The Interaction of Bovine Adrenodoxin with CYP11A1 (Cytochrome P450\textsubscript{sec}) and CYP11B1 (Cytochrome P450\textsubscript{11\mu})

ACCELERATION OF REDUCTION AND SUBSTRATE CONVERSION BY SITE-DIRECTED MUTAGENESIS OF ADRENODOXIN*

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The kinetics of protein-protein interaction and heme reduction between adrenodoxin wild type as well as eight mutants and the cytochromes P450 CYP11A1 and CYP11B1 was studied in detail. Rate constants for the formation of the reduced CYP11A1-CO and CYP11B1-CO complexes by wild type adrenodoxin, the adrenodoxin mutants Adx-(4–108), Adx-(4–114), T54A, T54S, and S112W, and the double mutants Y82F/S112W, Y82L/S112W, and Y82S/S112W (the last four mutants are Δ113–128) are presented. The rate constants observed differ by a factor of up to 10 among the respective adrenodoxin mutants for CYP11A1 but not for CYP11B1. According to their apparent rate constants for CYP11A1, the adrenodoxin mutants can be grouped into a slow (wild type, T54A, and T54S) and a fast group (all the other mutants). The adrenodoxin mutants forming the most stable complexes with CYP11A1 show the fastest rates of reduction and the highest rate constants for cholesterol to pregnenolone conversion. This strong correlation suggests that C-terminal truncation of adrenodoxin in combination with the introduction of a C-terminal tryptophan residue enables a modified protein-protein interaction rendering the system almost as effective as the bacterial putidaredoxin/CYP101 system. Such a variation of the adrenodoxin structure resulted in a mutant protein (S112W) showing a 100-fold increased efficiency in conversion of cholesterol to pregnenolone.

Cytochromes P450 (CYP11A1, CYP11B1, and CYP11B2)\textsuperscript{1} of the inner mitochondrial membrane catalyze various hydroxylation steps in the biosynthesis of steroid hormones (mineralocorticoids, glucocorticoids, and androgens) in vertebrates (1, 2). The electrons required for these hydroxylation reactions are provided by NADPH and are transferred to the cytochromes P450, which mediate the final oxygen activation, via a small electron transport chain. Comparable with the components of the soluble bacterial cytochrome P450 systems, such as P450\textsubscript{cam} (CYP101) and P450\textsubscript{terp} (CYP108), the proteins of this electron transport system are an FAD-containing NAD(P)H-de-pendent reductase and an iron-sulfur protein of the [2Fe-2S] ferredoxin type.

Adrenodoxin, the ferredoxin of the adrenal gland (3), has been studied intensively by chemical modifications (4–6) and later by a great number of site-directed mutagenesis studies (7–13). Recently crystal structures of a truncated form, Adx-(4–108) (14), and of full-length Adx (15) have been solved. In contrast to the bacterial systems, the reductase and the cytochromes of the mitochondrial systems are membrane-associated or membrane-bound proteins, respectively (1, 16).

The reduction kinetics of the soluble bacterial cytochrome P450\textsubscript{cam} is well understood (17–20), whereas little is known about the kinetics of the membrane-bound mitochondrial cytochromes P450 (21–23). Nevertheless previous investigations point to considerable differences in the reaction mechanisms and in the regulation in these two types of cytochrome P450 systems.

To get deeper insight into the mechanism causing these differences, we intended to create adrenodoxin mutants with an increased efficiency in terms of substrate conversion and reduction rate of CYP11A1 and CYP11B1. According to sequence alignments (24, 25), serine in position 112 of bovine adrenodoxin corresponds to the last amino acid in putidaredoxin, tryptophan in position 106. Tryptophan in position 106 of putidaredoxin has been shown previously to be of crucial importance for binding and electron transfer to CYP101 (20, 26). Consequently, a set of four truncated adrenodoxin mutants with a C-terminal tryptophan residue in position 112 was prepared to find out how this residue affects the reactivity of adrenodoxin.

For comparison we used two truncated adrenodoxin mutants (Adx-(4–114) and Adx-(4–108)), which like the wild type do not contain a tryptophan, to elucidate the effect of the deleted C terminus. Both truncated mutants are known to display an increased substrate conversion activity (27), but a detailed characterization of their reduction behavior has not been performed as yet.

Furthermore, adrenodoxin mutants T54A and T54S (11) were included in this study with the intention to investigate whether a substitution in the [2Fe-2S] cluster surrounding (which is completely unrelated to the above-mentioned adrenodoxin deletions and mutations) could be able to affect affinity and/or reactivity in the adrenodoxin/mitochondrial P450 (i.e. CYP11A1 or CYP11B1) system in a similar manner.

MATERIALS AND METHODS

Biochemicals and Reagents—Pfu DNA Polymerase was from Stratagene. NADPH was purchased from SERVA, and cholesterol was from Sigma. All other chemicals were of the highest purity commercially available.

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\#The abbreviations used are: CYP11A1, cytochrome P450 11A1; CYP11B1, cytochrome P450 11B1; CYP101, cytochrome P450\textsubscript{cam}; Adx, adrenodoxin; HPLC, high pressure liquid chromatography.
Increased Efficiency of Adrenodoxin Mutants

Bacterial Strains and Plasmids—Escherichia coli strains HB101 and BL21 were used as host strains for the heterologous expression of adrenodoxin. The cDNA-containing plasmid was pKKHC (28).

Protein Expression and Purification—A set of four adrenodoxin mutants was designed using wild type adrenodoxin and three Tyr-S2 mutations and Y82P (10). A tryptophan residue was introduced in position 112 by polymerase chain reaction-based site-directed mutagenesis, and simultaneously the 16 C-terminal amino acids of the wild type sequence were deleted.

The 3′ polymerase chain reaction primers consisted of the following sequences: 5′-GGGGAACTTACCAACCGGCTACGGTACTCG-3′ (reverse primer) and 5′-GTCAGCTCTGCATAGTGTTGG-3′ (forward primer). The cDNA clone and the adrenodoxin cDNA are pAM-based; in addition, the tryptophan codon is italicized, and the HindIII site is underlined. Oligonucleotides for polymerase chain reaction were purchased from Biotez (Berlin, Germany). Ligation, transformation, plasmid preparation, and dideoxy sequencing were carried out according to standard protocols (29).

Bacteria were grown as previously reported (30) with slight modifications. The expression of adrenodoxin was purified after enzymatic cell lysis as described previously, and the final concentration of adrenodoxin was determined using ε455 = 9.8 mM−1 cm−1 (31). The purity of the adrenodoxin preparation was estimated by determining the Q-value (A414/A373).

Adrenodoxin reductase was heterologously expressed and purified as described elsewhere (32). The molar extinction coefficient used for concentration estimation was ε373 = 10.9 mM−1 cm−1 (33). The adrenodoxin concentration estimation was carried out from bovine adrenals, and their concentrations were estimated by carbon monoxide difference spectra assuming ε340–440 = 91 mM−1 cm−1 (34). The high spin content of CYP11A1 was determined using ε935 = 92 mM−1 cm−1 (35).

SDS-polyacrylamide gel electrophoresis was done using standard procedures (36). Western blot analysis of adrenodoxin was performed according to Sambook et al. (29) using rabbit polyclonal antibodies against adrenodoxin (1:10,000) (27). The interaction of adrenodoxin with CYP11A1 promotes the binding of cholesterol, which causes a shift of the low spin (417 nm) to the high spin (393 nm) form of the cytochrome heme iron. The absorbance changes were plotted versus the adrenodoxin concentration and fitted by numerical integration using the program CHEMSIM (37).

Results

Production of Adrenodoxin S112W and Double Mutants with Additional Changes in Position 82—The mutants were produced by site-directed mutagenesis using the primers described under “Materials and Methods.” Dideoxy sequencing revealed that the adrenodoxin cDNA contained only the desired mutations except for mutant S112W where an additional silent mutation had occurred in codon 87 (AGA → AGG), which, however, does not change the coded amino acid.

Expression and Purification—The mutant proteins were expressed into the cytoplasm of E. coli BL21. The specific content of cells per liter of culture was 4.3–5.3 g. Production of the heterologous proteins was verified by SDS-polyacrylamide gel electrophoresis and Western blot analysis (data not shown).

The efficiency of each purification step is reflected by the absorbance at 276 nm. The final yield of each adrenodoxin mutant after purification is given in Table I. All four tryptophan mutants gave lower yields in purification than the wild type protein due to a slightly different behavior of the mutants during the hydrophobic interaction chromatography step, i.e. the salt gradient used for wild type purification (starting from 2.2 M sodium cholate, and 0.3% Tween 20).

### Table I

| Adrenodoxin species | Q-values determined after different steps of chromatography | Final yield |
|---------------------|------------------------------------------------------------|-------------|
|                      | HIC | EIX | GF | mmol/liter culture |
| Wild type            | 0.51 | 0.90 | 0.93 | 850 |
| S112W               | 0.45 | 0.60 | 0.63 | 530 |
| Y82L/S112W         | 0.49 | 0.64 | 0.68 | 725 |
| Y29S/S112W         | 0.51 | 0.66 | 0.68 | 700 |
| Y29S/S112W         | 0.26 | 0.55 | 0.68 | 525 |

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ammonium sulfate down to 1.0 M) was not sufficient to completely elute the respective mutant protein from the column.

Spectral Characterization—UV/visible spectra of the investigated adrenodoxin mutants clearly display differences in the UV region at 280 nm as compared with the wild type protein due to the newly introduced tryptophan residue (Fig. 1). Also the contribution of the amino acid in position 82 (Tyr, Phe, and nonaromatic, respectively) to the absorbance around 280 nm is detectable, whereas a difference between Y82S/S112W and Y82L/S112W could not be observed.

Redox Potentials—The redox potentials of wild type and mutant adrenodoxins are displayed in Table II. Interestingly the redox potential of all four tryptophan mutants is more than 60 mV lower as compared with the wild type protein. It is readily noted that these values are within the same range as those of the adrenodoxin mutants that do not contain a tryptophan but either are truncated or contain an amino acid substitution in the immediate cluster environment.

$K_S$ Values—The binding affinity of adrenodoxin to its natural redox partner CYP11A1 has been determined for the adrenodoxin mutants (Table III). Differences in $K_S$ of the wild type as compared with other publications (27) could be attributed to the different buffer conditions used for the titrations, i.e. the buffer we used contained 0.1 M potassium chloride, whereas in former studies a potassium phosphate buffer without additional salt was used to determine the $K_S$ values. The ionic strength is known to largely affect the interaction between adrenodoxin and CYP11A1 (22). Moreover, in contrast to most former studies where different buffers were used for the determination of binding constants, steady-state kinetic constants, and pre-steady-state kinetic constants, respectively, we used the same buffer system for the binding studies, the CYP11A1-dependent conversion of cholesterol, and the stopped flow assays to assure similar assay conditions for all experiments and thus to allow the determination of affinities relevant in the reduction reaction. An example for these titrations is given in Fig. 2.

C-terminal truncation of adrenodoxin leads to an increased affinity that is even more pronounced together with a C-terminal tryptophan residue and is dramatically enhanced for the combination of truncation, aromatic amino acid in position 82

![Figure 1: Absorbance spectra of adrenodoxin wild type and the different mutants.](image1)

![Figure 2: Adx-induced low spin (418 nm) to high spin (393 nm) conversion of CYP11A1 followed by differential spectroscopy.](image2)

### Table II

| Adrenodoxin species | Redox potential |
|---------------------|----------------|
| Wild type           | −270           |
| S112W               | −334           |
| Y82F/S112W          | −322           |
| Y82L/S112W          | −333           |
| Y82S/S112W          | −332           |
| Adx(4–114)          | −342 (7)       |
| Adx(4–108)          | −344 (12)      |
| T54A                | −329 (11)      |
| T54S                | −340 (11)      |

### Table III

| Adrenodoxin species | $K_S$ (μM) |
|---------------------|------------|
| Wild type           | 5.2 ± 0.2  |
| S112W               | 0.61 ± 0.01|
| Y82F/S112W          | 0.63 ± 0.04|
| Y82L/S112W          | 2.1 ± 0.3  |
| Y82S/S112W          | 2.3 ± 0.1  |
| Adx(4–114)          | 4.3 ± 0.4  |
| Adx(4–108)          | 2.6 ± 0.1  |
| T54A                | 7.5 ± 1.3  |
| T54S                | 6.6 ± 0.7  |
tion, which allowed the extrapolation to apparent maximal velocity constants for the respective adrenodoxin species. Thus, the different adrenodoxin mutants can be compared with respect to their efficiency to reduce the cytochrome.

Table IV displays the maximal velocity constants ($k_{\text{app, max}}$) derived from the fits shown in Fig. 3. For six mutants (Y82S/S112W, Y82L/S112W, Adx-(4–108), Adx-(4–114), T54A, and T54S) and the wild type, a monoexponential function could be fitted to the data, whereas the time courses for the other mutants (Y82F/S112W and S112W) were best described by biexponential functions. The first, rapid phase of the biexponential covered about 33% of the total reaction amplitude and was used to calculate $k_{\text{app, max}}$ (see below). Examples for the different time courses are given in Fig. 4. The second, slow phase (see Table IV) appeared similar for S112W and Y82F/S112W.

Kinetics of CYP11B1 Reduction—Rate constants determined from the stopped flow measurements are shown in Table IV. All time courses were best described by biexponential functions. Interestingly in this study no significant difference between the adrenodoxin species used is observed.

Substrate Conversion—Conversion of cholesterol to pregnenolone was performed according to Hannemann et al. (40). $k_{\text{cat}}$ and $K_m$ values for the different adrenodoxin species are listed in Table V. Data clearly show an increased $k_{\text{cat}}$ and, correspondingly, a lowered $K_m$ for the truncated mutants, particularly for the two mutants S112W and Y82F/S112W. The $k_{\text{cat}}$ value is increased 6.6-fold (S112W) and 9.4-fold (Y82F/S112W), whereas the $K_m$ is decreased 9-fold (S112W) and 9.7-fold (Y82F/S112W). All truncated mutants display similar $K_m$ values, being lower by a factor of about 10 as compared with the wild type. In contrast, the $K_m$ values for the “full-length mutants” are not changed in this dramatic way, being 2-fold higher (T54A) and 1.6-fold lower (T54S) than the wild type. Besides mutants S112W and Y82F/S112W (see above), a considerable effect on $k_{\text{cat}}$ is observable only for the truncated mutant Adx-(4–108) where the $k_{\text{cat}}$ is 2.8-fold increased.

The data obtained from CYP11B1-dependent conversion of 11-deoxycorticosterone to corticosterone are summarized in Table VI. The large differences between the adrenodoxin species observed for CYP11A1-dependent conversion of cholesterol to pregnenolone are not detected in the CYP11B1 reaction. The differences in $k_{\text{cat}}$ are marginal (largest difference factor is 1.8 for T54S and wild type) when comparing wild type adrenodoxin and the mutant species. The $K_m$ values differ by a factor of 2.4 for adrenodoxin Y82F/S112W as compared with the wild type, which is the most pronounced difference observed.

DISCUSSION

An interesting and so far not understood feature in cytochrome P450-dependent reactions is the observation that most of the bacterial systems display 100–1000-fold higher turnover rates as compared with microsomal and mitochondrial ones. Attempts to improve the efficiency of mitochondrial P450 systems should thus lead to deeper insight into the mechanism of protein-protein interactions and reduction kinetics in this important class of enzymes.

We used wild type adrenodoxin and a set of eight mutant proteins in stopped flow experiments as well as in differential spectroscopy to investigate the kinetics and thermodynamics of the protein-protein interaction and the reduction rate of CYP11A1 and CYP11B1 by adrenodoxin. The electron transfer was indirectly monitored at 450 nm where CO binding to P450, which occurs upon reduction of the protein-bound heme, can be detected.

To correlate the initial reduction process determined in stopped flow experiments with the subsequent redox cycles (one more cycle in the case of the CYP11B1-dependent conver-
TABLE IV

Determined maximal rate constants for the reduction of CYP11A1 and CYP11B1 with Adx and its mutants

| Adrenodoxin species | CYP11A1 | Exponential equation used for fit | $k_{app, max}$ | \( \text{Exponential equation used for fit} \) | $k_{app, max}$ |
|---------------------|---------|----------------------------------|----------------|----------------------------------|----------------|
|                     |         | \( s^{-1} \)                      |                | \( s^{-1} \)                      |                |
| Wild type           |         | Mono                             | 2.0 ± 0.5      | Mono                             | 0.37 ± 0.06    |
| Adx-(4-108)         |         | Mono                             | 4.2 ± 0.3      | Mono                             | 0.48 ± 0.09    |
| Adx-(4-114)         |         | Mono                             | 2.9 ± 0.2      | Mono                             | 0.53 ± 0.02    |
| Y82L/S112W          |         | Mono                             | 3.2 ± 0.3      | Mono                             | 0.34 ± 0.03    |
| Y82S/S112W          |         | Mono                             | 3.6 ± 0.4      | Mono                             | 0.33 ± 0.04    |
| Y82F/S112W          |         | Mono                             | 19.2 ± 1.0     | Mono                             | 0.51 ± 0.01    |
| S112W               |         | Bi                               | 0.40 ± 0.07    | Bi                               | 0.50 ± 0.02    |
| T54A                |         | Multi-exponential                 | 23.3 ± 3.4     | Bi                               | 0.38 ± 0.07    |
| T54S                |         | Mono                             | 0.9 ± 0.1      | Bi                               | 0.45 ± 0.04    |

TABLE V

CYP11A1-dependent conversion of cholesterol to pregnenolone: \( k_{cat} \) values and \( K_m \) values for the different adrenodoxin species

| Adrenodoxin species | CYP11B1 | Exponential equation used for fit | $k_{app, max}$ | \( \text{Exponential equation used for fit} \) | $k_{app, max}$ |
|---------------------|---------|----------------------------------|----------------|----------------------------------|----------------|
|                     |         | \( s^{-1} \)                      |                | \( s^{-1} \)                      |                |
| Wild type           |         | Mono                             | 11 ± 0.2       | Mono                             | 0.37 ± 0.06    |
| S112W               |         | Bi                               | 74 ± 4         | Bi                               | 0.48 ± 0.09    |
| Y82F/S112W          |         | Mono                             | 105 ± 2.7      | Mono                             | 0.53 ± 0.02    |
| Y82L/S112W          |         | Mono                             | 16 ± 0.6       | Mono                             | 0.34 ± 0.03    |
| Y82S/S112W          |         | Mono                             | 9 ± 0.9        | Mono                             | 0.33 ± 0.04    |
| Adx-(4-114)         |         | Mono                             | 14 ± 0.6       | Mono                             | 0.51 ± 0.01    |
| Adx-(4-108)         |         | Bi                               | 31 ± 1.4       | Bi                               | 0.50 ± 0.02    |
| T54A                |         | Multi-exponential                 | 10 ± 1.8       | Bi                               | 0.38 ± 0.07    |
| T54S                |         | Mono                             | 14 ± 0.9       | Bi                               | 0.45 ± 0.04    |

Fig. 4. Comparison between a biexponential and a monoexponential time course. In both cases the concentration of the adrenodoxin was 16 \( \mu \)M. A shows 110 s of the monoexponential reaction of the wild type adrenodoxin. The inset displays the first 10 s of the reaction. B shows 20 s of the monitored biexponential reaction of the mutant S112W. The inset displays the 1st s of the reaction.

TABLE VI

CYP11B1-dependent conversion of 11-deoxycorticosterone to corticosterone: \( k_{cat} \) values and \( K_m \) values for the different adrenodoxin species

| Adrenodoxin species | CYP11B1 | Exponential equation used for fit | $k_{app, max}$ | \( \text{Exponential equation used for fit} \) | $k_{app, max}$ |
|---------------------|---------|----------------------------------|----------------|----------------------------------|----------------|
|                     |         | \( s^{-1} \times 10^{-3} \) | \( \mu \)M     | \( s^{-1} \times 10^{-3} \) | \( \mu \)M     |
| Wild type           |         | 70 ± 2.3                         | 2.39 ± 0.12    | 29                               |
| S112W               |         | 97 ± 5.8                         | 1.05 ± 0.05    | 92                               |
| Y82F/S112W          |         | 107 ± 9.1                        | 0.97 ± 0.03    | 110                              |
| Y82L/S112W          |         | 81 ± 10.5                        | 0.99 ± 0.05    | 81                               |
| Y82S/S112W          |         | 81 ± 8.9                         | 1.85 ± 0.16    | 44                               |
| Adx-(4-114)         |         | 108 ± 5.4                        | 2.01 ± 0.23    | 54                               |
| Adx-(4-108)         |         | 114 ± 12.8                       | 2.39 ± 0.14    | 48                               |
| T54A                |         | 97 ± 15.9                        | 2.79 ± 0.54    | 35                               |
| T54S                |         | 131 ± 7.5                        | 1.43 ± 0.09    | 91                               |

The components of the bacterial putidaredoxin/CYP101 system are homologous to the mitochondrial adrenodoxin/CYP11A1 and adrenodoxin/CYP11B1 system and have been extensively studied due to the ready availability of the different proteins (19, 26, 42–44). Therefore, it became the best understood and commonly used model system for electron-transfer investigations in ferredoxin-mediated P450 reactions, although experiments revealed considerable differences between the respective systems. (i) They differ by about 1 order of magnitude in the velocity of formation of the reduced CO complex displayed rate constants of 2.5–3 s\(^{-1}\) for CYP11A1 reduction (22) and 35 s\(^{-1}\) for the CYP11B1 system (45). (ii) The aromatic character
of the C-terminal tryptophan in position 106 of putidaredoxin (26, 44) is indispensable for the strong binding of putidaredoxin and CYP11B1, whereas wild type adrenodoxin has no tryptophan at all and also comprises a more extended C-terminal end (adrenodoxin consists of 128 amino acids). (iii) Putidaredoxin and adrenodoxin are unable to substitute for one another in the respective reductase reaction (5) or to serve as an effector for substrate turnover in the corresponding system of the other one (18), whereas the respective reduced P450-CO complexes could be generated (46).

To design more efficient adrenodoxin mutants, i.e. proteins with putidaredoxin-like properties, we constructed a set of four truncated adrenodoxin mutants with a C-terminal deletion of 16 amino acids and introduced a tryptophan in position 112, which according to sequence alignments corresponds to tryptophan 106 in putidaredoxin. For comparison, two truncated adrenodoxin mutants, Adx-(4–114) and Adx-(4–108), which do not contain a tryptophan, and the two full-length adrenodoxin mutants, T54A and T54S, showing a substitution in the surrounding of the [Fe–S] cluster, were used.

The redox potentials of all the truncated forms of adrenodoxin as well as of the full-length mutants containing a replacement in position Thr-54 are similar (Table II) and differ from that of the wild type by about −60 mV. Therefore, a contribution of the amino acid in position 82 to the redox potential can be ruled out in concordance with former studies where full-length adrenodoxin Tyr-82 mutants showed wild type-like midpoint potentials (10). Consequently the drop in midpoint potential is due to the deletion of the adrenodoxin C terminus in the truncated mutants and/or the presence of a tryptophan residue. The latter explanation, however, seems unlikely since the redox potential of putidaredoxin is significantly higher (47) as compared with wild type adrenodoxin. Additionally, site-directed mutagenesis studies on putidaredoxin proved that the amino acid at position 106 (Trp in the wild type protein was mutated to Phe, Leu, Lys, Val, Tyr, respectively) or even its absence (mutant Δ106) does not significantly modulate the redox potential (20). Moreover, the truncated adrenodoxin mutants Adx-(4–114) and Adx-(4–108) also exhibit lowered redox potentials.

In the Thr-54 mutants, on the other hand, a change in the hydrophobicity of the cluster surrounding may account for the lowered redox potential; in fact, this explanation might hold true for the truncated mutants as well considering a structural rearrangement within the molecule due to deletion of the C terminus that is transmitted to the environment of the cluster. Indeed, Burova et al. (48) described a more compact overall structure of adrenodoxin mutant Adx-(4–108) as determined by calorimetric experiments and limited proteolysis indicating that differences between the wild type Adx and the truncated Adx exist in solution (48). The lowered redox potentials of the truncated mutants should improve their ability to reduce CYP11A1, which, in fact, has been observed (Tables II and IV). In this context, it is interesting to note that the two Thr-54 mutants, despite their lowered redox potentials, behave wild type-like in the kinetic assays (discussed below).

The ability of the adrenodoxin mutants to reduce CYP11A1 and CYP11B1 and to promote the cytochrome P450-dependent substrate conversion as well as the affinity of the adrenodoxin mutants to CYP11A1 was studied by performing different spectroscopic measurements using steady-state and stopped flow kinetics.

The data we provide clearly show dramatic differences in the interaction of the designed adrenodoxin species with CYP11A1 (Table III), whereas it was not possible to determine the respective binding constants for CYP11B1 due to the inherent instability of this cytochrome in the low spin state. Wild type adrenodoxin as well as the adrenodoxin mutants T54A and T54S (which are identical in length with the wild type protein) bind weaker by a factor of 8.5–12.3 to CYP11A1 than the C-terminally truncated mutants S112W and Y82FS112W. The other mutants (Adx-(4–114), Adx-(4–108), Y82S/S112W, and Y82L/S112W), which are also lacking 14–20 amino acids at the C terminus, display increased affinities to CYP11A1 as well. Obviously truncation of the C-terminal end of adrenodoxin enhances the affinity of the iron-sulfur protein to its redox partner CYP11A1. Thus, one explanation for the function of the C-terminal end of adrenodoxin in vivo might be the modulation of binding strength to a moderate level to permit an optimal interaction of adrenodoxin with its various reaction partners.

The mutant adrenodoxins S112W as well as Y82FS112W, besides improved affinity to CYP11A1, also show enhanced activity in the cholesterol conversion reaction (see Table V) with increased $k_{cat}$ values (6.6- and 9.4-fold) and decreased $K_m$ values (9- and 9.7-fold) resulting in a 50–100-fold increase of the reaction efficiency ($k_{cat}/K_m$). Looking at the other mutants of this study, only adrenodoxin-(4–108) displays a higher $k_{cat}$, being increased by about 2.8-fold as compared with the wild type. Interestingly the four tryptophan mutants differ largely in their binding affinity to CYP11A1, which could be attributed to the mutation in position 82 on the surface of adrenodoxin where an aromatic residue seems to be necessary for an amplification in binding strength (Table III). On the other hand, the $K_m$ values of all truncated mutants are within the same range and are decreased by a factor of −10 as compared with wild type adrenodoxin. This lowered $K_m$ in CYP11A1-dependent cholesterol conversion is paralleled by decreased $K_p$ values of the respective CYP11A1-adrenodoxin complexes. Surprisingly the differences found for the CYP11A1-dependent substrate conversion mediated by adrenodoxin mutants S112W and Y82FS112W in comparison to the adrenodoxin wild type could not be observed with CYP11B1 as the final electron acceptor (see Table VI). Only minor differences between the adrenodoxin species were detected in the CYP11B1-dependent conversion of 11-deoxycorticosterone to corticosterone with a maximum difference in $k_{cat}/K_m$ of 3.8-fold for adrenodoxin mutant Y82FS112W. These data can be interpreted in two ways: either none of the structural alterations in the different adrenodoxin mutants affect the interaction with CYP11B1, or the rate-limiting step of the CYP11B1-dependent substrate conversion reaction occurs after the interaction of adrenodoxin with the cytochrome. In the latter case, substrate hydroxylation or product release from the enzyme could be considered rate-limiting. In fact, Imai et al. (49) demonstrated that the rate-limiting step in the CYP11B1-dependent aldosterone production is the release of the product from the enzyme. If this was also true for corticosterone formation, possible alterations in the interaction of the different adrenodoxin species with CYP11B1 would not be detected in this experimental system. It has to be kept in mind, however, that the interactions between the different components of the P450 systems described here are at least partially mediated by electrostatic forces. Therefore, the salt concentration used in the assays most probably affects binding affinities and efficiency of the respective system so that former results (11, 27) obtained under conditions different from ours show comparable tendencies indeed but cannot be related directly to the values presented in this work.

The rate constants determined for the formation of the reduced CYP11A1-CO complex by the various adrenodoxin species differ by up to a factor of 10 (Table IV) that again is in contrast to the values observed for the CYP11B1 reduction where all the different adrenodoxin species behave quite sim-
CYP11A1 by adrenodoxin mutants S112W and Y82F/S112W. In position 82 of adrenodoxin is also required for this potential stacking interactions with Trp-112 of the adrenodoxin mutants and CYP11A1 seems to be possible and may result in a conformational change of the cytochrome P450. In a computer model is supported by the fact that the second rate constant is faster reduction of CYP11A1 (as detected by CYP11A1 interaction between CYP11A1 and adrenodoxin is paralleled by an aromatic residue in position 82 of adrenodoxin appears to be important for an additional increase of the rate constant that again is in concordance with the amplified binding affinity of those adrenodoxin mutants to CYP11A1 and with their accelerated kinetics of the CYP11A1-dependent substrate conversion. Moreover, biexponential time courses of CYP11A1 reduction could only be observed for adrenodoxin S112W and Y82F/S112W, i.e. those tryptophan mutants containing an aromatic residue in position 82 (which is on the surface of adrenodoxin). This result might be explained by the formation of at least two different complexes of adrenodoxin and CYP11A1 with only one being productive while the other one is unable to transfer electrons (Fig. 5). From the smaller amplitude (see Fig. 4) of the first, rapid phase, it can be concluded that the productive complex is thermodynamically less favored. This mechanistic model is supported by the fact that the second rate constant is independent from adrenodoxin concentration as it would be assumed for a rearrangement of an unproductive complex to form the productive configuration. The formation of additional hydrophobic interactions between the respective adrenodoxin mutants and CYP11A1 seems to be possible and may result in a conformational change of the cytochrome P450. In a computer model (50) two aromatic residues (Trp-401 and Phe-411) are located on the CYP11A1 surface close to the site of ionic interaction with adrenodoxin (51). These two residues might form stacking interactions with Trp-112 of the adrenodoxin mutants (S112W and Y82F/S112W). Obviously an aromatic amino acid in position 82 of adrenodoxin is also required for this potential interaction as no biphase behavior was observed with the other adrenodoxin species.

The CYP11A1 reduction courses of all other adrenodoxin species tested were apparently monoeXponential, i.e. no second phase was observed. This means that either no unproductive adrenodoxin-CYP11A1 complex exists with these proteins, or the rearrangement of an unproductive complex into the productive complex is as fast or even faster than the reduction of CYP11A1 mediated by the respective adrenodoxins. It would, therefore, be of great interest to obtain three-dimensional information on the adrenodoxin-CYP11A1 complex(es) and to elucidate the structural basis of the formation of different (i.e. productive and unproductive) forms.

A comparison of the rate constants for the formation of the reduced CYP11A1-CO complex, e.g. 23 s⁻¹ for the fastest (S112W) mutant, with the k_{cat}/K_{m} value of pregnenolone formation (0.07 s⁻¹ for S112W, see Table V) shows that these rate constants cannot be directly correlated. The same holds true for the relation of these values of all other adrenodoxin species. This leads to the conclusion that the rate-limiting step in the CYP11A1-dependent substrate conversion, although being correlated to adrenodoxin binding and CO reduction rate, remains to be elucidated.

Taken together the truncated adrenodoxin mutants containing a tryptophan at the C-terminal end show the tendency to a more “putidaredoxin-like” behavior, i.e. a stronger binding to the redox partner CYP11A1, an enhanced substrate conversion activity, and a faster formation of the reduced CYP11A1-CO complex. The introduced mutations lead to a significant increase in substrate specificity (i.e. k_{cat}/K_{m}) by 2 orders of magnitude.

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Fig. 5. Reaction scheme proposed for the reduction of CYP11A1 by adrenodoxin mutants S112W and Y82F/S112W. A_red is the reduced adrenodoxin species and C is CYP11A1. A_redC_p represents the productive complex being able to reduce the cytochrome. A_redC_u represents the unproductive complex, which has to undergo a rearrangement to yield the productive form; this rearrangement is assumed to be slow. Initially about 33% of the complex are assembled in the productive form and 66% in the unproductive, but obviously thermodynamically favored, configuration. A_red oxidized adrenodoxin species; C_red reduced CYP11A1.
Increased Efficiency of Adrenodoxin Mutants

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The Interaction of Bovine Adrenodoxin with CYP11A1 (Cytochrome P450_{c18}) and CYP11B1 (Cytochrome P450_{11B}): ACCELERATION OF REDUCTION AND SUBSTRATE CONVERSION BY SITE-DIRECTED MUTAGENESIS OF ADRENODOXIN

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