Specific Aspects of Electron Transfer from Adrenodoxin to Cytochromes P450ccc and P45011β*

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An analysis of the electron transfer kinetics from the reduced [2Fe-2S] center of bovine adrenodoxin and its mutants to the natural electron acceptors, cytochromes P450ccc and P45011β, is the primary focus of this paper. A series of mutant proteins with distinctive structural parameters such as redox potential, microenvironment of the iron-sulfur cluster, electrostatic properties, and conformational stability was used to provide more detailed insight into the contribution of the electronic and conformational states of adrenodoxin to the driving forces of the complex formation of reduced adrenodoxin with cytochromes P450ccc and P45011β and electron transfer. The apparent rate constants of P450ccc reduction were generally proportional to the adrenodoxin redox potential under conditions in which the protein-protein interactions were not affected. However, the effect of redox potential differences was shown to be masked by structural and electrostatic effects. In contrast, no correlation of the reduction rates of P45011β with the redox potential of adrenodoxin mutants was found. Compared with the interaction with P450ccc, however, the hydrophobic protein region between the iron-sulfur cluster and the acidic site on the surface of adrenodoxin seems to play an important role for precise complementarity in the tightly associated complex with P45011β.

The key step of different biological reactions such as oxidative phosphorylation, photosynthesis, respiration, drug metabolism, and many other processes taking place in living organisms is an electron transfer. Electron transfer reactions commonly occur between protein-bound prosthetic groups of the electron donors and acceptors by protein-protein interaction (1). However, only a little is known about the geometry of the protein-protein complex required for successful electron transfer and about the rate-limiting processes.

Bovine adrenodoxin is of particular interest because of its specific role as an electron carrier associated with steroid hydroxylation reactions. Adrenodoxin is a member of the ferredoxin family of electron-transferring non-heme iron proteins. It is a low molecular mass protein (14 kDa), negatively charged at neutral pH values and containing the [2Fe-2S] cluster as redox-active group. Adrenodoxin mediates the transfer of electrons from NADPH via adrenodoxin reductase to the heme iron of the active group. Adrenodoxin mediates the transfer of electrons from NADPH via adrenodoxin reductase to the heme iron of active group. Adrenodoxin mediates the transfer of electrons from NADPH via adrenodoxin reductase to the heme iron of active group.

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The abbreviations used are: CYP11A1, cytochrome P45011A1 (P450ccc); CYP11B1, cytochrome P45011B1 (P45011β); HPLC, high performance liquid chromatography.
Adrenodoxin Interactions with Cytochromes P450scc and P45011B

Reduction potentials of the free species and any differences in the association free energies for the one-electron reduced complex (16). Theoretical dependence of electron transfer rate on redox potential has been shown to be valid in many model systems (17). However, electron transfer reactions of cytochrome P450 are very complicated and could meet very different driving forces in different reaction steps (18). Another point that should be emphasized is that most experimental data on electron transfer in steroid hydroxylase system were obtained for CYP11A1. The general conclusions derived from these experiments are not necessarily true for CYP11B1 if the different physicochemical, immunochemical, and catalytic characteristics of cytochromes CYP11A1 and CYP11B1 are considered.

To provide more detailed insight into the contribution of electronic and conformational states to the driving forces for the one-electron reduced complex formation we performed studies with special attention to the exclusive role of adrenodoxin. For this purpose a series of adrenodoxin mutants possessing distinctive structural characteristics (i.e., redox potential, microenvironment of the iron-sulfur chromophore, electrostatic parameters, conformational stability) was used as electron transfer mediators.

EXPERIMENTAL PROCEDURES

Preparation and Quantitation of Proteins—The wild type adrenodoxin and the site-directed mutants were generated as originally described by Beckert et al. (8, 9). Expression in Escherichia coli and purification of recombinant proteins were carried out as reported previously (8). The absorbance ratios A276/A370 for wild type, His-56, and Asp-76 mutant adrenodoxin preparations were higher than 0.9. Absorbance ratios of A414/A370 for the Tyr-82 mutants were higher than 1.0 because of the absence of tyrosine 82. The concentrations of adrenodoxin proteins were determined using ε414 = 9.8 (mM cm)⁻¹ (19).

Adrenodoxin reductase and cytochromes CYP11A1 and CYP11B1 were purified according to the procedure of Akhrem et al. (20) with slight modifications. The concentration of adrenodoxin reductase was determined spectrophotometrically using an extinction coefficient of 11.3 (mM cm)⁻¹ at 450 nm (21). The concentrations of cytochromes P450 were estimated according to Omura and Sato (22).

Analytical Methods—EPR and CD spectra were recorded as described previously (8).

Fluorescence emission spectra of protein solutions were taken with an RF-5001 PC spectrophotometer by exciting at 270 nm. Measurements were carried out in 10 mM phosphate buffer, pH 7.4, at room temperature. Protein concentrations used were in the range 0.03–0.2 mg/ml, where all geometric effects may be eliminated (9, 23).

Redox potentials of the wild type adrenodoxin and the mutants were measured by the pulse photooxidation method with safranin T as indicator and mediator according to Sligar and Gunasal (24). Potentials were quantitated in a 3-ml reaction mixture containing 10 mM EDTA, 0.3 mM glucose, 2.5 mM safranin T, and 100 μM of an oxygen-scavenging system (4 mg/ml glucose oxidase and 2 mg/ml catalase) in 100 mM potassium phosphate buffer, pH 7.5, using the procedure described by Beckert et al. (8). All data were analyzed according to the Nernst equation.

Enzyme Activity Assays—The assays involving the measurements of the kinetics of interaction between adrenodoxin and adrenodoxin reductase using cytochrome c as an electron acceptor were carried out in the cytochrome c reduction mixture (1 ml) containing 0.2 μM adrenodoxin reductase, 100 μM horse heart cytochrome c, and adrenodoxin or adrenodoxin mutants (variable amounts) in 33 mM potassium phosphate buffer, pH 7.4. The reaction was started by the addition of 120 μM NADPH. The reduction of cytochrome c was monitored at 550 nm, and the activity was calculated on the basis of an extinction coefficient of 20 (mM cm)⁻¹ at 550 nm for cytochrome c.

11β-Hydroxylase activity measurements were performed as described previously (8). The reaction mixture (0.5 ml) consisted of CYP11B1 (0.4 μM) and adrenodoxin reductase (0.4 μM), adrenodoxin or adrenodoxin mutants (variable amounts), deoxycorticosterone (100 μM), and the NADPH-regenerating system (64 μM glucose 6-phosphoric acid disodium salt and 2 units of glucose-6-phosphate dehydrogenase) in 50 mM potassium phosphate buffer, pH 7.4, with 1 mM EDTA, 0.1 mM dithioerythritol, 0.01% (w/v) Tween 80. The conversion of deoxycorticosterone to corticosterone was carried out at 37 °C for 10 min after initiation of the reaction with NADPH. The reaction was stopped with dichloromethane, which simultaneously extracts the steroids. The extracted steroids were then dried under a nitrogen stream and analyzed by reverse phase HPLC with 70% methanol as a solvent at a flow rate of 1 ml/min. Corticosterone and deoxycorticosterone were used as external standards.

The cholesterol side chain cleavage activity of the adrenodoxin mutants was measured in the reconstituted assay system catalyzing the conversion of cholesterol to pregnenolone according to the procedure of Sugano et al. (25) with modifications described by Beckert et al. (8). The incubation mixture contained, in a final volume of 1 ml, 20 mM potassium phosphate buffer, pH 7.4, 0.3% Tween 20, 100 μM cholesterol, 1 μM CYP11A1, 0.5 μM adrenodoxin reductase, adrenodoxin (variable amounts), and the NADPH-regenerating system. The reaction was started by adding 60 μM NADPH. After a 10-min incubation at 37 °C, the reaction was stopped by heating to 98 °C. For conversion of steroids into the 3-on-4-ene form, 50 μl of cholesterol oxidase (0.4 unit) in 20 mM potassium phosphate buffer, pH 7.4, containing 1% sodium cholate and 100 μM corticosterone as an internal standard was added to the reaction mixture. The internal standard was necessary because of the additional isomerase activity of cholesterol oxidase, which makes the exact determination of the transformed cholesterol amounts impossible, but not that of pregnenolone formation. After incubation at 37 °C for 10 min, the steroids were extracted with dichloromethane. The samples were analyzed by reverse phase HPLC with a gradient solvent system of 70–100% methanol. Cholestenone, progesterone, corticosterone, and pregnenolone were used as standards.

Differential Spectral Titration of His-56 Mutants—Titration was performed according to Kido and Kimura (14). Binding of oxidized adrenodoxin to the oxidized CYP11A1 was followed spectrophotometrically by a high spin shift of the P450 heme iron, caused by adrenodoxin-induced cholesterol binding, using 2.3 μM CYP11A1 in 50 mM potassium phosphate buffer, pH 7.4, with 0.03% Tween 20 and 25 μM cholesterol at room temperature. Defined amounts of adrenodoxin were added from a stock solution. Calculation of concentrations of free adrenodoxin was done as described by Coghlan and Vickery (5).

Kinetic parameters were determined by least squares linear regression analysis of the Lineweaver-Burk plot. The standard deviations of the kinetic and binding constants were calculated from data of three or four separate experiments.

Stopped Flow Experiments—An investigation of the first electron transfer reaction from adrenodoxin to cytochromes P450 was carried out using a single channel stopped flow ASVD spectrometer SX-17MV (Applied Photophysics) according to Lambeth and Kriensiger (26) with some modifications. The reduction assays were performed in two different manners. In a first set of experiments one test tube contained 0.5 μM adrenodoxin reductase, varying amounts of adrenodoxin, and 1 μM CYP11A1 in 50 mM potassium phosphate buffer, pH 7.4, with 0.03% Tween 20 and 25 μM cholesterol, or with 0.01% Tween 80, 1 mM EDTA, and 25 μM deoxycorticosterone, respectively. The second test tube contained the reducing component, NADPH (1 mg/ml) in the respective reaction solution.

The reaction mixtures were treated with carbon monoxide, and an oxygen scavenging system was added. Electron transfer reaction was followed by monitoring the absorbance of the ferrous-carbon monoxide complex at 450 nm, an increase of which was shown to be a precise measurement of cytochrome P450 reduction by reduced adrenodoxin (27). Electron transfer rate constants were obtained by a computerized exponential fit of the data.

In the other case, the reduction assay was essentially similar to the above mentioned one, with the only exclusion that NADPH was first incubated with the adrenodoxin/adrenodoxin reductase mixture in the first test tube, and the oxidized cytochrome P450 (CYP11A1 or CYP11B1) complexed with the steroid was in the second test tube. The extent to which the reduction of adrenodoxin by adrenodoxin reductase proceeds after incubation with NADPH was checked spectrophotometrically using the method described by Kimura (19).

RESULTS AND DISCUSSION

The main concern of the present study was to relate the essential structure-function properties of adrenodoxin, which were modified by site-directed mutagenesis, to the specific aspects of electron transfer in steroid hydroxylase systems. The surface near to the [2Fe-2S] cluster of adrenodoxin is of considerable interest since here the flavoprotein must supply its electrons, and yet it is at this part of the adrenodoxin surface...
that the P450 cytochrome must interact. Tyr-82 mutants (i.e. Y82F, Y82S, Y82L) were chosen taking into consideration two points. On the one hand, mutations of tyrosine 82 were shown to affect differentially interactions of adrenodoxin with cytochromes CYP11A1 and CYP11B1 (8); and on the other hand, Tyr-82 seems to be located close to the conserved His-56 residue, which is immediately adjacent to one of the cysteine ligands of the [2Fe-2S] cluster (9). In addition, adrenodoxin proteins with amino acid substitutions in position 56 (i.e. H56T, H56Q, and H56R) were used for our studies, since His-56 has been shown to contribute to the integrity of the protein region between the iron-sulfur cluster and the highly conserved acidic region of adrenodoxin, which is important for binding to both adrenodoxin reductase and P450 (9). Moreover, His-56 seems to play a key role in the stabilization of adrenodoxin structure as became evident from recent calorimetric studies on folding and stability of adrenodoxin (28).

Taking into account the role of complementary electrostatic interactions for the association of electron transfer proteins, mutant D76E of adrenodoxin with a substitution in the highly conserved acidic region was further selected. This conservative change maintained the negative charge in position 76 but altered the exact positioning of the carboxylate moiety potentially inducing disturbances of specific short range protein-protein interactions as was shown for a heterologous system consisting of human placenta ferredoxin, bovine adrenodoxin reductase, and CYP11A1 (6).

The wild type and the mutants were expressed in similar yields as holoprotein and exhibited absorption CD and EPR spectra characteristic for the correctly assembled iron-sulfur cluster, indicating that incorporation of the cluster was not disturbed upon amino acid replacements (data not shown).

The redox potentials of the mutant proteins provide more detailed insight into the local environment of the iron-sulfur center in these proteins (29). The effect of amino acid replacement near the cluster is sharply defined in the case of His-56 substitution. Replacement of His-56 by Gln, Arg, or Thr led to marked changes in the redox potentials of the mutants being 28, 65, and 66 mV, respectively, lower compared with that of the wild type (Table I). These changes demonstrate that histidine in position 56 affects the ligand field around the iron-sulfur cluster. Some lowering (8 mV) in the redox potential was estimated for mutant Y82S (~282 mV), indicating that introduction of the small polar serine residue instead of the hydrophobic tyrosine residue could produce conformational changes of the cluster environment since the microenvironment of the iron-sulfur chromophore of native adrenodoxin is known to be largely hydrophobic (30). Redox potentials obtained for other Tyr-82 mutant proteins, in which tyrosine was changed to the hydrophobic phenylalanine or leucine (i.e. Y82F and Y82L), were found to be not significantly different from those of the wild type (~274 mV). The redox potential of the D76E mutant remains unchanged, confirming the previous suggestion that residues in this position are most likely not located in the immediate vicinity of the cluster (6).

Theoretical analysis of electron transport reactions, based on the outer sphere mechanism, predicts that a relationship should exist between the rate constant of the electron transfer and the redox potential difference between donor and acceptor (17). Indeed, a clear correlation was shown to exist between the rate constant for the reduction and the redox potential of the reactants in a structurally homologous series of electron transfer proteins (31). The regulation of the CYP11A1 redox potential by substrates and the influence of these changes on the rate of the electron transfer from reduced adrenodoxin to cytochrome have been investigated by Lambeth and Kriensgiri (26).

Here we have investigated in more detail the relationship among structural properties of adrenodoxin mutants and the
The kinetics of interaction among adrenodoxin and adrenodoxin reductase were assayed using cytochrome c as an electron acceptor. The reduction of cytochrome c was monitored at 550 nm. Catalytic and binding properties of the adrenodoxin mutants in CYP11A1- and CYP11B1-dependent substrate conversion were studied by analyzing the products of the respective hydroxylation reaction, pregnenolone and corticosterone.

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| Adrenodoxin | Cytochrome c reduction assay | Cholesterol side chain cleavage | 11β-Hydroxylation activity |
|-------------|-----------------------------|--------------------------------|---------------------------|
|             | relative $K_m$ | relative $K_m$ | relative $K_m$ |
| WT          | 1.0           | 1.0            | 1.0          |
| Y82F        | 0.7           | 0.7            | 0.2          |
| Y82S        | 0.9           | 1.4            | 2.7          |
| Y82L        | 1.0           | 1.2            | 0.4          |
| H56Q        | 2.1           | 1.2            | 6.1          |
| H56T        | 2.8           | 1.2            | 4.1          |
| H56R        | 5.0           | 3.6            | 6.3          |
| D76E        | 11.2          | 2.6            | 3.8          |

*WT, wild type.

| Adrenodoxin | CYP11A1 spectral binding |
|-------------|---------------------------|
|             | relative $K_d$ |
| WT          | 1.0 |
| Y82F        | 0.8 |
| Y82S        | 2.7 |
| Y82L        | 1.6 |
| H56Q        | 1.0 |
| H56T        | 2.6 |
| H56R        | 4.8 |
| D76E        | 10.2 |

*WT, wild type.

The second set of experiments was performed to avoid a possible contribution of adrenodoxin reduction or oxidation by adrenodoxin reductase to the measured rate of electron transfer between adrenodoxin and CYP11A1. For this purpose, adrenodoxin was preincubated with adrenodoxin reductase and NADPH in the first test tube. The other test tube contained oxidized cytochrome CYP11A1. The molar ratios of proteins were identical to those for the preceding stopped flow assays. Analyses of the adrenodoxin absorption spectra after incubation with adrenodoxin reductase and NADPH in the oxygen-free solution indicated that at the start point of the stopped flow measurement, when the mixture containing adrenodoxin reductase, NADPH, and adrenodoxin or its mutants was mixed with oxidized cytochrome, the wild type adrenodoxin as well as the mutant proteins were fully reduced by adrenodoxin reductase (data not shown), thus being appropriately dissociated from the flavoprotein (32). In this case the rate constants of electron transfer from the reduced adrenodoxin to the oxidized cytochrome must be totally adrenodoxin-dependent since the flavin- to iron-sulfur electron transfer is not rate-limiting under these conditions. As evident from Fig. 1B, the wild type adrenodoxin and the mutants, which have unchanged redox potentials (Table I) and no significantly changed affinity to adrenodoxin reductase and to CYP11A1 (Tables II and III) (i.e. Y82F, Y82L), revealed about the same values of the reduction rates as in the preceding stopped flow assay. This observation
CYP11A1, whereas H56T has a slight increase in binding energy. Binding studies using optical difference spectroscopy revealed that oxidized H56Q exhibits the same affinity for oxidized adrenodoxin as the wild type. However, the rate constants for the CYP11A1 reduction as a function of the redox potential differences on electron transfer rates. Approximately linear correlation ($r = 0.86$) between redox potentials and rate constants for reduction could be shown in this protein group. In contrast, for the mutant proteins Y82S, H56R, and D76E structural and electrostatic effects become more important, masking the effect of redox potential differences on electron transfer rates.

An investigation of the first electron transfer from adrenodoxin to cytochrome CYP11B1 catalyzing 11$\beta$ hydroxylation of deoxycorticosterone was carried out taking into consideration stopped flow data obtained for CYP11A1. For these measurements adrenodoxin was incubated with NADPH and adrenodoxin reductase prior to mixing with the oxidized CYP11B1 substrate complex. As for CYP11A1 reduction we obtained a clear dependence of the first-order rate constants of electron transfer on adrenodoxin concentration (Fig. 3). However, the $k_{app}$ values were lowered nearly 2-fold using CYP11B1 at the same stoichiometry of the reaction components, coinciding with earlier suggestions about a less effective transfer of reducing equivalents to CYP11B1 compared with CYP11A1 (33). Additionally, as shown in Fig. 4, no correlation of the reduction rates of CYP11B1 on the redox potential of the adrenodoxin mutants was found. Moreover, the reduction rates of the D76E mutant are approximately the same compared with those of the wild type, whereas they are 5-fold lower in the reduction assay with CYP11A1. In addition, CYP11B1, al-
though not sensitive to the replacement D76E, seems to be more sensitive than CYP11A1 to the properties of the amino acids substituting positions 56 and 82. Analyzing the data shown in Fig. 3 revealed that interaction between CYP11B1 and adrenodoxin is substantially of hydrophobic nature. The hydrophobic protein region between the iron-sulfur cluster and the acidic site on the surface of the adrenodoxin seems to play an important role for the precise complementarity of the tightly associated complex with CYP11B1.

Successively, we have examined comparatively how the kinetics of the first electron transfer is related to catalytic and binding properties of the adrenodoxin mutants in cytochrome c reduction and CYP11A1- or CYP11B1-dependent substrate conversion. These studies have demonstrated that amino acid substitutions that alter the electrostatic properties of adrenodoxin (i.e. Y82S, H56R, D76E) decreased the activity and affinity of these mutants for both adrenodoxin reductase and CYP11A1 as evidenced by raised $K_m$ and $K_V$ values (Tables II and III). As expected, the effect of imbalance of the electrostatic interactions is most pronounced for mutant D76E, exhibiting a more than 10-fold decrease in the affinity to adrenodoxin reductase as measured by a respective increase in the $K_m$ value of the cytochrome c reduction (Table II). Moreover, although adrenodoxin mutant D76E exhibits a 10-fold increase in the $K_m$ value for CYP11A1 as evident from the spectral binding studies (Table III), the $K_m$ value for this mutant in the CYP11A1 substrate conversion assay reveals under our experimental conditions only a 2.6-fold increase compared with the wild type (Table II). Of particular interest is the similarity of the electrostatic effects caused by H56R and D76E replacements concerning adrenodoxin interaction with adrenodoxin reductase and CYP11A1. Replacement of His-56 with arginine residue results in a 5-fold decreases in binding affinities for both adrenodoxin reductase and CYP11A1 (Tables II and III).

Analysis of the enzymatic activity of adrenodoxin mutants with respect to CYP11B1-dependent substrate conversion suggests differences in the binding requirements of this protein compared with CYP11A1. Close examination of the $K_m$ values indicates that the adrenodoxin mutants with charged or polar residues in positions 56 and 82 (i.e. H56Q, H56R, and Y82S) show decreased affinities to the CYP11B1. Interestingly, increases of the $K_m$ values for mutants H56R, H56Q, and H56T are greater than that of mutant D76E (Table II). On the other hand, replacing Tyr-82 with the more hydrophobic residues leucine or phenylalanine increased the ability of these mutants to interact with CYP11B1 as evidenced in 2.5-5-fold lower $K_m$ values.

However, the interpretation of the $K_m$ values obtained for CYP11A1 and CYP11B1 becomes more complicated since the turnover of these systems, being totally adrenodoxin-dependent, could become dependent on the interaction between adrenodoxin and adrenodoxin reductase, at least for the mutants possessing affected affinity to adrenodoxin reductase. As was clearly demonstrated in our stopped flow investigations, such flavin- to iron-sulfur electron transfer limitation would mask the measured kinetic constants for cytochrome reduction.

The $V_{max}$ values remain unchanged for all mutants in cytochrome c reduction and cholesterol or deoxycorticosterone conversion assays (data not shown), demonstrating that the efficiency of substrate conversion at infinite adrenodoxin concentrations is not influenced by amino acid substitution in positions 56, 82, or 76. Since the second electron transfer appears to be rate-determining for P450-dependent mixed function oxidations (13), it seems likely that the kinetic constants are primarily determined by second electron transfer.

In conclusion, it has been shown that a linear correlation between the redox potential of various adrenodoxin mutants and the apparent rate constant of CYP11A1 reduction can be observed. The correlation does not hold true for mutants changing the electrostatics and/or structure of the interaction site (i.e. H56R, D76E). Such a correlation could also be obtained for CYP11B1 reduction. Our data led us to conclude that for adrenodoxin/CYP11B1 interaction hydrophobic properties of the interaction site are more important.

Interestingly, in some cases the rate of the first electron transfer does not reflect the rate of the efficiency of substrate conversion. This indicates that either the first and second electron transfers are differentially regulated (implying different requirements for the adrenodoxin/P450 electron transfer complex) or that another step (e.g. product release) is rate-limiting in these systems.

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