The amino acid in position 49 in bovine adrenodoxin is conserved among vertebrate [2Fe-2S] ferredoxins as a hydroxy function. A corresponding residue is missing in the cluster-coordinating loop of plant-type [2Fe-2S] ferredoxins. To probe the function of Thr-49 in a vertebrate ferredoxin, replacement mutants T49A, T49S, T49L, and T49Y, and a deletion mutant, T49Δ, were generated and expressed in Escherichia coli. CD spectra of purified proteins indicate changes of the [2Fe-2S] center geometry only for mutant T49Δ, whereas NMR studies reveal no transduction of structural changes to the interaction domain. The redox potential of T49Δ (−370 mV) is lowered by −100 mV compared with wild type adrenodoxin and reaches the potential range of plant-type ferredoxins (−305 to −455 mV). Substitution mutants show moderate changes in the binding affinity to the redox partners. In contrast, the binding affinity of T49Δ to adrenodoxin reductase and cytochrome P-450 11A1 (CYP11A1) is dramatically reduced. These results led to the conclusion that Thr-49 modulates the redox potential in adrenodoxin and that the cluster-binding loop around Thr-49 represents a new interaction region with the redox partners adrenodoxin reductase and CYP11A1. In addition, variations of the apparent rate constants of all mutants for CYP11A1 reduction indicate the participation of residue 49 in the electron transfer pathway between adrenodoxin and CYP11A1.

[2Fe-2S] ferredoxins are found in all organisms from archaea and bacteria to higher plants and animals and function as mediators of electron transfer in a range of multicomponent redox systems (1). The redox active prosthetic group in this class of ferredoxins is characterized by an iron-sulfur cluster, consisting of two non-heme iron ions ligated to thiolate side chains of four cysteines of the polypeptide, bridged by two inorganic sulfide ions.

[2Fe-2S] ferredoxins are classified by structure and function into plant-type ferredoxins and vertebrate-type ferredoxins. Vertebrate-type [2Fe-2S] ferredoxins, present in oxygenase systems of bacteria and vertebrates, transfer electrons from a NAD(P)H-dependent ferredoxin reductase to different cytochrome P450 enzymes (2). In vertebrates, ferredoxins of the [2Fe-2S] protein family are present in adrenal cortex, placenta, liver, kidney, and brain (3), where they participate in cytochrome P450-catalyzed hydroxylation reactions to produce steroid hormones, vitamin D metabolites, and bile acids.

Bovine Adx as a member of vertebrate [2Fe-2S] ferredoxins, functions as electron mediator from the isalloxazin system of adrenodoxin reductase (AdR) to the heme iron of two cytochromes P450, CYP11A1 and CYP11B1, localized in the inner mitochondrial membrane of the adrenal cortex (4). CYP11A1 converts cholesterol to pregnenolone, and CYP11B1 catalyzes the 11β-hydroxylation of 11-deoxycorticosterone and 11-deoxycorticisol and the production of aldosterone.

The crystal structures of a truncated bovine Adx (5) and of a full-length Adx (6) determined at 1.85- and 2.5-Å resolution, respectively, display a compact (α+β) fold typical for [2Fe-2S] ferredoxins. The polypeptide chain is organized into a large core domain, containing the iron-sulfur cluster, and a smaller interaction domain. This small 35-amino acid comprising domain includes the acidic region between residues 72 and 79, which was shown to be responsible for the recognition of the redox partners AdR and cytochrome CYP11A1 (7). The identification of corresponding positively charged residues on the interaction partners AdR and CYP11A1 (8, 9) confirmed a recognition model mainly based on electrostatic interactions.

The ability to accept and donate electrons during interaction with redox partners is tightly connected to the redox potentials of iron-sulfur proteins. The redox potential differs widely in [2Fe-2S] proteins and is correlated to the ferredoxin type. In general, plant-type ferredoxins display lower redox potentials (between −305 and −455 mV) (10) than vertebrate-type ferredoxins (−235 to −273 mV). The protein sequence alignment of the region around the [2Fe-2S] cluster ligands (Fig. 1) between plant-type ferredoxins and vertebrate-type ferredoxins displays a difference between the two ferredoxin types in the length of the metal-binding loop proximate to the reducible iron atom. The loop in vertebrate-type ferredoxins contains five residues, and the conserved alcoholic amino acid in the middle of the loop consists of a threonine or serine (Fig. 1). In plant-type ferredoxins, the loop is one amino acid shorter and a corresponding conserved residue is missing. In Adx, a threonine is positioned in the middle of the loop between the cysteine cluster ligands Cys-46 and Cys-52. In order to understand the particular role of the amino acid Thr-49 for the functional properties of the [2Fe-2S] cluster, a series of mutants of Adx was generated in which Thr-49 was replaced by alanine (T49A), serine (T49S), leucine (T49L), and tyrosine (T49Y) or Thr-49

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1 The abbreviations used are: Adx, adrenodoxin; AdR, adrenodoxin reductase; CYP11A1, cytochrome P-450 11A1; CYP11B1, cytochrome P-450 11B1; CYP101, cytochrome P-450 cam; HPLC, high performance liquid chromatography; CD, circular dichroism; NMR, nuclear magnetic resonance; Pdx, putidaredoxin.
was deleted (T49Δ). Recombinant proteins were purified from *E. coli* and characterized in detail by biochemical and biophysical methods.

### EXPERIMENTAL PROCEDURES

#### Expression and Mutagenesis—Adx proteins were produced using the T7-expression system consisting of *E. coli* strain BL21(DE3)pLysE and vector pET3d (19). Mutations in the Adx cDNA were introduced by polymerase chain reaction according to Landt et al. (20). Oligonucleotides containing the appropriate restriction sites and mutations were synthesized by BioTez GmbH.

**Protein Purification**—Recombinant Adx and AdR were purified as described (21, 22). Protein concentration was calculated using ε_{280} = 9.8 (mm cm)^{-1} for Adx (23) and ε_{280} = 11.3 (mm cm)^{-1} for AdR (24). Isolation of CYP11A1 and CYP11B1 from bovine adrenal glands was performed according to Akhrem et al. (25) with slight modifications.

**Spectroscopic Methods**—Absorption spectra in the UV-visible region were recorded at room temperature on a Shimadzu double-beam spectrophotometer UV1010PC.

Samples for NMR spectroscopy (5 mg each) of Adx and T49Δ in 10 mM potassium phosphate buffer were dried at room temperature under vacuum and dissolved in 0.5 ml of D_2O. The pH was adjusted to 8.2 with a 50 mM potassium phosphate buffer. Both samples were left at 4 °C for approximately 72 h to exchange the amide protons for deuterium. 1H NMR spectra were recorded on a Bruker DRX 500 NMR spectrometer at 299 K. The residual water signal was suppressed with low power irradiation.

CD spectra were recorded as described previously (26) on a Jasco 715 spectropolarimeter. Temperature-dependent measurements were carried out at a heating rate of 50 °C/h from 20 to 65 °C with a temperature increment of 0.2 °C, monitoring the decrease of the circular dichroism signal at 440 nm.

### RESULTS

**Expression of Mutants**—To confirm that oligonucleotide-directed mutagenesis was limited to the predicted sites, Adx cDNA inserts in the expression vector pET3d were sequenced using the cycle sequencing method. All Adx proteins were expressed as holoproteins in the cytoplasm of *E. coli* strain BL21(DE3)pLysE, SDS-PAGE and Western blotting revealed specific bands at 14 kDa for all proteins. The expression yields of mutants T49A, T49S, T49L, and T49Y were each in the range of wild type adrenodoxin (50–100 mg/100 ml of *E. coli* culture). Yields of mutant T49Δ were reduced to one third of the wild type level. The final purity index (A_{414}/A_{270}) of the proteins was always higher than 0.9.

**CD Spectroscopy**—To measure the effect of mutations on the structure of the iron-sulfur cluster region CD spectroscopy was applied, a method that sensitively detects conformational changes in optically active substances like iron-sulfur cluster containing proteins. No significant changes were observed either in the near UV or visible region for the substitution mutants (Fig. 2). In contrast, deletion mutant T49Δ showed in the indicated regions shifted CD signals and in part lower amplitudes of the peaks compared with the wild type. Deletion of Thr-49 shifted the local maxima at 440 and 342 nm to 428 and 348 nm, respectively. In addition, a new shoulder appeared at 455 nm in the T49Δ spectrum. The signal changes in the range of 310–650 nm clearly reflect a rearrangement in the proximity of the iron-sulfur cluster of this mutant.

**NMR Spectroscopy**—To study the mutational effect of the deletion of Thr-49 on the aromatic region of T49Δ, 1H NMR spectra of T49Δ and Adx were recorded under the same conditions and compared with each other (Fig. 3). Both spectra show the same chemical shifts for the various aromatic residues (assignment according to Beckert et al. (Ref. 34)), indicating that the three-dimensional global structure of the protein was maintained after deletion of the amino acid Thr-49.

**Thermal Denaturation**—To analyze the effect of mutations on the protein stability, thermal unfolding of Adx was performed. A buffer system that prevents the destruction of the [2Fe-2S] cluster during heating (35) allows unfolded proteins to
regain the original absorption spectrum after renaturation (36). Wild-type Adx and all substitution mutants renatured after a 12-h incubation at 4 °C in the sulfide-containing buffer. In contrast, mutant T49A denatured irreversibly.

The CD signal of melting curves of all analyzed proteins recorded at 440 nm decreased in a sigmoidal manner (Fig. 4), indicating a significant role of this position in modulating the redox potential.

**Thermodynamic parameters of unfolding for wild type Adx and Thr-49 mutants**

Thermal unfolding parameters were derived from heat denaturation curves measured by CD spectroscopy at 440 nm. Denaturation was performed at a heating rate of 50 °C/h between 20 and 65 °C in a glycine buffer system (pH 8.5) containing 2-mercaptoethanol, Na2S, and ascorbic acid. Standard deviations were calculated for each protein from three independent determinations.

| Protein   | Td   | \(\Delta H T_d^a\) | \(\Delta T_d\) | \(\Delta (\Delta H T_d^a)\) |
|-----------|------|--------------------|----------------|---------------------------|
| Wild type | 49.3 | 367 ± 17           | 0              | 0                         |
| T49Δ      | 36.0 | 212 ± 1.2          | −13.3          | 2                         |
| T49A      | 50.2 | 391 ± 2            | +0.9           | +24                       |
| T49S      | 48.8 | 369 ± 3            | −0.5           | −9                        |
| T49L      | 49.4 | 358 ± 10           | +0.1           | −9                        |
| T49Y      | 46.8 | 353 ± 8            | −2.5           | −14                       |

* Enthalpy parameters for mutant T49Δ are not presented because its unfolding is not reversible.

**Affinity to the Redox Partner AdR**—In order to determine the extent to which the mutations affect the interaction with AdR, an assay involving cytochrome c as an artificial electron acceptor was used. Under the conditions employed, cytochrome c is in large excess, and the derived \(K_m\) values are essentially equivalent to dissociation constants (\(K_m\)) of the AdR-Adx complex (39). Table III shows that the affinity of the mutants T49A, T49S, and T49L to AdR is in the same range as the wild type. Replacement of Thr-49 with tyrosine resulted in a 3-fold in-
Role of Thr-49 in Bovine Adrenodoxin

FIG. 5. Correlation of the denaturation enthalpy of Adx mutants with the size of the amino acid in position 49. The calculated thermal denaturation enthalpy \( \Delta H(T_d) \) for wild type (wt) Adx and mutants in position 49 is shown. The inset presents the dependence of the \( \Delta H(T_d) \) values on the side chain volume of the amino acid present in position 49 (correlation coefficient 0.7). Side chain volumes of amino acids were taken from Zamyatin (37).

TABLE II
Redox potentials of Adx and Thr-49 mutants

| Protein | Redox potential* \( mV \) |
|---------|--------------------------|
| Wild type | -276 |
| T49A | -370 |
| T49A | -278 |
| T49S | -276 |
| T49L | -270 |
| T49Y | -271 |

* Standard deviation for T49A is ±20 mV, for all other proteins ±5 mV.

crease in complex formation, whereas the deletion of Thr-49 caused a 120-fold increase of the \( K_d \) value.

Affinity to the Redox Partner CYP11A1—Binding of the oxidized form of adrenodoxin to its electron acceptor CYP11A1 is comparable to that of the reduced form (40), thus allowing to analyze the affinities of wild type and mutant adrenodoxin to CYP11A1 by optical difference spectroscopy. Compared with wild type Adx, mutants T49S, T49L, and T49Y exhibit \( K_d \) values in the same range, mutant T49A shows a nearly 8-fold increased \( K_d \) value, and the \( K_d \) value of mutant T49A was not detectable (Table III).

Reconstitution of Enzymatic CYP11A1 Activity—The effect of mutations in position 49 on the enzymatic activity of the native electron acceptor CYP11A1 was investigated by HPLC analysis of the substrate conversion of cholesterol into pregnenolone (Table III). The values for maximal substrate conversion, \( V_{max} \), are quite similar for the wild type and the mutants T49A, T49S, and T49Y. The \( V_{max} \) value for mutant T49L is decreased by 30%. A substrate conversion with the deletion mutant T49D was not detectable. The \( K_m \) values for the substrate conversion are similar for the wild type and mutant T49S and slightly increased for mutants T49A, T49L, and T49Y, whereas no \( K_m \) for the reaction with the mutant T49A could be calculated.

Reduction of CYP11A1-Substrate Complex by Adx—To investigate the effect of mutations at position 49 on the electron transfer rate to CYP11A1, stopped-flow experiments in oxygen-free atmosphere were performed. In the experiments adrenodoxin, preincubated with AdR and NADPH in the first test tube, was rapidly mixed with oxidized CYP11A1 present in the second test tube. Under these conditions the rate constants of electron transfer from the reduced Adx to the oxidized CYP11A1 are fully Adx-dependent since the flavin to iron-sulfur electron transfer is not rate-limiting. The apparent rate constant, \( k_{app} \), measured for wild type Adx is 0.098 s\(^{-1}\) (Table IV). The value for mutant T49S is slightly increased (0.140 s\(^{-1}\)), whereas the value for mutant T49A is slightly decreased (0.063 s\(^{-1}\)). The rate constants for mutants T49L and T49Y are 3- and 6-fold reduced, respectively. Substitution of the threonine in position 49 by a tyrosine resulted in a \( k_{app} \) value increased by a factor of 3.

Calculation of the Electron Coupling Map of Adx—A global coupling map of the Adx molecule was calculated with the program Pathway (version 0.97) using Fe-1 of the [2Fe-2S] cluster as donor and all other atoms as acceptors. The coupling map was displayed on the surface of the three-dimensional structure of the truncated mutant of adrenodoxin, consisting of amino acids 4–108, with the program InsightII (Fig. 6). A small region with high coupling values can be identified on the surface of the molecule. All amino acids with high coupling are located in the loop covering the iron-sulfur center comprising the residues between position 47 and 54. The highest coupling values were calculated with the program Harlem for the residues Thr-54 (6.4 × 10\(^3\)), Thr-49 (5.3 × 10\(^3\)), Leu-50 (4.0 × 10\(^3\)), and Glu-47 (3.7 × 10\(^3\)), indicating a high probability that these amino acids are involved in electron transfer from the redox active iron-sulfur cluster to the Adx surface.

DISCUSSION

The importance of the threonine residue in position 49 in bovine Adx for its function as electron mediator has been investigated using site-directed mutagenesis and detailed analysis of the obtained mutants. Special attention has been attributed to structural and stability properties, to the redox potential, to the electron transfer function, and to the influence on binding affinities to the redox partners.

In the mature Adx, Thr-49 is located in a 5-residue-containing loop between the [2Fe-2S] cluster coordinating cysteines Cys-46 and Cys-52. This amino acid is conserved in [2Fe-2S] ferredoxins of the vertebrate-type as threonine or serine. In plant-type ferredoxins a corresponding residue is missing, and
Role of Thr-49 in Bovine Adrenodoxin

Interaction of wild type Adx and mutants with AdR was assayed following the reduction of cytochrome c at 550 nm. Enzymatic activities of wild type Adx and mutants in CYP11A1-dependent substrate conversion were studied by HPLC analysis of hydroxylation product formation. Binding of oxidized Adx to oxidized CYP11A1 was followed spectrophotometrically by the high spin shift of the P450 heme iron in the Soret region, caused by Adx induced cholesterol binding.

TABLE III

| Protein | 
|---------|
| **Cytochrome c reduction assay** | 
| 
| | 
| **K_m** | **V_max** |
| Wild type | 0.31 ± 0.03 | 259 ± 3 |
| T49Δ | 38.6 ± 14.4 | 263 ± 43 |
| T49A | 0.37 ± 0.02 | 309 ± 5 |
| T49S | 0.29 ± 0.2 | 333 ± 9 |
| T49L | 0.2 ± 0.035 | 250 ± 5 |
| T49Y | 0.095 ± 0.005 | 336 ± 28 |

**Cholesterol side chain cleavage**

| Protein | 
|---------|
| 
| | 
| **K_m** | **V_max** |
| Wild type | 0.67 ± 0.2 | 0.53 ± 0.05 |
| T49Δ | n.d.* | n.d. |
| T49A | 1.34 ± 0.3 | 0.61 ± 0.05 |
| T49S | 0.31 ± 0.2 | 0.57 ± 0.05 |
| T49L | 1.57 ± 0.4 | 0.37 ± 0.03 |
| T49Y | 0.98 ± 0.2 | 0.58 ± 0.05 |

**Spectral binding**

| Protein | 
|---------|
| 
| | 
| **K_m** |
| Wild type | 24 ± 1 |
| T49Δ | 197 ± 9 |
| T49A | 66 ± 7 |
| T49S | 51 ± 2 |
| T49L | 24 ± 3 |

*Expressed by nanomoles of cytochrome c reduced/min.
†Expressed by nanomoles of pregnenololone produced/min/nmol of CYP11A1.

**TABLE IV**

Reduction rate of CYP11A1

The velocity of formation of the CYP11A1-CO complex upon transfer of the first electron by wild type Adx or mutant proteins was measured in a stopped flow assay. The apparent rate constants and standard deviations were calculated from six determinations.

| Protein | **k_{app}** | 
|---------|
| | g⁻¹ s⁻¹ |
| Wild type | 0.098 ± 0.005 |
| T49Δ | 0.016 ± 0.0008 |
| T49A | 0.063 ± 0.003 |
| T49S | 0.140 ± 0.007 |
| T49L | 0.050 ± 0.002 |
| T49Y | 0.346 ± 0.020 |

**FIG. 6.** Electronic coupling between the iron-sulfur cluster and the surface atoms in the Adx molecule. A global electron coupling map was calculated with the program Pathway (version 0.97). The Fe-1 of the [2Fe-2S] cluster was set as electron donor, and all other atoms and bonds functional as electron acceptors. The calculated data were transferred to the protein structure of Adx and visualized with the program InsightII (MSI Inc.). Red indicates a high coupling rate; blue represents a low coupling rate. The amino acid Thr-49 is indicated on the surface in white.

The iron-sulfur cluster loop contains only 4 residues.

To determine the influence of amino acid substitutions or a deletion in position 49 on the structural properties of the protein, CD spectra have been recorded. As concluded from unchanged spectra, the introduction of alanine, serine, leucine, or tyrosine instead of threonine did not lead to changes in the environment of the iron-sulfur cluster. This observation is in agreement with computer modeling studies, which indicated that the introduction of the respective amino acids does not cause any steric hindrance in its surrounding (data not shown). Differences in the spectroscopic properties of wild type Adx and mutant T49Δ were discernible in the CD spectra of the proteins. Significant changes in both the near-UV and visible region sensitively reflect conformational changes in the surrounding of the optically active iron-sulfur cluster of mutant T49Δ. The CD spectrum of this mutant shows shifted CD signals, in part lower amplitudes of the peaks compared with the wild type, and the appearance of a new shoulder at 455 nm in the region of the largest CD maximum. Thus, shortening of the cluster loop by only one amino acid causes rearrangements in the active center, whereas the CD signals of substitution mutants seem not to be sensitive to a larger or smaller side chain volume of the amino acid in position 49.

For further analysis of the extent of the apparent structural changes of mutant T49Δ, 1H NMR spectroscopy was applied. The 1H assignments of residues His-10, His-56, His-62, and Tyr-82 have been confirmed previously (34) and display the same chemical shift in wild type Adx and in the mutant T49Δ. Residue Tyr-82 is part of the interaction domain, and residue His-56 forming hydrogen bonds to Tyr-82 and Ser-88 connects the core domain with the interaction domain and transmits conformational changes upon reduction of Adx. Since NMR signals of both residues indicate the same chemical environment in the mutant and in the wild type, a transduction of conformational changes in the cluster region of mutant T49Δ to the interaction domain can be excluded. The deletion, therefore, has no obvious effect influencing the structure around the residues on the acidic domain described as critical for the interactions studied.

In the tertiary structure of Adx, the iron-sulfur redox center is located close to the protein surface, where Thr-49 is positioned at the boundary separating the redox center from the aqueous phase (Fig. 7). Several potential hydrogen bonds around the iron sulfur cluster have been identified (5). In the immediate vicinity of Thr-49, the backbone amide groups of Glu-47 (E47N to S1), Gly-48 (G48N to C46S), and Ala-51 (A51N to C46S) may form a hydrogen bond to the sulfur atom of Cys-46 of the iron-sulfur cluster. The importance of these hydrogen bonds was directly studied by thermal unfolding of the proteins in a CD spectropolarimeter.
The nearly unchanged conformational stability of the iron-sulfur cluster in substitution mutants compared with wild type Adx is in agreement with the suggestion that the hydrogen bond network in the neighborhood of position 49 was not affected by the mutations. In contrast, the reduced $T_m$ value for the deletion mutant indicates that the stability of the iron-sulfur cluster vicinity is dramatically affected by lost or by weaker hydrogen bonds. This finding is supported by the irreversibility of the unfolding of mutant T49A, which is in contrast to reversible folding properties of the other Adx mutant proteins.

The deletion of the threonine in position 49 and the accompanying distortion of the hydrogen bond network results in a dramatic redox potential drop from $-276$ mV (wild type) to $-370$ mV (T49Δ). In contrast, all substitution mutants display an unchanged midpoint redox potential. The results of redox potential determinations suggest that the amino acid in position 49 stabilizes the hydrogen bond network and tunes the microenvironment of the iron-sulfur cluster and therefore the redox potential in bovine Adx. An effect of hydrogen-bonding interactions between [2Fe-2S] cluster ligand atoms and side chain or main chain donor atoms on the oxidation-reduction potentials of Adx (41) and Anabaena ferredoxin (42) has been observed previously. The redox potentials of bovine Adx mutants T54A and T54S are reduced by 55 and 34 mV, respectively. In this case, a hydrogen bond from the side chain hydroxyl group of Thr-49 to the sulfur atom of Cys-52 is affected in the mutant proteins and results in structural changes in the vicinity of the iron-sulfur cluster. In Anabaena ferredoxin mutant A45S, the presence of an additional hydrogen bond in the metal-binding loop from the side chain hydroxyl group of Ser-45 to the sulfur atom of Cys-41 seems to be responsible for the higher midpoint potential of mutant ferredoxin ($-382$ mV) relative to the wild type ($-406$ mV). The redox potential for the Adx deletion mutant T49Δ ($-370$ mV) reaches the potential range of plant-type ferredoxins ($-305$ to $-455$ mV), which is in general lower as in vertebrate-type ferredoxins. Since the deletion in mutant T49Δ results in a 4-residue-containing [2Fe-2S] cluster loop as present in plant-type ferredoxins, similar structures may be formed, which are a prerequisite to determine the redox potential in this range. Further crystallographic studies solving the detailed structure of the [2Fe-2S] center vicinity of the mutant T49Δ will present more insight into the general field of redox potential determination in ferredoxins.

The affinity of Adx to the redox partners CYP11A1 and AdR is particularly affected by mutations in position 49. The most pronounced effects can be observed for the deletion mutant T49Δ, which shows a 120-fold decrease in binding affinity to AdR and a drop to zero in the binding affinity to CYP11A1. Smaller effects were measured after replacement of Thr-49 with tyrosine or alanine. Mutant T49Y shows a 3-fold increase in complex formation with AdR, and mutant T49A shows an 8-fold decrease in complex formation with CYP11A1. Measurement of the binding constants of the other mutants to AdR and CYP11A1 revealed only slight changes or similar results compared with the wild type. Since binding constants for the substitution mutants are only changed for the largest and smallest introduced residues (T49A, T49Y), the surface contact via Thr-49 seems to be optimized for interaction with different redox partners. Dramatically changed binding properties of the deletion mutant, caused by structural rearrangements in the cluster region, suggest a crucial role of Thr-49 for direct interaction with binding partners or for the formation of an interacting surface represented by the [2Fe-2S] cluster loop. Corresponding to Thr-49 in Adx, in the bacterial ferredoxin putidaredoxin (Pdx) residue Ser-44 was substituted in a mutagenesis study (43). The authors suggested that substitution of Ser-44 with the smaller amino acid, glycine, increases solvent accessibility to the cluster and simultaneously induces distortion around the ferrous iron as indicated by shifting the electron spin resonance signal to a higher magnetic field. This mutation causes the same spectral changes of Pdx as the formation of the ternary complex Pdx-CYP101-CO. In the complex the change in the CYP101 active site upon CO binding is transmitted to Pdx within the complex and produces a conformational change of the iron-sulfur active center. Ser-44 in Pdx, homologous to Thr-49 in Adx, is part of a surface loop around the [2Fe-2S] center including the residues Asp-34, Asp-38, and Ser-42 involved in redox partner binding (44). Both proteins, the vertebrate ferredoxin Adx and the bacterial ferredoxin Pdx, comprise therefore a corresponding recognition domain involved in protein-protein interaction during the general function of electron transfer.

The efficiency of the cholesterol side chain cleavage is not significantly changed with Thr-49 substitution mutants. However, no CYP11A1-dependent enzymatic activity can be detected in reactions with the deletion mutant T49Δ, which is in agreement with the finding that no Adx T49Δ-CYP11A1 complex formation is detectable under the applied conditions. Stopped-flow analysis of the first electron transfer from T49Δ to CYP11A1 revealed a 6-fold reduced electron flux and indicates a very low binding affinity, which might be under the detection limit of optical titration experiments, which were used for estimation of the binding constant. Furthermore, the results of stopped-flow measurements support the one hand a reduced electron transfer to CYP11A1 for mutants that contain no alcoholic residue in position 49, and on the other hand an 3-fold increased electron transfer after substitution of threonine by the aromatic tyrosine residue. Interestingly, although the redox potential of mutant T49Y and the binding constant for CYP11A1 are not changed, the introduced tyrosine residue seems to form a preferred route for the electron transfer between Adx and CYP11A1, which is in good agreement with previous observations that aromatic residues enhance electron transfer in proteins (45). The results of these experiments indicate that residue 49 is involved in the electron transfer pathways from Adx to CYP11A1. This indication is in accordance with the calculated electron coupling between the [2Fe-2S] cluster and the surface of Adx, which pointed out a hot spot in the [2Fe-2S] cluster loop containing residue 49. The experimental and theoretical evidences obtained by stopped-flow experiments indicate that the redox partner interaction with this protein is particularly affected by mutations in position 49.
measurements and electron transfer calculations imply that residue 49 is positioned at the boundary separating the [2Fe-2S] redox center from the surface of the interacting redox partner protein during complex formation. The nature of the influence of Thr-49 in this position on the complex formation and electron transfer, which can be a direct interaction with binding partners or a structure-forming function in the [2Fe-2S] cluster loop, remains unclear and is subject to further studies.

Taken together, residue Thr-49 of bovine Adx plays an important role in the stabilization of the hydrogen bond network of the [2Fe-2S] center. Deletion of this residue markedly decreases the redox potential of the protein indicating that this residue determines the redox potential in Adx. Structural re-arrangements of the cluster vicinity in this mutant also led to decreased stability and dramatically reduced binding affinities of the redox partner proteins AdR and CYP11A1. Introduction of the smaller alanine and the larger tyrosine in this position of the redox partner protein during complex formation. The nature of the redox partner protein during complex formation and electron transfer, which can be a direct interaction with binding partners or a structure-forming function in the [2Fe-2S] cluster loop, remains unclear and is subject to further studies.

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