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Kinetic and optical biosensor study of adrenodoxin mutant AdxS112W displaying an enhanced interaction towards the cholesterol side chain cleavage enzyme (CYP11A1)

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Abstract In mammals, steroid hormones are synthesized from cholesterol that is metabolized by the mitochondrial CYP11A1 system leading to pregnenolone. The reduction equivalents for this reaction are provided by NADPH, via a small electron transfer chain, consisting of adrenodoxin reductase (AdR) and adrenodoxin (Adx). The reaction partners are involved in a series of transient interactions to realize the electron transfer from NADPH to CYP11A1. Here, we compared the ionic strength effect on the AdR/Adx and Adx/CYP11A1 interactions for wild-type Adx and mutant AdxS112W. Using surface plasmon resonance measurements, stopped flow kinetic investigations and analyses of the product formation, we were able to obtain new insights into the mechanism of these interactions. The replacement of serine 112 by tryptophan was demonstrated to lead to a dramatically decreased k_{off} rate of the Adx/CYP11A1 complex, resulting in a four-fold decreased K_d value and indicating a much higher stability of the complex involving the mutant. Stopped flow analysis at various ionic strengths and in different mixing modes revealed that the binding of reduced Adx to CYP11A1 seems to display the limiting step for electron transfer to CYP11A1 with pre-reduced AdxS112W being much more efficient than wild-type Adx. Finally, the dramatic increase in pregnenolone formation at higher ionic strength using the mutant demonstrates that the interaction of CYP11A1 with Adx is the rate-limiting step in substrate conversion and that hydrophobic interactions may considerably improve this interaction and the efficiency of product formation. The data are discussed using published structural data of the complexes.

Keywords Adrenodoxin · Adrenodoxin reductase · CYP11A1 · Stopped flow · Biacore · Mitochondrial steroid hydroxylase system

Abbreviations

AdR	Recombinant wild-type adrenodoxin reductase
Adx	Recombinant wild-type adrenodoxin
CYP	Cytochrome P450
CYP11A1	Cytochrom
P450scc	Side chain cleavage enzyme from bovine adrenals
NADPH	Nicotinamide adenine dinucleotide phosphate
FAD	Flavine adenine dinucleotide
SPR	Surface plasmon resonance

Introduction

Mitochondrial cytochrome P450 systems are involved in the biosynthesis of steroid hormones. CYP11A1 catalyzes the rate-limiting step in the biogenesis of steroid hormones in adrenal mitochondria (Bernhardt 1996; Miller 1995; Bernhardt and Waterman 2007). In this reaction, the side chain of the substrate cholesterol is cleaved off to yield pregnenolone and isocaproic aldehyde at the expense of three molecules of NADPH and three molecules of oxygen (Shikita and Hall 1974). Another mitochondrial cytochrome P450, CYP11B1, converts 11-deoxycortisol to cortisol, the most important glucocorticoid in humans, and CYP11B2 catalyzes the biosynthesis of aldosterone, the most important mineralocorticoid. The reduction equivalents

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necessary for oxygen activation and substrate hydroxylation in all the three mitochondrial P450 systems are carried via a small electron transfer chain containing two additional redox-active proteins (Bernhardt and Waterman 2007; Hannemann et al. 2007). These reduction equivalents, provided by NADPH, are transferred to a FAD-containing adrenodoxin reductase (AdR) that transfers electrons to the one electron carrier adrenodoxin (Adx), the ferredoxin of the adrenal gland (Grinberg et al. 2000; Ewen et al. 2010). The [2Fe2S] cluster-containing Adx subsequently transfers electrons, one at a time, to the heme iron of CYP11A1, CYP11B1 and CYP11B2. Thus, Adx plays a central role in these important steps of steroid biosynthesis by participation in various transient interaction between the redox partners (Keizers et al. 2010).

During the last 2 decades, much work has been done to obtain a better understanding of the mechanism and kinetics of this system (Schiffler et al. 2001; Schiffler and Bernhardt 2003; Uhlmann et al. 1994; Beckert and Bernhardt 1997; Muller et al. 2003; Archakov and Ivanov 2011). Electrostatic interactions were shown to play a prominent role in this process (Brandt and Vickery 1993; Vickery 1997; Schiffler et al. 2004). In addition, tyrosine 82 (Beckert and Bernhardt 1997) and the loop region of Adx between amino acids 47 and 51 (Hannemann et al. 2001; Zöllner et al. 2002) were shown to strongly affect protein interactions in mitochondrial P450 systems. Nevertheless, the nature of the rate limiting step in this system is still a matter of controversy and remains to be elucidated. Using rapid mixing techniques we were able to follow the reduction of all three protein components of the CYP11A1 system within one measurement and determined the apparent rate constants for the reduction of AdR by NADPH, of Adx by reduced AdR and of CYP11A1 by reduced Adx as shown before (Schiffler et al. 2004). Moreover, variation of the ionic strength revealed a clear difference in the redox behavior of Adx towards its redox partners. To get a deeper insight into the mechanism of the Adx redox partner interactions, especially the role of electrostatic contributions, in this article we investigated the function of an Adx mutant lacking the C-terminal amino acids 113–128 and bearing a replacement of the amino acid in position 112 from serine to tryptophane, thus bringing a highly hydrophobic amino acid into the C-terminal region, which is crucial for the redox partner interactions (Uhlmann et al. 1994). Tryptophan was chosen, since it comprises the corresponding amino acid in the homologous position of the bacterial ferredoxin, putidaredoxin, which is a more efficient electron transfer protein than Adx. It was shown that this replacement led to an increased efficiency of Adx towards CYP11A1-dependent cholesterol conversion (Schiffler et al. 2001; Schiffler and Bernhardt 2003).

Materials and methods

Biochemicals and reagents

All chemicals used were of the highest purity commercially available.

Bacterial strains and plasmids

The protease-deficient *Escherichia coli* strain BL21DE3 was used as host strain for the heterologous expression of AdR and Adx. The Adx cDNA was a generous gift of Dr. Waterman, Nashville, TN. The Adx cDNA-containing plasmid was pKKHC (Porter and Larson 1991). The plasmid containing the coding sequence for AdR was kindly provided by Dr. Sagara (Sagara et al. 1987).

Protein expression and purification

Bacteria were grown as previously reported (Uhlmann et al. 1992) with slight modifications as described in (Schiffler et al. 2004). Recombinant Adx was purified after sonification as described, and the final concentration of Adx was determined using $\varepsilon_{414} = 9.8\ \mathrm{mM^{-1}\ cm^{-1}}$ (Huang and Kimura 1973). The puritiy of the Adx preparation was estimated by determining the relative absorbance of the protein at 414 and 273 nm, Q-value (A_{414}/A_{273}).

AdR was heterologously expressed and purified as described elsewhere (Sagara et al. 1993). The molar extinction coefficient used for concentration estimation was $\varepsilon_{450} = 10.9\ \mathrm{mM^{-1}\ cm^{-1}}$ (Chu and Kimura 1973). CYP11A1 was isolated from bovine adrenals, and the concentration was estimated by carbon monoxide difference spectra assuming $\varepsilon_{450-490} = 91\ \mathrm{mM^{-1}\ cm^{-1}}$ according to (Omura and Sato 1964).

Stopped flow measurements

Rapid mixing experiments were carried out with a single channel stopped-flow spectrophotometer SX-17MV equipped with PEEK tubings (Applied Photophysics) at 15°C. The system was made anaerobic as described previously (Schiffler et al. 2001, 2004). The reaction buffer consisted of 50 mM HEPES (pH 7.3), varying amounts of potassium chloride and 0.05% of Tween 20. The pH of the buffer was checked after salt addition and remained constant. Two different modes of mixing were used as specified below.

To follow the whole cascade of reactions within one measurement (i.e., reduction of AdR, Adx and CYP11A1) syringe A contained only NADPH (2 mM) and syringe B contained three proteins in the following concentrations, AdR (4 μM), Adx (8 μM) and CYP11A1 (4 μM). Upon

reduction, all three proteins showed marked changes in their absorbance at 450 nm.

To follow specifically the reduction of CYP11A1, syringe A contained CYP11A1 (4 μM), while syringe B contained NADPH (400 μM), AdR (4 μM) and Adx (8 μM). The mixture in syringe B was allowed to age for 10 min to assure a complete reduction of the Adx. The solutions in the two syringes were CO-saturated prior to loading into the driving syringes. Formation of the reduced cytochrome was monitored by the respective CO complex, giving rise to an absorbance maximum at 450 nm by recording 4,000 data points with a split time base of 10/200 s. The dead time for the measurements was 1.3 ms with the SX-17MV. Three to seven reaction traces were overlaid, and mono- or biexponential functions were fitted to the averaged curves in order to determine apparent rate constants for reduction of AdR by NADPH, of Adx by AdR and of CYP11A1 by Adx.

Optical biosensor measurements

Analysis of the binding of oxidized Adx or AdxS112W to AdR_{ox} or to $CYP11A1_{ox}$ was performed by using a Biacore2000 system. Immobilization of Adx to the carboxy methylated dextran matrix of a CM5 sensor chip was carried out as described previously (Zöllner et al. 2002; Schiffler et al. 2004). In order to activate the carboxy groups on the sensor chip, a solution containing a 0.2 M *N*-ethyl-*N*′-dimethylaminopropyl-carbodiimide (EDC) and 0.05 M *N*-hydroxysuccinimide (NHS) was injected onto the chip with a flow rate of 5 μl/min. Afterwards free amino groups of each Adx species were used to covalently couple approximately 200 RU (resonance units) of the respective protein to the sensor chip matrix. The coupling procedure was completed by injecting 50 μl of a 1 M ethanolamine hydrochloride solution in order to block any remaining free ester groups. The binding analysis was carried out by injecting different AdR_{ox} or $CYP11A1_{ox}$ concentrations in the range of 10 to 1,000 nM with a flow rate of 10 μl/min and recording the refractive index changes. All protein-containing solutions were prepared in Biacore HBS-EP buffer (0.01 M HEPES buffer pH 7.4, 0.15 M NaCl with 0.005% Surfactant P20). Afterwards still bound AdR or CYP11A1 was removed by injecting 5 μl of a 1 mM NaOH solution. The regeneration performance was evaluated by analyzing the baseline response after up to 30 binding cycles with 1 μM AdR and 1 μM CYP11A1, respectively.

Analysis of the curves and the determination of K_d values were achieved by using the BIAcore evaluation software 3.1.

CYP11A1-dependent substrate conversion assay

CYP11A1-dependent conversion of cholesterol to pregnenolone under varying salt concentrations was performed as described (Sugano et al. 1989) with slight modifications (Schiffler et al. 2004). The cholesterol side chain cleavage activity was assayed for 10 min at 37°C in a reconstituted system. The mixture contained 0.5 μM CYP11A1, varying amounts of Adx (from 0.2 to 3 μM), 0.5 μM AdR and 100 μM cholesterol. The reaction was started by adding NADPH. The buffer we used for the substrate conversion assay was the same as described for the stopped flow assays but, in addition, contained an NADPH regenerating system. This regenerating system consisted of glucose-6-phosphate dehydrogenase, glucose-6-phosphate and magnesium chloride. The reaction was stopped by boiling the samples. After cooling down to 37°C, cholesterol oxidase was added to convert the steroids to the respective 3-on-4-ene form (i.e., progesterone and cholestenone). The reaction was carried out for 10 min and stopped by adding chloroform to extract the steroids. The samples were analyzed by reversed phase HPLC on a JASCO system using a C-18 column (3.9 × 150 mm, waters) heated in a peltier oven to 40°C. The mobile phase used for separation was a mixture of acetonitrile:isopropanol (30:1).

Results and discussion

Biacore measurements

Using surface plasmon resonance we followed the binding of Adx and AdxS112W to AdR and CYP11A1 in the oxidized state using a Biacore 2000 device. The data given in Table 1 show significant differences in the interaction of Adx and AdxS112W with AdR and CYP11A1, respectively. As shown in Table 1, binding of Adx as well as AdxS112W to AdR and CYP11A1 is characterized by a fast *on*-rate, whereby binding to CYP11A1 displays a much higher *on*-rate than binding to AdR. This is in accordance with previous data (Schiffler et al. 2004; Berwanger et al. 2010). In contrast to the *on*-rates, the *off*-rates for all complexes studied are in the same order of magnitude and do not differ so much. The biggest difference in the *off*-rates observed is that between the AdxWT/CYP11A1 complex and the AdxS112W/CYP11A1 complex with a factor of 3.4. This indicates that the AdxS112W mutant causes a significantly increased stability of the complex. This is also reflected in the K_d values demonstrating that the complex between AdxS112W and CYP11A1 is about four-fold stronger than the respective complex with wild-type Adx and 177-fold more stable than the complex between AdxS112W and AdR. The dissociation constant for the complex between human ferredoxin reductase and ferredoxin was determined by Brandt and Vickery, using the cytochrom c method (Brandt and Vickery 1993), to be in the range of 20–100 nM, which is in very good agreement

Table 1 Binding parameters for the different reaction partners of the bovine CYP11A1 system determined from measurements with a Biacore 2000 system as described in "Materials and methods"

Protein:protein-interaction	k_{on} [M^{-1} s^{-1}]	k_{off} [s^{-1}]	K_d (k_{off}/k_{on}) [M]	rel. K_d
Adx_WT/AdR	4,434	0.0038	8.56E−07	1
Adx_S112W/AdR	5,105	0.00307	6.01E−07	0.7
Adx_WT/CYP11A1	691,000	0.00926	1.34E−08	1
Adx_S112W/CYP11A1	798,000	0.00271	3.39E−09	0.25

The standard error is ±5% of the displayed values

with our surface plasmon resonance data for the binding of oxidized AdR to Adx provided in this study. The slightly higher stability of the AdxS112W/AdR complex compared with the AdxWT/AdR complex might be due to additional hydrophobic contacts. According to the crystal structure of the complex between AdR and Adx (Muller et al. 2001), there might be an additional interaction site between the tryptophan in position 112 of AdxS112W and phenylalanine in position 348 of AdR. This could lead to a stacking effect that enhances the binding between AdR and Adx.

Taken together, the SPR experiments clearly demonstrate that mutant AdxS112W causes a slightly higher stability of the Adx/AdR complex and a four-fold higher stability of the Adx/CYP11A1 complex.

Stopped flow kinetic investigations

The reduction of the protein components of the CYP11A1 system was studied performing stopped-flow measurements. In these measurements NADPH was mixed with the oxidized protein components, and the reaction was followed at 450 nm as described in "Materials and methods". In this mixing mode, we observed three phases, and each of them was best described by a mono-exponential equation as described before (Schiffler et al. 2004). An example of such a reaction trace is shown in Fig. 1. The three phases can easily be attributed to the sequential reduction of all three protein components of the CYP11A1 redox chain. The first two phases (i.e., the reduction of AdR and Adx, respectively) are missing when using pre-reduced Adx. Mathematical analysis of the three phases allowed the determination of the reduction rates for AdR, AdxS112W and CYP11A1 at different ionic strengths from one and the same measurement.

The first phase observed in these measurements is the reduction of AdR by NADPH. The apparent rate constants for reduction of AdR at different salt concentrations derived from these measurements are in the range of 30–50 s^{-1} as previously shown (Schiffler et al. 2004). Although the rate constant was not expected to be dependent on the Adx species used, the constants differ slightly in their salt-dependence between AdxWT and AdxS112W (data not shown). These differences in salt sensitivity can be explained by a direct influence of the ferredoxin on the reduction of the reductase by NADPH, maybe via conformational changes.

In the second phase observed upon mixing the three oxidized proteins with NADPH, the reduction of Adx by AdR can be followed. As can be seen in Fig. 2, the difference between the wild-type Adx and the mutant S112W is modest, and a salt sensitivity can only be detected in the range between 0 and 100 mM KCl. The optimum of salt is in the range of 100 mM potassium chloride in the buffer corresponding to the physiological concentration of potassium ions in a mammalian cell. The slight change in the sensitivity to salt using mutant AdxS112W might be due to the additional hydrophobic stabilization of this interaction via S112W and Phe348 of AdR as discussed before. Since the *on*-rate for the complex formation between Adx or AdxS112W and AdR is 4,434 and 5,105 M^{-1} s^{-1} (Table 1), suggesting a fast binding event, the rather slow reduction of Adx should be due to either slow electron transfer from flavin to the iron-sulfur cluster or to some type of gating/rearrangement of the ferredoxin. Previous studies by the Lambeth group have shown that the electron transfer from the flavin to the iron-sulfur cluster is the rate-limiting step in the reduction of Adx by NADPH (Lambeth et al. 1979).

The third phase displays the most dramatic differences between AdxWT and AdxS112W. Surprisingly, CYP11A1 reduction by AdxS112W is much slower over almost the whole range of salt concentrations studied (Fig. 3). The fastest CYP11A1 reduction by AdxS112W at 200 mM potassium chloride proceeds with an observed velocity equal to only 1/5 of the optimum CYP11A1 reduction by wild-type Adx. Only at this KCl concentration is the mutant Adx faster at reducing CYP11A1 than AdxWT. The relative difference in reduction velocity between AdxWT and AdxS112W demonstrates an exponential drop ranging from 39-fold faster with no additional salt in the buffer to a 0.7-fold slower reduction rate observed for wild-type Adx when using 200 mM salt. These differences reflect the obviously different binding behavior of these two Adx species towards their reaction partners. As can be seen in Table 1, the *on*-rates determined for the oxidized Adx/CYP11A1

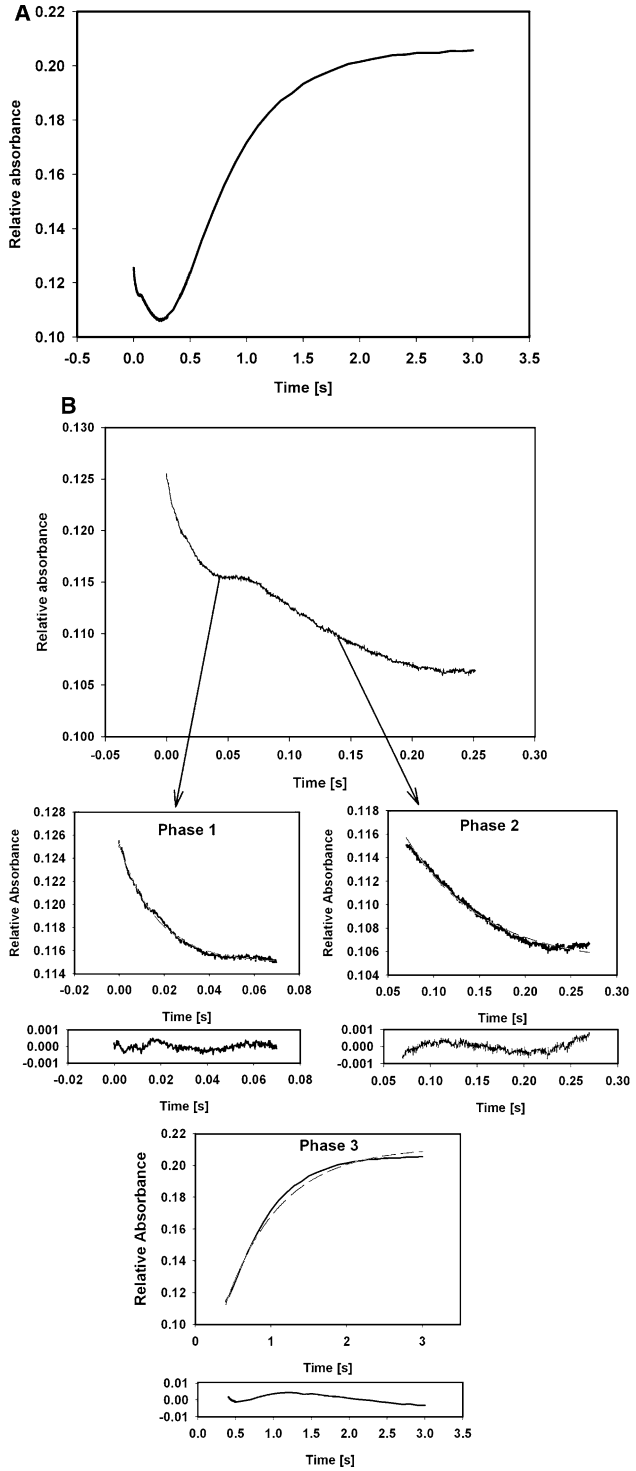

Fig. 1 Exemplary sequential reduction of AdR, Adx and CYP11A1 followed within one shot. NADPH from one syringe was mixed with the oxidized proteins of the CYP11A1 system. **a** Original reaction trace followed at 450 nm displaying the three different reaction phases. **b** The different reaction phases were fitted separately as displayed using either mono-exponential or bi-exponential fits. Phase 1 corresponds to the reduction of AdR by NADPH, whereas Phase 2 shows the reduction of Adx by reduced AdR. Phase 3 displays the formation of the CYP11A1-CO complex formed upon reduction of the cytochrome by Adx. The residual plots for each fit are given below the trace

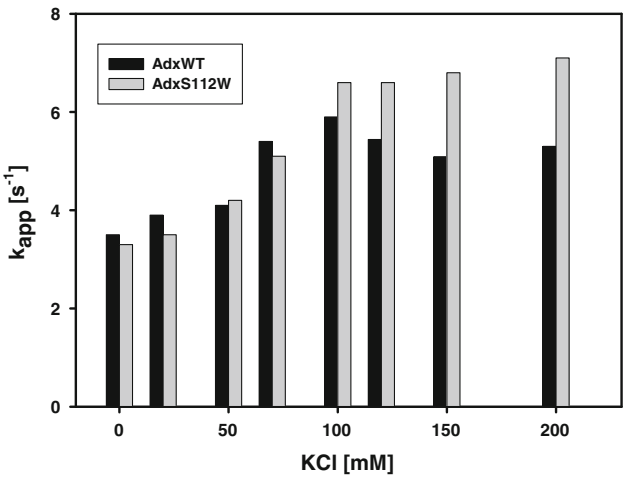

Fig. 2 Velocity of reduction of Adx by AdR at different ionic strength derived from "one shot" measurements. Adx is reduced in the presence of oxidized CYP11A1. The standard deviation is ±5%

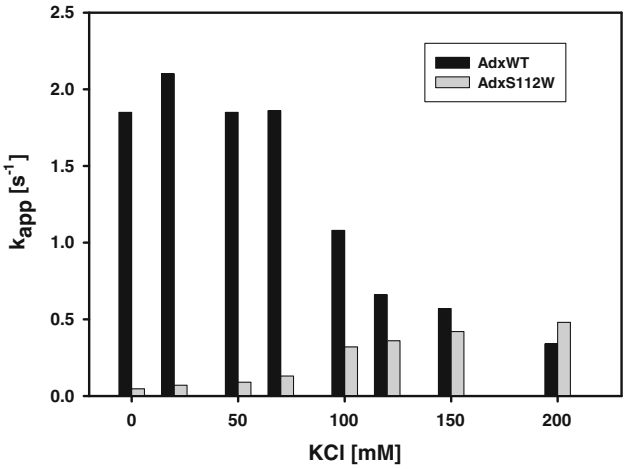

Fig. 3 Velocity of reduction of CYP11A1 by Adx at different ionic strengths derived from "one shot" measurements. The standard deviation is ±5%

complex are 155-fold higher than for the AdR/Adx complex, and the K_d-values for the two possible complexes differ by a factor of 177 (i.e., the S112W/CYP11A1 complex is much more stable than the S112W/AdR complex). In our stopped flow measurements, complexes of the oxidized proteins, with a preference for Adx/CYP11A1 complexes, were already pre-formed when mixing NADPH with the mixture of the three oxidized proteins. This explains the different CYP11A1 reduction velocities of the two Adx species. The effect of salt sensitivity of the CYP11A1 reduction by AdxWT is a five-fold decrease of the reduction rate observed upon increasing salt concentration and a ten-fold increase of the observed reduction rate for AdxS112W. Moreover, the overall reduction rates observed with this mode of mixing compared with the one using pre-reduced Adx (see below) do not differ significantly

for AdxWT but differ by a factor of between 20 and up to a 374 for AdxS112W.

When applying the mixing mode, where pre-reduced Adx was used, the reaction traces obtained are different compared with the reaction traces seen when Adx was not pre-reduced. In this mixing mode, the traces of reduction of CYP11A1 by wild-type Adx were best described by a mono-exponential equation as shown before (Schiffler et al. 2004), while the reduction of CYP11A1 by the mutant species AdxS112W was best described by a bi-exponential fit. These measurements are also in good agreement with previous observations using these Adx species (Schiffler et al. 2001). We also determined the influence of the ionic strength on the reaction between pre-reduced Adx and CYP11A1. As shown in Fig. 4, the velocity of reduction of CYP11A1 by AdxWT is strongly dependent on the ionic strength of the reaction buffer with decreasing apparent rate constants upon increasing ionic strength. Following the same reaction using Adx mutant S112W, the salt dependence of the CYP11A1 reduction appears to be less pronounced, and the observed apparent rate constants are more or less constant over a broad range of salt concentrations. Moreover, the relative velocity of CYP11A1 reduction by AdxS112W compared with AdxWT grows exponentially with increasing salt concentration from 6.8 up to 89. The dramatic difference in the reduction velocity of CYP11A1 by AdxS112W using the different mixing modes can be explained by a limiting step in the binding of AdxS112W to either CYP11A1, which is more probable, or to AdR, which seems to be less likely, or by structural rearrangements of the complex between oxidized AdxS112W and CYP11A1. As shown in Table 1, the SPR measurements demonstrate that the binding of AdxS112W to CYP11A1 is stronger than the binding of AdxWT to CYP11A1. The

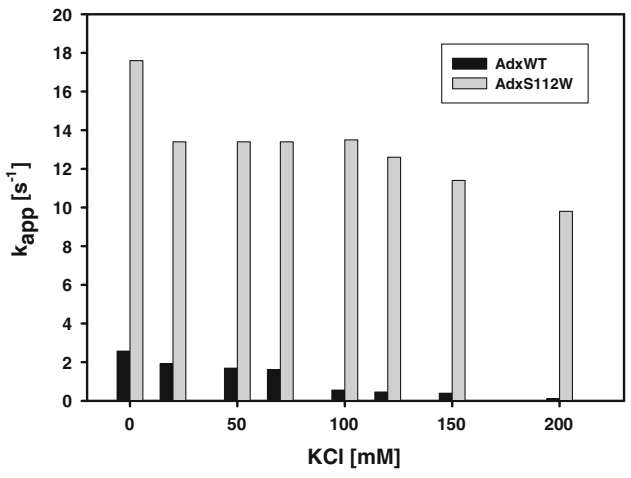

Fig. 4 Apparent rate constant of the reduction of CYP11A1 by pre-reduced Adx under different ionic strength. Adx was aged in one syringe together with NADPH and AdR to yield reduced Adx. The standard deviation is ±5%

difference in reduction velocities may be due the ionic nature of the interaction between Adx and CYP11A1 (Grinberg et al. 2000), which becomes disturbed with higher salt concentrations (Schiffler et al. 2004; Berwanger et al. 2010). On the contrary, there seems to be no such limitation for mutant AdxS112W, suggesting an additional interaction site between Adx and CYP11A1 that is not affected by salt. The tryptophan in position 112 could form stacking interactions with the tyrosine in position 82 of Adx, which was shown to participate in binding (Beckert and Bernhardt 1997). Indeed, position 112 of Adx is in close proximity to position 82 of Adx in the crystal structure of the AdR/Adx complex (Muller et al. 2001) More important, however, would be (a) stacking interaction(s) between W112 of the mutant Adx and (an) aromatic residue(s) of CYP11A1. According to the published structure of a CYP11A1 model (Usanov et al. 2002; Vijayakumar and Salerno 1992), hydrophobic interactions between the respective adrenodoxin mutants and CYP11A1 seem very likely to contribute to the stabilization of the complex. Two aromatic residues (Trp-401 and Phe-411) are located on the CYP11A1 surface close to the site of ionic interaction with adrenodoxin (Wada and Waterman 1992; Usanov et al. 2002). These two residues might form stacking interactions with Trp-112 of the adrenodoxin mutant S112W. Obviously an aromatic amino acid in position 82 of adrenodoxin is also required for this potential interaction. Unfortunately, the very recently published structure of the Adx (partial structure)/CYP11A1 complex (3NA0.pdb) does not allow reaching any conclusions about this site, since only residues Phe43–Gln93 of Adx are shown.

Taken together, the data from the stopped flow measurements, where the reduction of the proteins is followed, confirm the assumption that the off-rate of the Adx/CYP11A1 complex formation is the rate-limiting step in the CYP11A1 reduction whereby the reduction of the cytochrome by Adx is highly dependent on the affinity of the interaction partners.

Substrate conversion assay

The conversion of cholesterol to pregnenolone was monitored to link the results of the redox experiments described above to the overall reaction catalyzed by CYP11A1. Substrate conversion assays following the product formation of pregnenolone gave significant differences in the catalytic efficiency (i.e., k_{cat}/K_m) between AdxWT and AdxS112W over the whole range of salt concentrations used in our study (Fig. 5). The most pronounced differences between AdxWT and AdxS112W can be observed in the range of higher salt concentrations (>50 mM KCl) showing a decrease in the efficiency of substrate conversion when using Adx (Schiffler et al. 2004) and giving an up to 14-fold

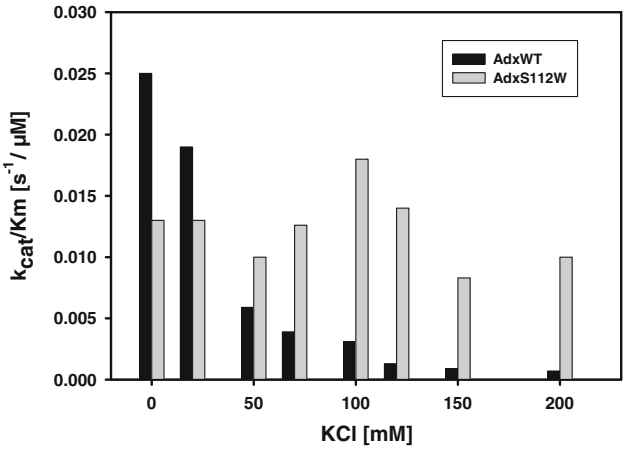

Fig. 5 K_{cat}/K_m (i.e., the catalytic efficiency) of the CYP11A1 dependent conversion of cholesterol to pregnenolone. The dependence on the ionic strength is shown. Recombinant AdR and Adx were used as well as CYP11A1 purified from adrenals. The standard deviation is ±5%

higher catalytic efficiency for AdxS112W compared with Adx at 200 mM salt concentration (see Fig. 5). Overall, the substrate conversion catalyzed by AdxS112W, however, does not show a strong dependence on the ionic strength of the reaction mixture. This observation reflects very well the situation of CYP11A1 reduction using pre-reduced Adx where also no strong changes in the reduction velocity at various salt concentrations were observed when using AdxS112W. Thus, it can be concluded that the protein-protein interaction, especially the interaction between Adx and CYP11A1, is critical for the overall activity of this enzyme system. This is in accordance with the decreased k_{off}-rate and K_d value of the AdxS112W/CYP11A1 compared with the Adx/CYP11A1 complex as measured using SPR (Table 1).

Conclusions

Our results convincingly demonstrate that the detailed analysis of mutant AdxS112 W, which was shown in previous investigations to increase the substrate conversion of CYP11A1, led to new insights into the mechanism of the transient interactions among the components of the mitochondrial steroid hydroxylating CYP11A1 system, consisting of a reductase, AdR, a ferredoxin, Adx, and a cytochrome, CYP11A1. Applying real-time binding studies using surface plasmon resonance, we at first were able to support the previous observation that the interaction between AdR and Adx is less stable than that between Adx and CYP11A1. Moreover, a significant effect of the replacement of serine 112 by tryptophan on the k_{off} rate of the Adx/CYP11A1 complex was shown to lead to a four-fold decrease of the K_d value. This demonstrates that the replacement of serine in position 112 by tryptophan (and the truncation of the protein from 128 to 112 amino acids) considerably increases the stability of the Adx/CYP11A1 complex, whereas that of the Adx/AdR complex was much less affected. A rational explanation for this observation was deduced from structural data of both complexes. The salt dependence of the reduction of CYP11A1 demonstrated that electrostatic interactions play a pivotal role in this process. However, when pre-reduced AdxS112W is used, there was only little influence of the ionic strength on the reduction velocity, supporting the preferential use of hydrophobic contacts in this case. Moreover, a dramatic increase in the reduction rate was observed for the AdxS112W mutant compared with wild-type Adx. A possible explanation could be that the complex of reduced AdxS112W with CYP11A1 is much more stable and/or induces a more active conformation compared to wild-type Adx.

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