The role of threonine 54 in adrenodoxin for the properties of its iron-sulfur cluster and its electron transfer function

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The amino acid in position 54 of adrenodoxin is strongly conserved among ferredoxins, consisting of a threonine or serine. Its role was studied by analyzing mutants T54S and T54A of bovine adrenodoxin. Absorption, circular dichroism, fluorescence, and electron paramagnetic resonance spectra of mutant T54S show that this substitution has no influence on the formation and stability of the ferredoxin. The redox potential of this mutant, however, was lowered by 55 mV as compared with native adrenodoxin, indicating a role for this residue in redox potential modulation. Incorporation of the iron-sulfur cluster was not impaired in the T54A mutant, although structural features of the oxidized protein were considerably changed. The decreased stability of the T54A mutant as compared with the wild type and mutant T54S indicates that a hydrogen bond donor at this position stabilizes the protein. Both mutations have been shown to be functionally active. Replacement of threonine 54 by serine or alanine, however, leads to rearrangements at the recognition sites for its redox partners. This is reflected by decreased $K_m$ and $K_r$ values of both mutants for the cytochromes P450, whereas only T54A displayed a decreased $K_m$ value in cytochrome c reduction. Substrate conversion was accelerated (2.2- and 2.4-fold for mutants T54A and T54S, respectively) in the CYP11B1, but not in the CYP11A1-dependent reaction.

Ferredoxins are ubiquitous iron-sulfur proteins present in bacteria, plants, and animals. They take part in a broad variety of electron transfer reactions. In plants and algae a ferredoxin of the [2Fe-2S] type passes electrons from photosystem I to NADP⁺ via a ferredoxin reductase in the process of carbon assimilation (1). The hydroxylase systems of vertebrates and some bacteria utilize ferredoxin to transfer an electron from an NAD(P)H-dependent reductase to various cytochromes P450 (2-5). The [2Fe-2S] ferredoxins of bacteria, e.g. putidaredoxin, lhiA, or terpE, are components of the hydrogenation systems for camphor, linalool, or α-terpinene, respectively, the carbon sources of these organisms (3-5). In the adrenal cortex, adrenodoxin is involved in steroid hormone biosynthesis. It passes electrons to the mitochondrial cytochromes P450 CYP11A1, CYP11B1, and CYP11B2, converting cholesterol to pregnenolone, catalyzing the 11β-hydroxylation of 11-deoxy-cortisol and 11-deoxycorticosterone, and producing aldosterone, respectively (6-9).

The way in which the redox partners interact during electron transfer is still a matter of controversy. In the "shuttle" model (10-15), adrenodoxin sequentially forms binary complexes with the reductase and the cytochrome. A ternary complex of adrenodoxin reductase, and CYP11A1 is proposed in the second model (16-20). Very recently, the necessity of two molecules of adrenodoxin for one-electron transfer has been suggested (21) which is supported by former results obtained with CYP11B1 (22).

Recognition and interaction of adrenodoxin with its redox partners is mainly based on electrostatic interactions (23-25). However, tyrosine 82 of adrenodoxin was shown to participate in binding to CYP11A1 and CYP11B1, but not to adrenodoxin reductase (26). Furthermore, transmission of conformational changes of the cluster to tyrosine 82 and the acidic residues of the binding region of adrenodoxin for its redox partners has been shown to be mediated by histidine 56 (27). Deletion of the C-terminal part of adrenodoxin up to amino acid 109 have been shown to influence binding affinity and electron transfer to CYP11A1 and CYP11B1, but not interaction with adrenodoxin reductase (28).

The ability to accept and donate electrons is tightly connected to the redox potentials of iron-sulfur proteins. Generally, the features determining or modulating a certain redox potential are not well understood yet, but seem to be related at least in ferredoxins to the water accessibility of the iron-sulfur center, negative charges close to the cluster and on the protein surface, and hydrogen bonding pattern (extent, strength, and specific position) (29). Some of these features should differ in the [2Fe-2S] proteins which cover a considerable redox potential range. The cysteine ligands to the cluster (cysteines 46, 52, 55, and 92 in adrenodoxin (30)) are homologous in these proteins and therefore cannot account for their different properties. On the other hand, the single free Cys-95 in adrenodoxin is not able to replace Cys-92 when the latter residue was substituted by serine (30) as it was described for the [4Fe-4S] cluster of Azotobacter vinelandii ferredoxin I (31). Replacement of any of the cysteine cluster ligands in adrenodoxin with serine lead to the formation of apoproteins (27, 30). In contrast, the [2Fe-2S] ferredoxin of Clostridium pasteurianum (32), Anaabaena vegetative ferredoxin (33), and the [2Fe-2S] center of the Escherichia coli fumarate reductase (34) are stable when the cysteine cluster ligands are replaced by serine and in the case of fumarate reductase also by aspartic acid (35). So far, little is known about structures which stabilize the cluster and the conformation in ferredoxins (36). Very recently, a method to facilitate calorimetric studies on folding and stability of adrenodoxin was developed (37).

The different consequences of cluster ligand substitutions in adrenodoxin and other ferredoxins are of particular interest.
Role of Threonine 54 in Adrenodoxin

MATERIALS AND METHODS

Reagents and Biochemicals—Taq DNA polymerase and restriction endonucleases were from Amersham Buchler KG. Horse heart cytochrome c and NADPH were obtained from Boehringer Mannheim. The steroids were purchased from Sigma. All other reagents were of the highest purity grade commercially available.

Bacterial Strains, Plasmids, and Oligonucleotides—E. coli strain HB101 was used as host strain. For expression of the adrenodoxin mutants, the plasmid pKAdx (30) was used. The 3’ polymerase chain reaction primers with appropriate cloning sites were chemically synthesized by BioTez GmbH and consisted of the following sequences (base replacement is labeled): 5’-GGACCTTGGCTTTCAATTTGACTGTTGACCTCA-3’ (T54S), 5’-GGACCTTGGCTTTCAATTTGACTGTTGACCTCA-3’ (T54A), and 5’-GGGGAAGCCTTGGCTTTCAATTTGACTGTTGACCTCA-3’ (T54C).

Plant type ferredoxins

Plum sativum

Vertebrate type ferredoxins

Adrenodoxin

Placenta Ferredoxin

Kidney Ferredoxin

Pulidaredoxin

E. coli Ferredoxin

Terpredoxin

Fig. 1. Amino acid sequences of various vertebrate and plant type ferredoxins around three cysteine ligands to the [2Fe-2S] centers (positions 46, 52, and 55 of adrenodoxin). The conserved threonine residue corresponding to position 54 in adrenodoxin is labeled. The cysteine cluster ligands are indicated by their position numbers in the ferredoxins. The sequences were taken from Refs. 5 (adrenodoxin, kidney ferredoxin, pulidaredoxin, terpredoxin), 53 (plantaferredoxin, centraferredoxin, kidney ferredoxin, putidaredoxin, terpredoxin), 53 (plantaferredoxin, centraferredoxin, kidney ferredoxin, putidaredoxin, terpredoxin), and 63 (Plataxon, S. quaricauda).

Since the [2Fe-2S] center is assumed to adopt a similar tetrahedral structure in these proteins having homologous sequences around the ligand cysteines, the plant type ferredoxins display a higher degree of overall sequence similarity than the vertebrate type ferredoxins. As it is obvious from Fig. 1, the residue corresponding to position 54 of adrenodoxin is always occupied by either a threonine or a serine, residues which are known to be hydrogen bond donors within the protein or to water molecules that contribute to the protein stability.

In order to understand the particular role of threonine 54 for the assembly of the iron-sulfur cluster and for determining its functional properties, we used mutants of adrenodoxin in which Thr-54 was replaced by serine (T54S) or alanine (T54A). The absorbance ratio A414/A280, expressing the ratio of holo-adrenodoxin to total protein, was used as a purity index for adrenodoxin. The mutants were analyzed by SDS-polyacrylamide gel electrophoresis in a 15–20% gradient gel, followed by Western blotting using a polyclonal antibody against bovine adrenodoxin. Determination of the amino acid composition and N-terminal sequencing of recombinant protein were performed according to published procedures (28). Isolation of adrenodoxin reductase, CYP11A1 and CYP11B1 from bovine adrenals, as well as estimation of the respective concentrations and specific activities, were carried out as described previously (28).

Spectroscopic Methods—EPR measurements of the dithionite-reduced proteins were carried out under the following conditions: microwave power, 10 mW; modulation amplitude, 10.084 G; time constant, 0.32 ms; sweep time, 167.772 s; temperature, –163 °C on a Bruker ESP300E spectrometer. Absorption spectra were recorded at room temperature on a Shimadzu double-beam spectrophotometer UV2101PC. Adrenodoxin fluorescence emission was measured on a Shimadzu RF-5001PC spectrofluorophotometer at a 0.5-cm fluorescence cuvette. The absorbance of the single tyrosine S2 could be directly observed since adrenodoxin does not contain tryptophan. The excitation wavelength was 275 nm.

CD spectra were recorded on a Jasco J 720 spectropolarimeter. Samples contained 100 μM adrenodoxin in 10 mM potassium phosphate buffer (pH 7.4) in a 1-cm cuvette for measurements in the 250–650 nm range and 20 μM adrenodoxin in 2 mM potassium phosphate buffer (pH 7.4) in a 0.1-cm cuvette for measurements in the 184–260 nm range. The spectrum of the respective potassium phosphate buffer was recorded as a baseline (measurement conditions were: bandwidth, 1 nm; response, 2 s; step, 0.5 nm). Temperature-dependent measurements were carried out at a heating rate of 50°C/h from 20 to 80°C with a temperature increment of 0.25°C, monitoring the decrease of the decrease of the circular dichroism signal at 440 nm. Data were analyzed and Tm and ΔHm were determined using a nonlinear regression program kindly provided by Dr. O. Ristau (MDC, Berlin).

Redox Potential Measurements—The redox potentials of adrenodoxin and the mutants were determined using the dye photoreduction method (41). Samples contained 30 μM EDTA for proton supply, 0.25 mM Safranin O as mediator and indicator, 2 μM adrenodoxin, and an oxygen removing system consisting of 35 mM glucose, 50 units/ml glucose oxidase, and 10,000 units/ml catalase in 100 mM potassium phosphate buffer (pH 7.5). For measuring the redox potential of adrenodoxin in complex with CYP11A1 according to Ref. 12, 15 μM CYP11A1 was added to the reaction mixture. The samples were flushed with N2 and left in the dark for 30 min. The system was reduced step by step by varying the irradiation time with a heat-filtered 500 watt xenon lamp, and the respective absorption spectrum in the 380–540 nm range was recorded. Data were analyzed using the Nernst equation.

Enzyme Assays—Activity of the adrenodoxin mutants was tested in reconstituted systems, under conditions in which the measured activity was a function of the active adrenodoxin concentration (28). Cytochrome c reduction was assayed in 50 mM potassium phosphate buffer (pH 7.5), 0.1% Tween 20 at room temperature. Reaction mixtures contained 0.05 μM adrenodoxin reductase, 65 μM cytochrome c, and varying concentrations of adrenodoxin. The reaction was initiated by addition of 140 μM NADPH. The absorbance change at 550 nm was monitored, and the activity determined using ε414 = 20 mm−1 cm−1.

CYP11A1-dependent conversion of cholesterol to pregnenolone was performed as described (28). Reaction mixtures consisted of 20 mM potassium phosphate buffer (pH 7.5), 0.3% Tween 20, 0.5 μM adrenodoxin reductase, 0.5 μM CYP11A1, 100 μM cholesterol, specified amounts of adrenodoxin, a NADPH generating system consisting of 15–20% gradient gel, and 10 mM glucose 6-phosphate dehydrogenase, and 60 μM NADPH. After the reaction, the steroids were converted into their corresponding 3-one-4-en forms by adding 2 units/ml cholesterol oxidase, extracted, and analyzed by reverse-phase high performance liquid chromatography. Cholesterolene and progesterone were used as external standards.

CYP11B1 assays contained 0.4 μM adrenodoxin reductase, 0.4 μM CYP11B1, 100 μM deoxycorticosterone, varying concentrations of adrenodoxin, the NADPH generating system, as described, in 50 mM potassium phosphate buffer (pH 7.5), 0.1 mW dithiothreitol, and 60 μM...
NADPH and were performed as described (28). Dichloromethane, which acts to extract the steroids, was used to stop the reaction. The amount of corticosterone produced was determined by high performance liquid chromatography with corticosterone and 11-deoxycorticosterone as external standards.

Stopped-flow Assays—The transfer of the first electron from adrenodoxin to cytochrome P450 was recorded at 20 °C using a single channel stopped-flow ASVD spectrometer SX-17MV (Applied Photophysics) as described in Ref. 28. Test tube A contained 1 \( \mu M \) cytochrome P450 saturated with its respective substrate, 1 \( \mu M \) adrenodoxin reductase, and 3 \( \mu M \) adrenodoxin. Test tube B contained 2 \( \mu M \) NADPH and an oxygen removing system consisting of glucose oxidase, catalase, and glucose. The samples were treated with carbon monoxide and loaded into the respective driving syringes. The total volume per shot was 2 \( \times \) 50 \( \mu l \). Reduction of the cytochrome P450 was followed by measuring the increase in 450 nm absorbance of its ferrous carbon monoxide complex.

Optical Titrations—Binding of adrenodoxin to CYP11A1 was measured by optical difference spectroscopy using tandem cuvettes. In the sample cuvette, 0.5 \( \mu M \) CYP11A1 in 50 \( \mu M \) potassium phosphate buffer (pH 7.5), 0.1% Tween 20, 15 \( \mu M \) cholesterol was titrated with adrenodoxin. Binding of cholesterol to CYP11A1, promoted by binding of adrenodoxin, causes a conversion of the cytochrome heme group from its low (absorption peak at 417 nm) to its high spin form (absorption peak at 393 nm). The absorbance changes were plotted according to Scatchard (42) as a function of free adrenodoxin concentration, which was calculated according to the following equation assuming a single binding site of adrenodoxin on CYP11A1: [adrenodoxin]free = [adrenodoxin]total - (\( \Delta A/\Delta g_{\text{M}} \))(CYP11A1).

RESULTS

Expression and Characterization of Adrenodoxin Thr-54 Mutants—Adrenodoxin was expressed into the cytoplasm of E. coli HB101 using the expression vector pKKA6x (30). In the same system, cDNAs of adrenodoxin mutants containing a single base replacement have been expressed, which were obtained by polymerase chain reaction cloning. The replacements were confirmed by nucleotide sequencing. Mutant proteins were further analyzed by N-terminal sequencing and determination of their amino acid composition which was very close to the predicted value of each amino acid deduced from the sequence (43). The threonine replacement by serine and alanine, respectively, was clearly detectable in the mutants (data not shown).

Western blotting revealed specific bands (14 kDa each) for adrenodoxin and the mutants (not shown). All proteins were expressed to almost the same level (50–100 mg/liter of E. coli culture). The final purity index (\( A_{420}/A_{270} \)) of the proteins was always higher than 0.92.

Properties of the Iron-Sulfur Cluster—To determine the effect of a single amino acid replacement at position 54 of adrenodoxin on the structure of the iron-sulfur cluster, EPR, UV/vis, CD, and fluorescence spectra of the proteins were recorded. Oxidized adrenodoxin is not EPR active since both iron atoms are in the high-spin \( Fe^{3+} \) form (total spin = 0), whereas EPR spectra of reduced adrenodoxin (\( Fe^{2+}/Fe^{3+} \)) are characterized by two g values: \( g_1 = 1.94 \) and \( g_2 = 2.03 \) (30, 44, 45). Unchanged EPR spectra of mutants T54S and T54A provide evidence that the mutants were expressed as holoproteins with the reduced [2Fe-2S] center being assembled similar to that of the wild type (not shown).

Absorption spectra of oxidized [2Fe-2S] ferredoxins are characterized by a number of absorption maxima in the visible and near UV region. The typical peaks of adrenodoxin, caused by the [2Fe-2S] cluster in its specific surrounding, occur at 455, 414, and 320 nm (6). UV/vis spectra of wild type adrenodoxin and mutant T54S were indistinguishable. In mutant T54A, however, a bathochromic shift of the peak at 320 to 340 nm could be observed (Fig. 2). These apparent structural differences between the oxidized forms of wild type adrenodoxin and mutant T54S on the one hand and T54A mutant on the other hand were further analyzed by CD spectroscopy since it sensitively reflects conformational changes in optically active substances like a protein iron-sulfur cluster. In fact, wild type adrenodoxin and mutant T54S again exhibit similar spectra (Fig. 3, A-C), whereas mutant T54A shows shifted CD signals and in part lower amplitudes of the peaks as compared with wild type adrenodoxin. The signal changes in the 310–650 nm range clearly reflect a rearrangement in the immediate vicinity of the cluster of this mutant (Fig. 3, A and B). Moreover, the T54A replacement seems to slightly affect the general polypeptide backbone conformation (the local maximum at 195 nm shifts to 191 nm; Fig. 3C) and to display an influence on the CD properties of the aromatic amino acids since the molar circular dichroic absorption \( \Delta \varepsilon \) of this mutant decreases from 1.4 \( \times 10^{-2} \) (wild type) to \( -3.2 \times 10^{-2} \) cm/mol at 297 nm (Fig. 3B).

The fact that wild type adrenodoxin, as well as the mutants, contain only a single tyrosine and four phenylalanines, and lack tryptophan, suggests an involvement of the tyrosine in the observed changes because phenylalanine residues are known to contribute marginally to the spectral characteristics of a protein. In order to confirm the latter observation, we recorded the fluorescence spectra of the adrenodoxin mutants. While the tyrosine fluorescence emission peak occurs at its characteristic position (305 nm) (46, 47) in both the wild type and the mutants, the intensity of this peak is increased by 250% in mutant T54A as compared with the wild type and mutant T54S (Fig. 4).

Thermal Stability—The threonine at position 54 is located close to the iron-sulfur cluster of adrenodoxin, and therefore its substitution might affect the conformational stability of the protein. To check the characteristics of thermal denaturation of the mutants, unfolding of the proteins upon increasing temperatures was followed by measuring their circular dichroism. Single wavelength melting curves were recorded at 440 nm, where the maximum circular dichroism of the iron-sulfur clus-
ter is observed, by slowly increasing the temperature from 20 to 80 °C. The circular dichroism signal decreases in a sigmoidal manner upon increasing temperatures, allowing an exponential fit of the data. Thermal denaturation of adrenodoxin first

Fig. 3. CD spectra of adrenodoxin and the Thr-54 mutants. A, visible region; B, near UV region; C, far UV region. Samples consisted of 100 μM adrenodoxin in 10 mM potassium phosphate buffer (pH 7.4) for A and B, and 20 μM adrenodoxin in 2 mM potassium phosphate buffer (pH 7.4) buffer for C. wt, wild type.

Fig. 4. Fluorescence emission spectra of Thr-54 mutants of adrenodoxin. The fluorescence emission of adrenodoxin, which contains a single tyrosine at position 82 and no tryptophan, was measured on a Shimadzu RF-5001 PC spectrophotometer. Each sample contained 100 μM adrenodoxin in 10 mM potassium phosphate buffer (pH 7.4). The excitation wavelength was 275 nm. wt, wild type.

was followed in a potassium phosphate buffer (pH 7.5) usually used for the enzyme assays (see “Materials and Methods”). In this system, changes of the CD peak are irreversible on cooling. Application of a buffer system containing 2-mercaptoethanol, Na₂S, and ascorbic acid in glycine buffer (pH 8.5), which was very recently developed for calorimetric studies on adrenodoxin (37), prevents the irreversible destruction of the iron-sulfur cluster. The thermal transition temperatures (Tₘ) of adrenodoxin depend on the buffer system used and indicate a slightly lower overall stability of adrenodoxin and the mutants in potassium phosphate buffer (pH 7.5) due to the absence of Na₂S which is considered to stabilize the protein (37) (Fig. 5). The Tₘ values of mutant T54A are 5.5 °C (potassium phosphate) and 2.6 °C (glycine buffer system) lower than those of the wild type (Table I). Considering the denaturation enthalpy ΔHₘ of the reversible denaturation process in the glycine buffer system, mutant T54A again displays a considerably lower value than the wild type (51 kJ/mol; Table I). Statistical significance of differences between mutant T54A and wild type adrenodoxin has been shown by Student’s t test. Both Tₘ and ΔHₘ indicate mutant T54A to be less stable than the wild type. In contrast, mutant T54S and wild type adrenodoxin have similar Tₘ and ΔHₘ.


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The thermodynamic parameters of the adrenodoxin mutants in different buffer systems are shown in Table I. The parameters and standard deviations were derived from heat denaturation curves of adrenodoxin (two independent experiments for each mutant) at a heating rate of 50 °C/h from 20 to 80 °C measured by CD spectroscopy at 440 nm. Thermal denaturation was followed in a potassium phosphate buffer used for enzyme assays, and in a glycine buffer, containing 2-mercaptoethanol, Na2S, and ascorbic acid. As thermal denaturation in the first buffer system was irreversible on cooling, no ΔHm value of this process could be calculated.

| Adrenodoxin        | Potassium phosphate buffer (pH 7.5), Tm (°C) | Glycine buffer system (pH 8.5), Tm (°C) | ΔHm (kJ/mol) |
|--------------------|---------------------------------------------|----------------------------------------|--------------|
| Wild type          | 49.1 ± 0.3                                  | 52.3 ± 0.1                             | 336 ± 6      |
| T54S               | 49.4 ± 0.3                                  | 52.7 ± 0.1                             | 350 ± 15     |
| T54A               | 43.6 ± 0.4                                  | 49.7 ± 0.1                             | 285 ± 9      |

ΔHm values (Table I).

Redox Potentials of the Thr-54 Mutants—The redox potential of an electron transport protein is an important parameter for its specific function, i.e., its ability to accept and donate electrons. Redox potentials of the adrenodoxin mutants were determined using the dye Safranin T for reduction of adrenodoxin which is suitable since its midpoint potential (−292 mV) is close to that of adrenodoxin (40). The measured redox potential of wild type adrenodoxin (Table II) resembles the value for native adrenodoxin isolated from bovine adrenals (−273 mV) (41).

| Adrenodoxin | Redox potential of |
|-------------|--------------------|
| Wild type   | −274 mV            |
| T54S        | −292 mV            |
| T54A        | −286 mV            |

In contrast, reduction of CYP11B1 is 1.7 times the wild type rate when using adrenodoxin mutants T54S and T54A (Table IV). In contrast, reduction of CYP11B1 is 1.7 times the wild type rate when using adrenodoxin mutants T54S and T54A (Table IV).

Binding Affinity to CYP11A—Binding of the oxidized form of adrenodoxin to its electron acceptor CYP11A is comparable to that of the reduced form (12), thus allowing the analysis of the affinities of wild type and mutant adrenodoxin to CYP11A by optical difference spectroscopy. As compared with wild-type adrenodoxin, mutants T54S and T54A exhibit a nearly 3.6-fold and 1.3-fold decrease in the Kd values, respectively (Table V), which according to the Michaelis-Menten equation may contribute to the decreased Km values in the CYP11A assay.

DISCUSSION

Substitution mutants of adrenodoxin in which threonine 54 was replaced by serine or alanine were analyzed in order to study the role of this amino acid residue located between two cysteine ligands to the [2Fe-2S] cluster at a position which is strongly conserved (threonine or serine) among the ferredoxins of bacteria, plants, and animals. Attention has especially been paid to structural properties and stability of the mutants by analyzing their spectral features, to their redox potentials, and to their electron acceptor and donor functions.

The specific environment of a certain reduced [2Fe-2S] cluster is sensitively reflected by EPR spectroscopy as confirmed by the different EPR spectra of plant and vertebrate type [2Fe-2S] proteins, although their general structure is assumed to be similar. The EPR signals of the adrenodoxin mutants resemble those of the wild type and therefore indicate that the conformation of the reduced iron-sulfur center in adrenodoxin is not significantly affected by the substitutions at position 54 (data not shown). The structural properties of the proteins in oxidized form, however, point to the necessity of a hydroxyl group at position 54 for maintaining the native conformation of the oxidized iron-sulfur center. While mutant T54S and wild type adrenodoxin exhibit similar characteristics in optical, CD, and fluorescence spectroscopy (Figs. 2-4), substitution of the hydrophobic alanine at position 54 (mutant T54A) causes a rearrangement in the environment of the oxidized cluster (Figs. 2 and 3). This T54A mutation also affects the remote tyrosine residue near the negatively charged protein surface of adrenodoxin which is involved in binding of adrenodoxin reductase and cytochrome P450 (Fig. 4), and slightly influences the polypeptide backbone conformation (Fig. 3C). Alanine is not able to form a hydrogen bond as is the native threonine, and thus destabilizes the α priori highly flexible cluster area (52) of a ferredoxin by making it even more flexible, whereas a hydrogen bonding system would not significantly be disturbed by a T→S replacement. The diminished volume of serine as compared with threonine in mutant T54S does not lead to any...
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Interaction of the adrenodoxin mutants with adrenodoxin reductase was assayed following the reduction of cytochrome c at 550 nm. In order to characterize the enzymatic activities of the adrenodoxin mutants with the terminal electron acceptors CYP11A1 and CYP11B1, the products of the respective hydroxylation reaction, pregnenolone and corticosterone, were analyzed by HPLC. The \( K_m \) and \( V_{max} \) values and their standard deviation were calculated from five or six independent experiments and are relative to adrenodoxin concentration. Statistical significance of differences between the \( K_m \) values of mutant T54A and wild type adrenodoxin in the cytochrome c assay, the \( K_m \) values of both mutants and wild type adrenodoxin in the CYP11A1 assay, and the \( K_m \) and \( V_{max} \) values of both mutants and wild type adrenodoxin in the CYP11B1 assay has been shown by Student’s \( t \) test.

### Table III

| Adrenodoxin | Cytochrome c reduction assay | Cholesterol side chain cleavage assay | 11β-Hydroxylation assay |
|-------------|-------------------------------|--------------------------------------|-------------------------|
|             | \( K_m \) \( \mu M \) | \( V_{max} \) nmol cytochrome c reduced/min | \( K_m \) \( \mu M \) | \( V_{max} \) nmol pregnenolone produced/min nmol CYP11A1 | \( K_m \) \( \mu M \) | \( V_{max} \) nmol corticosterone produced/min nmol CYP11B1 |
| Wild type   | 5.7 ± 0.7 | 7.8 ± 0.9 | 1.18 ± 0.11 | 4.04 ± 0.5 | 1.40 ± 0.18 | 6.0 ± 0.5 |
| T54S        | 5.2 ± 0.5 | 8.8 ± 1.0 | 0.24 ± 0.02 | 4.10 ± 0.4 | 0.45 ± 0.04 | 14.2 ± 1.0 |
| T54A        | 2.0 ± 0.3 | 6.9 ± 0.7 | 0.43 ± 0.05 | 3.92 ± 0.4 | 0.74 ± 0.06 | 13.1 ± 0.9 |

### Table IV

| Adrenodoxin | Reduction of CYP11A1 | CYP11B1 |
|-------------|----------------------|---------|
|             | \( k_{app} (s^{-1}) \) | \( k_{app} (s^{-1}) \) |
| Wild type   | 1.21 ± 0.13 | 0.45 ± 0.06 |
| T54S        | 1.06 ± 0.10 | 0.75 ± 0.06 |
| T54A        | 0.83 ± 0.07 | 0.76 ± 0.08 |

### Table V

| Adrenodoxin | Spectral binding assay \( K_d \) \( \mu M \) |
|-------------|----------------------------------|
| Wild type   | 0.25 ± 0.03 |
| T54S        | 0.07 ± 0.01 |
| T54A        | 0.19 ± 0.02 |

The missing hydrogen bond and the rearrangement of the iron-sulfur cluster surrounding in mutant T54A results in a decrease of the thermal stability of this protein. Temperature-dependent CD measurements reveal lower \( T_m \) values (5.5 and 2.6 degrees; Table I) for mutant T54A in both buffer systems used. Also, the \( \Delta H_m \) value of this mutant for the reversible denaturation process is lowered by 51 kJ/mol as compared with that of the wild type (Table I). The stability of mutant T54A upon storage is also diminished as compared with the wild type, as indicated by a decrease in the absorbance ratio \( A_{340}/A_{270} \) from 0.9 to 0.8.

The three-dimensional structures of [2Fe-2S] ferredoxins carrying a threonine (53, 54) or a serine (55) at the position equivalent to threonine 54 in adrenodoxin allow speculation about the role of this residue in adrenodoxin. In putidaredoxin (53), T47-OH (being homologous to threonine 54 in adrenodoxin) is excluded from water molecules and located close (−3 Å) to the cluster ligand C45-S, suggesting a contact between these two amino acid residues. A similar hydrogen bond between T48-OH and C46-S (3.4 Å) was proposed for the [2Fe-2S] ferredoxin from the cyanobacterium Anabaena (52, 54).

### Table II

| Adrenodoxin | Kd \( K_m \) \( nmol/mmol \) | V \( V_{max} \) nmol prenenolone produced/min nmol CYP11A1 | Kd \( K_m \) \( nmol/mmol \) | V \( V_{max} \) nmol corticosterone produced/min nmol CYP11B1 |
|-------------|-----------------------------|-------------------------------------------------------------|-----------------------------|-------------------------------------------------------------|
| Wild type   | 0.25 ± 0.03 | 0.25 ± 0.03 | 0.25 ± 0.03 | 0.25 ± 0.03 |
| T54S        | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 |
| T54A        | 0.19 ± 0.02 | 0.19 ± 0.02 | 0.19 ± 0.02 | 0.19 ± 0.02 |

While the replacement of threonine 54 by serine does not affect the conformation of the cluster in its environment, the redox potential of mutant T54S is markedly lowered (Table II) in comparison to wild type adrenodoxin. Interestingly, the redox potentials of the [2Fe-2S] proteins of higher plants, which contain a serine at the position corresponding to threonine 54 of adrenodoxin (the only known exception is Equisetum ferredoxin II, see Fig. 1), are usually 50 mV lower than those of most ferredoxins from blue-green algae, in which this position is occupied by a threonine (except for Anabaena heterocyst ferredoxin, see Fig. 1) (59), although both groups belong to the class of plant type ferredoxins (33, 59). Substitution of T54S in adrenodoxin caused a similar redox potential shift (−55 mV; Table II) leading to the conclusion that the residue naturally occurring at this position (threonine or serine) is directly involved in redox potential tuning. Determination of the redox potential of mutant T54A revealed a value 11 mV lower than that of mutant T54S (Table II). Alkaline as a non-aromatic apolar residue has a low surface probability resulting from its hydrophobicity. It is effective in excluding the solvent close to the iron-sulfur center, thus leading to a low dielectric constant and a decrease in the redox potential of the protein (60). A generally higher redox potential as displayed by the vertebrate-type ferredoxins in comparison to plant-type ferredoxins permits a higher concentration of reduced [2Fe-2S] centers during turnover (61), which might be essential to prevent competition between oxidized and reduced ferredoxins for binding to its electron acceptor as it was described for adrenodoxin and CYP11A1 (14, 28).

The redox potential is closely related to the electron acceptor and donor functions of adrenodoxin, which are retained in the
mutants. Upon complex formation with CYP11A1, both mutativeferredoxin (52), indicating that the electron transfer rate is
chrome function of oxidized adrenodoxin mutants was tested in a cyto-
impaired by substitution of threonine 54. The electron acceptor
reaction. The capability of adrenodoxin to transfer electrons was not
reduction assay. There are only marginal changes in the kinetic parameters indicating that replacement of threo-
nine 54 by serine or alanine does not considerably affect inter-
action with adrenodoxin reductase.

The capacity of adrenodoxin to transfer electrons was not impaired by substitution of threonine 54. The electron acceptor function of oxidized adrenodoxin mutants was tested in a cyto-
chrome c reduction assay. There are only marginal changes in the kinetic parameters indicating that replacement of threo-
nine 54 by serine or alanine does not considerably affect inter-
action with adrenodoxin reductase.

The affinity of adrenodoxin to CYP11A1 (Table V) and the hydroxylating activities of CYP11A1 and CYP11B1 (Table III), however, were particularly affected by the T54S mutation, while mutant T54A showed less pronounced effects. This points to either different binding sites for adrenodoxin reductase and the cytochromes or the possibility that interaction with the cytochromes more sensitively reflects minimal changes of the adrenodoxin molecule. It is unlikely that threonine 54 interacts directly with cytochrome P450, as can be suggested from the three-dimensional models of adrenodoxin. However, threonine 54 seems to occupy a position which connects the environment of the cluster with the surface of the protein (Fig. 6).

Furthermore, the efficiency of the cholesterol side chain deavage with the threonine 54 mutants is nearly unchanged, whereas the conversion rate of 11-deoxycorticosterone to corticosterone using these mutants is accelerated more than 2-fold as compared with the wild type (Table III), pointing to an enhanced velocity of the second electron transfer to CYP11B1. The first electron transfer to CYP11B1, but not to CYP11A1, was also slightly accelerated using mutants T54S and T54A (Table IV). A similar discrimination between CYP11A1 and CYP11B1 was already described (28). In contrast, the ability to accept and donate electrons was not significantly affected by replacement of threonine 48 (the homologous residue to threonine 54 in adrenodoxin) by either serine or alanine in Anabaena vegetative ferredoxin (52), indicating that the electron transfer rate is
differentially regulated in plant and vertebrate type ferredoxins.

In conclusion, the alcoholic residue at the position corre-
sponding to residue 54 in adrenodoxin plays an important role in determining the redox potential of [2Fe-2S] proteins. Introduction of the more hydrophobic alanine at this position in adrenodoxin markedly decreases the stability of the holoprotein, indicating participation of the hydroxyl group of residue 54 in stabilizing the cluster by hydrogen bonds. Additionally, substitution of threonine 54 by alanine leads to changes in the spectral features of the oxidized protein, but not in the reduced state, indicating a remarkable rearrangement of the cluster surrounding upon reduction of the protein. Both mutants retained the functional capabilities of adrenodoxin. The observed variations in the kinetic parameters reflect the fact that substitution of threonine 54 results in a slight rearrangement at the surface of adrenodoxin and provide further evidence for discrimination not only between the reductase and the cyto-
chromes, but also between CYP11A1 and CYP11B1. The mechanisms which control the electron transfer rates to the different cytochromes P450 and in other ferredoxin-dependent redox systems are subject to further studies.

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