatic activity of the cytochrome P450-dependent steroid hydroxylases is based on their ability to activate molecular oxygen by reductive splitting of dioxygen. This multistep reaction requires the transfer of electrons from the flavoprotein adrenodoxin reductase (AR) via adrenodoxin (Adx) to the terminal cytochromes P450 as electron acceptors in dependence on the specific hydroxylation substrate (1–3). Several models for electron transfer have been discussed, including a shuttle model in which Adx forms consecutive 1:1 complexes (4) with AR and cytochrome P450scc and models requiring the formation of an organized 1:1 ternary complex (5) or a 1:2:1 quaternary complex (6) between AR, Adx, and cytochrome P450scc. Common to these models is a complex between AR and Adx during the first steps of electron transfer from the reductase to the cytochrome P450.

Recently, the crystal structures of two forms of bovine adrenodoxin (7, 8) and of adrenodoxin reductase (9) were determined. These structures revealed the general topology of the two proteins and the molecular environments of the [2Fe-2S] cluster of Adx and the FAD moiety of AR. Here, we report the 2.3-Å resolution crystal structure of a cross-linked 1:1 complex of full-length Adx and AR. This structure shows the geometry of an electron transfer complex of soluble, freely dissociable proteins from a higher eukaryote for the first time, highlights structural adaptations that accompany the binding of AR to Adx, and permits us to predict electron transport paths in their complex.

EXPERIMENTAL PROCEDURES

Sample Preparation—Recombinant bovine Adx and AR were purified and crystallized as described (10). The synthesized Adx differs from the wild-type protein by the exchange of Ser² for glycine and is composed of 128 amino acids, including the N- and C-terminal residues missing in the truncated adrenodoxin, Adx(4–108), studied earlier (7). Cross-linking of AR to Adx has also been described (11, 12). The native complex is formed at low ionic strength between the two proteins, and the cross-linking was carried out with the water-soluble coupling reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, purchased from Sigma). The mixture of Adx (1.2 μmol) and AR (300 nmol) was dialyzed for 18–20 h against 20 mM potassium phosphate, pH 7.4, for 3 h; 50–100 mM potassium phosphate, pH 7.2, followed by addition of an equal volume of fresh 8 mM EDC solution in distilled water and incubation at 4 °C in the dark with occasional stirring. After 8 h, excess of the reagent was removed on a Sephadex G-25 column equilibrated with 10 mM potassium phosphate, pH 7.4. The colored fraction was pooled and applied on a 2.4 cm DEAE-Fractogel column and washed with two gradient solutions as follows: 10–50 mM potassium phosphate, pH 7.4, for 3 h; 50–100 mM potassium phosphate, pH 7.2, for 3 h. The peak containing the covariant cross-linked complex of the recombinant Adx and AR was consequently purified on an AD-Sepharose column to remove residual AR and on an ADP-Sepharose column to remove unbound Adx. The cross-linking of AR to EDC was expected (11, 12) to yield an amide bond between the ε-amino group of Lys⁶⁶ in Adx and the γ-carboxyl group of Glu⁴ in AR.

X-ray Data Collection—Four x-ray diffraction data sets from three crystals were collected at 100 K on MAR345 imaging plates at beam lines BW7B (EMBL Outstation at DESY, Hamburg) and BW6 (MPG-ASMB, c/o DESY). Due to problems with spatial reflection overlaps

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Structure of Adrenodoxin Reductase-Adrenodoxin Complex

TABLE I

| Parameter                        | Values          |
|----------------------------------|-----------------|
| **Crystallographic data and structure refinement** |                  |
| Space group                      | P6,22           |
| Cell constants a/c (Å)           | 92.2/607.85     |
| Resolution (Å)                   | 2.3             |
| Observed/unique reflections      | 201,839/55,229  |
| Completeness (all data/outer shell) | 79/66%          |
| R<sub>work</sub> (all data/outer shell) | 0.049/0.12      |
| *(I/|I|)* (all data/outer shell)      | 18.5/11         |
| R/|R<sub>work</sub>|<sub>b</sub>       | 0.223/0.222/0.268 |
| Asymmetric unit                  |                 |
| Complexes/atoms                  | 2/9117          |
| Protein, chains/residues/atoms   | 4/132/8701      |
| Iron-sulfur clusters/atoms       | 2/8             |
| Sulfate ions/atoms               | 5/25            |
| FAD molecules/atoms              | 2/106           |
| Water molecules                  | 277             |

caused by the long c axis of 607.85 Å, the data reached only 79% completeness (85% at 2.5 Å resolution), although several detector settings were used. The data sets were processed by DENZO/SCALEPACK (13) and contained 55,229 unique reflexes after merging (Table I).

**Structure Determination and Refinement**—The structure of the AR-Adx complex was solved by molecular replacement using the coordinates of AR and Adx-(4–108) as deposited in the Protein Data Bank (codes 1jc and 1ay). Two complexes per asymmetric unit with a molecular mass of 64,927 Da each were assembled by placing the protein molecules into the unit cell using AMORE (14) and rigid-body refinement with the program CNS (15) resulting in R = 0.386, R<sub>work</sub> = 0.394 at 2.8 Å resolution. Application of a solvent mask, positional and atomic temperature factor refinement, several rounds of manual density fitting, and the addition of 5 sulfate ions and 277 water molecules reduced R to 0.223 and R<sub>free</sub> to 0.268 (Table I). 1132 residues out of 1176 could be buried within the electron density. Almost all modeled water positions are also occupied in the crystal structures of Adx-(4–108) and AR (9). The averaged main chain and side chain parameters are equal or better than those in a set of 118 structures used by PROCHECK (16), and the Ramachandran diagram is free of outliers.

**RESULTS AND DISCUSSION**

**Architecture of the AR-Adx Complex**—The hexagonal crystals used in this analysis are formed by cross-linked 1:1 complexes between AR and Adx, both in their oxidized form. Two complexes related by a noncrystallographic screw rotation are present in the asymmetric unit. Complex I contains residues 5–117 of Adx and 4–460 of AR, and complex II contains residues 5–110 of Adx and 5–460 of AR. Electron density for both complexes related by a noncrystallographic screw rotation are present. The averaged main chain and side chain parameters are equal or better than those in a set of 118 structures used by PROCHECK (16), and the Ramachandran diagram is free of outliers.

**Structure of the AR-Adx Complex**

**Reorientation of AR Domains during Complex Formation**—Both Adx and AR are two-domain proteins. Adx consists of a core domain containing the [2Fe-2S] cluster and a small interaction domain (7), and AR contains a FAD domain and a NADP domain of about equal size (9). Whereas no significant difference between Adx-(4–108) and Adx as present in the complex is detected, the two AR domains show a slightly different orien-
Arg240 and Lys27 of AR as reference contact points with Adx requires a 3.7° rotation (7.2° for complex II). Considering of Asp39 in redox-partner binding has been suggested earlier binding by the homologous putida redoxin (27). An involvement that has been implicated in cytochrome P450cam (CYP101) drawn with SETOR (20).

This figure and Figs. 3 (bottom) were parts of the secondary interaction region. Residues Asp 39 and Asp 41 contacting His 28 and Lys 27 of AR, during steroid biosynthesis must be regarded as very unlikely. Contacts to AR, the formation of an organized 1:1:1 complex between AR, Adx, and cytochrome P450scc for electron transport is known also to be involved in cytochrome P450scc binding (1).

Fig. 2. Least squares superposition of the FAD domains of AR (bottom) from the crystal structures of free AR (9) (black) and the AR-Adx complex (molecule 1 in red and molecule 2 in gold). In the complex, the NAD domains of AR (top) undergo a slight domain rearrangement relative to the FAD domains that pulls the regions in contact with Adx closer together. Arg211, Arg244, and Arg244 of AR are part of the primary and Lys27 is part of the secondary interaction region. This figure and Figs. 3 (bottom) were drawn with SETOR (20).

Complex Formation by Electrostatic Interactions—The AR-Adx complex displays a highly charged surface (Fig. 3, top) arising from interacting surfaces that are predominantly acidic (Adx) or basic (AR). Of the 580 Å² of solvent-accessible surface buried in the complex, 325 Å² are from hydrophobic side chains. Nearly half of the AR-Adx interface is composed of polar and charged residues engaging in a large number of hydrogen bonds and salt links. Hence, electrostatic interactions may be considered the primary driving force for complex formation in agreement with chemical modification (21) and site-directed mutagenesis experiments (1, 22–24) of AR and Adx.

Electrostatic interactions predominate in the two main interaction sites of the AR-Adx complex. In the primary interaction region (Fig. 3, bottom left), arginines 211, 240, and 244 of the NADP domain of AR are involved in numerous salt bridges with Adx carboxylate groups. Aspartates 72, 76, and 79 of the Adx interaction domain are binding partners to AR, whereas the acidic residues Glu73 and Glu74 of Adx are facing away from the interface. The electron density provides no evidence for a covalent cross-link formed at this interaction site. Acidic Adx residues located at the primary AR-Adx interaction region are involved also to be involved in cytochrome P450scce binding (1). Given the participation of several of these side chains in contacts to AR, the formation of an organized 1:1:1 complex between AR, Adx, and cytochrome P450scce for electron transport during steroid biosynthesis must be regarded as very unlikely.

A secondary interaction region is centered around the Adx residues Asp39 and Asp41 contacting His28 and Lys27 of AR, respectively (Fig. 3, bottom right). Again, these contacts are polar and are mainly formed by charged side chains. Asp39 and Asp41 are located in the core domain of Adx at a surface region that has been implicated in cytochrome P450cam (CYP101) binding by the homologous putida redoxin (27). An involvement of Asp39 in redox-partner binding has been suggested earlier (28) based on a comparison of the Adx structure with crystal structures of plant-type ferredoxins (29).

Fig. 3. Electrostatic interactions between AR and Adx. Top, surface drawings of AR (right), the AR-Adx complex in the orientation displayed in Fig. 1 (center), and Adx (left). Adx and AR are rotated relative to their orientation in the complex as indicated to emphasize the interacting surfaces. Surfaces are colored corresponding to the electrostatic potential calculated by the program DELPHI (25) for an ionic strength of 0.1 M. The deepest shades of blue and red correspond to potentials of ± 10 kT. Blue surface regions carry positive charge, and red surfaces are negatively charged. In the primary and secondary interaction sites, predominantly positively charged surface areas of AR are brought into close contact with predominantly negatively charged regions of the Adx surface. Surfaces were calculated and displayed with GRASP (26). Bottom left, salt bridges (dotted lines) connecting AR and Adx in the primary interaction region. Residues are labeled black in AR and red in Adx. Bottom right, secondary interaction region with brown colored 2Fo – Fc electron density contoured at 1σ. A salt bridge linking His28 of AR and Asp41 of Adx is indicated by the dotted line, and water molecules are shown as blue spheres. Note the covalent cross-link between AR Lys27 and Adx Asp41. The inset shows the green colored 2Fo – Fc, omit map, contoured at 1σ. The cross-linked side chains are 90° clockwise rotated around the long axis.

Covalent cross-linking of Adx and AR with carbodiimide prior to crystallization results in the formation of a peptide bond between the carboxylate function of Asp39 and the primary amino group of Lys27 as clearly revealed by electron density (see Fig. 3, bottom right). This finding is unexpected,
since the cross-linking procedure employed was reported (11, 12) to yield a covalent bond linking AR Glu\textsuperscript{4} and Adx Lys\textsuperscript{66}. Peptide sequencing and mass spectrometric analysis prove that the Glu\textsuperscript{4}-Lys\textsuperscript{66} cross-link is indeed not formed in the AR-Adx complex.\textsuperscript{2}

The suggested Glu\textsuperscript{4}–Lys\textsuperscript{66} cross-link is incompatible with the binding mode to AR of Adx reported here. It does not permit contacts between the proteins in the primary interaction region as supported by mutagenesis experiments (1, 22–24) and renders unlikely a close enough approach of the redox centers for electron transfer. For these reasons we are convinced that the reported complex, and not a complex cross-linked at AR Glu\textsuperscript{4}/Adx Lys\textsuperscript{66}, represents the functional interaction between AR and Adx.

Possible Electron Transfer Path—Efficient electron transfer between the redox centers requires spatial proximity. The closest approach of atoms belonging to the [2Fe-2S] cluster of Adx and the isoalloxazine ring of the FAD of AR is 10.3 Å (9.65 Å in complex II), well within the 14-Å threshold reported to define the limit of electron tunneling in a protein medium (30). The fractional packing density of protein groups found in natural multiredox center oxidoreductases of known structure (30). Thus, from proximity and packing density considerations alone, one may conclude that the geometry of the AR-Adx complex will support electron tunneling between the redox centers. The observed geometry is calculated by ETUNNEL (30) to support electron transfer rates of 10\textsuperscript{8} to 10\textsuperscript{9} s\textsuperscript{-1}. This is orders of magnitude above the experimentally determined (4) flavin-to-iron transfer rate of 3–4 s\textsuperscript{-1}. By assuming that the covalent cross-link does not force an unnaturally tight AR-Adx interaction, it may thus be concluded that the rate of the redox reaction in which AR and Adx are involved is not limited by electron transfer within the AR-Adx complex.

The program HARLEM, analyzing distinct protein structures with respect to tunneling probabilities (31), was further used to compute possible electron transfer routes between AR and Adx (Fig. 4). According to this analysis, electrons would most likely travel along covalent bonds, requiring two through-space jumps from the FAD isoalloxazine to AR Ile\textsuperscript{376} and from AR Thr\textsuperscript{377} to Adx Cys\textsuperscript{52}, one of the [2Fe-2S] ligands. However, alternative transfer paths and a possible involvement of water molecules located at the interface region cannot be ruled out.

In summary, the 2.3-Å crystal structure of the AR-Adx redox complex suggests modes of electron transfer between a soluble [2Fe-2S] ferredoxin and its cognate reductase. It reveals the importance of electrostatic interactions in complex formation, in agreement with the concept of “electrostatic steering” (1, 7), and demonstrates that a slight domain rearrangement in AR is required for a tight AR-Adx interaction.

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