Stripping down the mitochondrial cholesterol hydroxylase system –
A kinetic study

Burkhard Schiffler, Andy Zöllner, and Rita Bernhardt* 

Naturwissenschaftlich-Technische Fakultät III, FR 8.8-Biochemie, Universität des Saarlandes, P.O. Box 151150, D-66041 Saarbrücken, Germany

* corresponding author.
Tel.: + 49-681-302-4241 fax: + 49 681-302-4739; e-mail address: ritabern@mx.uni-saarland.de

Running title: CYP11A1 kinetic studies

Keywords: Adrenodoxin, adrenodoxin reductase, CYP11A1, stopped flow, ionic strength, mitochondrial hydroxylase system, steroids, NADPH, rapid mixing, kinetics, surface plasmon resonance, Biacore
**Abbreviations:**

- AdR = recombinant Adrenodoxin reductase wild type
- Adx = recombinant Adrenodoxin wild type
- CYP11A1 = cytochrome P450sc = side chain cleavage enzyme from bovine adrenals
- NADPH = nicotinamide adenine dinucleotide phosphate
- FAD = flavine adenine dinucleotide
- SPR = surface plasmon resonance
- \( k_{\text{app}} \) = apparent rate constant

**Abstract:**

The origin of steroid hormones in mammals is cholesterol that is metabolized by the mitochondrial CYP11A1 system. The cytochrome P450 is fed with reduction equivalents via a small electron transfer chain, consisting of NADPH, adrenodoxin reductase, and adrenodoxin. Though the redox behavior of the individual protein components has been studied previously, the kinetics of the system in its entirety has not yet been analyzed. In this study we combine surface plasmon resonance experiments to determine the binding constants for the different pairs of redox partners with measurements of the pre-steady state kinetics of the different reaction steps of this system and steady state kinetics. We could correlate the individual protein-protein interactions with the effect of distinct reduction-oxidation steps on the overall catalytic activity of the CYP11A1 system. For the first time, we were able to follow the reduction of each of the protein components of this system within one measurement when we mixed all oxidized protein components with NADPH. These measurements allowed the determination of the individual apparent rate constants for the reduction of all three proteins involved. In addition, variation of the ionic strength...
in these experiments revealed different optimum salt concentrations for the reduction of adrenodoxin reductase and adrenodoxin, respectively, and unraveled dramatically changing reduction rates of CYP11A1 by adrenodoxin.

**Introduction:**

The mitochondrial steroid hydroxylase CYP11A1 belongs to the superfamily of cytochrome P450 enzymes. It catalyzes the rate-limiting step in the biogenesis of steroid hormones in adrenal mitochondria (1,2). In this reaction, the side chain of the substrate cholesterol gets cleaved off to yield pregnenolone and isocaproic aldehyde at the expense of 3 molecules of NADPH and 3 molecules of oxygen (3). The reduction equivalents, provided by NADPH, are transferred to a FAD containing adrenodoxin reductase (AdR), which transfers electrons to the one electron carrier adrenodoxin (Adx), the ferredoxin of the adrenal gland (4). The [2Fe2S] cluster-containing Adx subsequently transfers electrons, one at a time, to the heme iron of CYP11A1. These electrons are necessary for the reductive activation of oxygen and finally to hydroxylate the substrate. For a complete conversion of cholesterol to pregnenolone six electrons have to be supplied.

About 40 years ago the group of Kimura (5-8) started to characterize single components of the system followed by a multitude of investigations concerning the adrenal steroid hydroxylase system and its different components by various groups (9-18). During the last decade much work has been done to get a better understanding of the kinetics of this system (4,19-23). Nevertheless, the nature of the rate-limiting step in this system is still a matter of controversy discussion and remains to be elucidated.
For the first time we were able to follow the reduction of all three protein components of the CYP11A1 system within one measurement using rapid mixing techniques. We determined the apparent rate constants ($k_{app}$) for the reduction of AdR by NADPH, of Adx by reduced AdR, and of CYP11A1 from one measurement. In addition, variation of the ionic strength revealed a clear difference in the redox behavior of Adx towards its redox partners. Since the interaction of Adx with its redox partners is mainly of electrostatic nature it is of great interest to investigate the salt dependency of these reactions. In combination with surface plasmon resonance (SPR) studies and substrate conversion experiments we were able to sketch a detailed picture of the kinetics of this system.

Materials and methods:

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. The Adx cDNA-containing plasmid was pKKHC (24). The plasmid containing the coding sequence for AdR was kindly provided by Dr. Sagara (25).

Protein expression and purification -

Bacteria were grown as previously reported (26) with slight modifications. Briefly, we used freshly transformed *E. coli* BL21DE3 to inoculate a pre-culture. The bacteria were allowed to grow in ampicillin containing nutrient broth medium at 37°C over night. These cultures were used to inoculate 4 liters of a main culture containing
ampicillin. IPTG was added to induce heterologous protein production and afterwards cultures were grown at 37°C for 16 h. Recombinant Adx was purified after sonification as described and the final concentration of Adx was determined using $\varepsilon_{414} = 9.8 \text{ mM}^{-1} \text{ cm}^{-1}$ (27). The purity of the Adx preparation was estimated by determining the relative absorbance of the protein at 414 nm and 273 nm, Q-value ($A_{414}/A_{273}$).

AdR was heterologously expressed and purified as described elsewhere (28). The molar extinction coefficient used for concentration estimation was $\varepsilon_{450} = 10.9 \text{ mM}^{-1} \text{ cm}^{-1}$ (8). CYP11A1 was isolated from bovine adrenals, and the concentration was estimated by carbon monoxide difference spectra assuming $\varepsilon_{450-490} = 91 \text{ mM}^{-1} \text{ cm}^{-1}$ according to (29).

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 (19). The reaction buffer consisted of 50 mM HEPES (pH 7.3), varying amounts of potassium chloride, and 0.05 % 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 only the reduction of AdR by NADPH in the stopped flow apparatus, syringe A contained AdR (20 µM) and the concentration of NADPH in syringe B was equimolar. In a second set of measurements we used a 100-fold excess of NADPH. The change of absorbance at 450 nm was monitored, corresponding to the maximum change in absorbance upon reduction of AdR.
In order to determine the reduction rate of Adx (40 µM) and AdR (20 µM), the two oxidized proteins were mixed with NADPH (400 µM). Kinetic traces were followed at 450 nm where both proteins possess an absorbance maximum (8,30). A second approach to determine the reduction rate for Adx was the mixing of pre-reduced AdR with oxidized Adx, following Adx reduction at 414 nm corresponding to the main absorbance change upon reduction (30).

To follow specifically the reduction of CYP11A1, syringe A contained CYP11A1 (4 µM) while syringe B was filled with NADPH (400 µM), AdR (4 µM), and Adx (8 µM). The mixture in syringe B was allowed to age for 10 minutes to assure a complete reduction of Adx. The solutions in the two syringes were CO-saturated prior to loading into the driving syringes. To follow the whole cascade of reactions (i.e. reduction of AdR, Adx and CYP11A1) syringe A contained only NADPH (400 µM) and syringe B was filled with the three proteins in the following concentrations, AdR (4 µM), Adx (8 µM), and CYP11A1 (4 µM).

The reaction was monitored at 450 nm recording 4000 data points with a split time base of 10/200 sec. The dead time for the measurements was 1.3 ms with the SX-17MV apparatus. In order to reduce the background, three to seven identical reaction traces of each measurement were overlaid and averaged. Data fitting was performed using the program SigmaPlot 2001. Mono- or bi-exponential functions were fitted to the averaged curves in order to determine apparent rate constants for the reduction of AdR by NADPH, of Adx by AdR, and of CYP11A1 by Adx.

Upon reduction, all three proteins show marked changes in their absorbance at 450 nm. To follow the reduction of AdR by NADPH and of Adx by AdR, respectively, in one measurement, the decrease of the absorbance at 450 nm was followed. Formation of the reduced cytochrome was monitored by the respective CO complex, giving rise to an absorbance maximum at 450 nm.
Optical biosensor measurements -

Analysis of the binding of oxidized Adx to AdR\textsubscript{ox} or to CYP11A1\textsubscript{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 (31). In order to activate the carboxy groups on the sensor chip a solution containing a 0.2 M N-ethyl-\textprime-N\textprime-dimethylaminopropyl-carbodiimide (EDC) and 0.05 M N-hydroxysuccinimide (NHS) was injected onto the chip with a flow rate of 5µl/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\textsubscript{ox} or CYP11A1\textsubscript{ox} concentrations in the range from 10 nM to 500 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). To remove still bound AdR or CYP11A1 we injected 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 500 nM AdR and 500 nM CYP11A1, respectively. The observed changes were below 10 % (data not shown), indicating a good regeneration performance. Analysis of the binding 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 (32) with slight modifications (20). The cholesterol side chain cleavage activity was assayed for 10 min at 37°C in a reconstituted system. The mixture contained CYP11A1 (0.5 µM), different amounts of Adx (from 0.2 µM to 3.2 µM), AdR (0.5 µM) and cholesterol (100 µM). The reaction was started by adding NADPH. The buffer 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. After addition of cortisol as an internal standard, the samples were analyzed by reversed phase HPLC on a JASCO system using a C-18 column (3.9 x 150 mm, waters) heated in a peltier oven to 40°C. The internal standard was necessary because of the additional isomerase activity of cholesterol oxidase, which makes the exact determination of the transformed cholesterol impossible but not that of pregnenolone. The mobile phase used for separation was a mixture of acetonitrile:isopropanol (30:1). To quantitate the peak areas of the internal standard and the product, the JASCO Borwin software was used. Further data evaluation was done using MS Excel.

Results:

In this paper, we performed binding studies to investigate the protein-protein interactions of the mitochondrial CYP11A1 system in combination with a detailed time
resolved kinetic analysis of all protein components of this system. Due to the ionic nature of the interaction process of Adx with AdR and CYP11A1, respectively, the salt dependency was also studied at varying ionic strength. Such variations of the ionic strength have been analyzed by Hintz and Peterson (33) and affect the reduction behavior of the CYP101 system. CYP11A1 substrate conversion assays were carried out to correlate the binding and the redox processes with the catalytic activity of the system under physiological conditions. We used recombinant bovine wild type AdR, recombinant bovine wild type Adx, and CYP11A1 purified from bovine adrenals.

The interactions of the oxidized reaction partners of the mitochondrial P450 system have been investigated with an optical biosensor system (SPR). As described in “Materials and Methods”, the ferredoxin component was coupled to a chip and either the flavoprotein or the cytochrome were passed over this chip. Complex formation between AdR and Adx is a fast process characterized by a $k_{on}$ rate of 4434 $M^{-1} s^{-1}$ and a $K_d$ being in the nanomolar range (Table 1). Binding studies concerning the interaction of Adx with CYP11A1 resulted in a pronounced difference as compared with the binding of Adx to AdR (Table 1). For Adx binding to CYP11A1, the $k_{on}$-rate was determined to be about 156-fold faster than the $k_{on}$-rate observed for AdR. Also, the $K_d$ values for the two different complexes studied vary dramatically. The Adx*CYP11A1 complex is 63-fold more stable than the AdR*Adx complex.

The behavior of the reduced proteins was analyzed by stopped flow experiments, which allows to follow the reduction kinetics of each component directly. Two different modes of rapid mixing were compared. In a first series of measurements, the purine nucleotide (NADPH) was mixed with the protein components of the electron transfer chain. A second approach used a mixture of pre-
reduced reductase and Adx, which were subsequently mixed with the oxidized terminal electron acceptor of this system, CYP11A1.

A typical reaction trace observed at 450 nm after mixing of NADPH (syringe A) with AdR, Adx and CYP11A1 (syringe B) is given in Figure 1. In the first 250 ms of the reaction a fast drop of the absorbance is observed. The duration of this process is depending on the ionic strength and can take up to 500 ms (data not shown). This decrease in absorbance occurs in two phases, a first one within 60 ms followed by a second somewhat slower drop (Figure 1, insert). The first and second phase are separated by a small plateau (constant absorbance) of 10-15 ms. These two phases of the reaction trace are in agreement with a sequential reduction of the FAD containing AdR by NADPH followed by the reduction of the [2Fe2S] cluster in Adx (see also below). AdR reduction as well as Adx reduction leads to a clear decrease of absorbance at 450 nm as documented for the individual proteins in previous studies (30),(12). The third phase of the reaction trace is characterized by a pronounced increase of absorbance (Figure 1). This rise at 450 nm is expected upon CO complex formation of the ferrous heme in CYP11A1. All three phases can be fitted very well to mono-exponential functions yielding one velocity constant for each distinct process.

The first 60 ms phase of the reduction (i.e. the reduction of AdR) is dependent on the ionic strength of the reaction mixture, as described above. Figure 2 (filled circles) shows the apparent velocity constant of the first phase observed at different potassium chloride concentrations. The highest $k_{app}$ value could be observed at 50 mM potassium chloride. Lower or higher salt concentrations lead to a decrease of the observed velocity in this first part of the overall reaction. If the ionic strength exceeds 150 mM, an even lower $k_{app}$ can be observed compared with conditions were no salt was added. The second phase (from about 85 ms to 250 ms) of the reaction trace followed at 450 nm confers to the reduction of Adx by AdR (see also below). The
changes of the apparent velocity constants for the reduction of Adx at different ionic strength are given in Figure 2 (open circles). The overall rate constants observed for the ferredoxin reduction are decreased by a factor of 10 as compared with the reduction of AdR described above. Interestingly the optimum $k_{app}$ value is observed at an ionic strength of 100 mM.

The third phase (starting from 250 ms) of the reaction trace observed at 450 nm is an increase of the absorbance (Figure 3, filled circles). This increase occurs upon CO complex formation of the ferrous CYP11A1. The $k_{app}$ values observed for the reduction of CYP11A1 decrease dramatically with increasing salt concentration. This observation is in good agreement with the ionic nature of the complex between Adx and CYP11A1 (34-38).

The third phase of the reduction process (i.e. the reduction of CYP11A1) as described above was compared to the reduction kinetics of CYP11A1 when mixed rapidly with the pre-reduced electron transfer components. Using pre-reduced ferredoxin (i.e. NADPH, AdR and Adx in syringe 1 versus CYP11A1 in syringe 2), the drop in absorbance at the beginning of the measurement (first and second phase) could not be observed (data not shown). Thus, the reduction of AdR and Adx (seen in the measurements above; cf. Figure 1) was completed here during the incubation of NADPH, AdR and Adx in syringe 1 before mixing with CYP11A1 in syringe 2. In this mixing mode, the traces of reduction of CYP11A1 by Adx were also best described by a mono-exponential equation. These measurements are in good agreement with previous observations using this mixing mode (19). The pronounced dependence on the ionic strength of CYP11A1 reduction is shown in Figure 3 (open circles). A more than 23-fold decrease of the apparent velocity constant with increasing ionic strength is observed. The data obtained from these measurements
are in the same range as the data for CYP11A1 reduction in the “one shot” measurements described above (Figure 3, filled circles).

To validate the data obtained in the “one-shot” measurements reported above (i.e. AdR reduction and Adx reduction), we performed separate stopped flow measurements with each of the components of the CYP11A1 system (Figures 4 and 5). In a first set of experiments, we investigated the reduction of AdR by NADPH under different conditions. Reduction of AdR by an excess of NADPH resulted in a bi-exponential reaction trace (Figure 4 A). The first fast phase had an apparent rate constant of 44.9 s$^{-1}$ while the second phase was slower with 5.9 s$^{-1}$. These results are in excellent agreement with previous data provided by Lambeth and Kamin (12).

On the other hand, when using equimolar concentrations of NAPDH and AdR, the reaction was clearly mono-phasic and the $k_{app}$ value derived from the reaction trace was 17.4 s$^{-1}$ (Figure 4 B).

In a next experiment we used NADPH, AdR and Adx (Figure 5A). When mixing an excess of NADPH in one syringe with AdR and Adx in the other syringe, the reaction is clearly bi-phasic with a first fast phase of 53.4 s$^{-1}$ and a second slower phase with an apparent velocity of 2.2 s$^{-1}$. The first phase is the reduction of AdR while the second phase is the reduction of Adx.

When using pre-reduced AdR in one syringe and oxidized Adx in the other syringe the process was clearly bi-phasic (Figure 5B). We observed a first fast phase with an apparent rate constant of 71.1 s$^{-1}$ followed by a second phase with a $k_{app}$ of 5.6 s$^{-1}$. These data are close to the data obtained upon mixing of NADPH with the two oxidized proteins. The second rate constant is also in good agreement with previous studies (31) and corresponds with the reduction of Adx by AdR. The first apparent rate constant observed in these measurements is in the range of those observed for the reduction of AdR. However, even in the presence of excess
NADPH, the reduction of AdR is incomplete, i.e. some AdR remains in the oxidized state. On the other hand, the \( \text{on-rate} \) for the complex formation between AdR and Adx was calculated to be \( 4434 \text{ s}^{-1} \text{ M}^{-1} \) from our SPR measurements suggesting a fast binding event. Owing to these two facts, the first phase observed in the pre-steady state experiments with pre-reduced AdR and oxidized Adx can be explained by the progressing reduction of AdR in the presence of Adx. In support of this observation, previous data of Lambeth (12) show ternary complex formation between the reduction equivalent delivering NADPH and the first two protein components of the CYP11A1 electron transfer chain (i.e. AdR and Adx) upon reduction of AdR.

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. The influence of different salt concentrations on the substrate conversion is depicted as \( k_{\text{cat}}/K_m \) (i.e. the catalytic efficiency, which is a measure for the efficiency of the overall reaction with a specific substrate) in Figure 6. As stated above (see Figure 3) Adx displays a dramatic difference in providing reduction equivalents to CYP11A1 under varying salt concentrations. Thus, a striking decrease in the efficiency of the CYP11A1 dependent cholesterol conversion upon increasing salt concentrations is observed, which corresponds to the mainly ionic interaction of the two proteins. This finding underlines that higher salt concentrations hinder the interaction of Adx with CYP11A1.

**Discussion:**

In this study we present for the first time a complete analysis of the adrenal mitochondrial CYP11A1 electron transfer system using a combination of different
methods. We used surface plasmon resonance, rapid mixing techniques, and CYP11A1 dependent cholesterol conversion to investigate the interactions of the components of the mitochondrial CYP11A1 system (i.e. NADPH, AdR, Adx, and CYP11A1) in detail. The combination of these methods, which allows to visualize different aspects of this system, variation of the ionic strength as well as different mixing techniques for the stopped flow measurements lead to surprising new insights into this physiologically important system.

Using the phenomenon of SPR, we followed the binding of the different reaction partners of the CYP11A1 system in the oxidized state using a Biacore 2000 device. The data given in Table 1 show tremendous differences in the interaction of Adx with AdR and CYP11A1. Binding of Adx with AdR as well as with CYP11A1 is characterized by a fast on-rate. Yet, the binding of CYP11A1 to Adx displays a 156-fold higher on-rate than the binding of AdR to Adx. In contrast to the on-rates, the off-rates for the two physiological relevant complexes studied are in the same order of magnitude and differ by a factor of 2.4 only. Consequently the $K_d$ value for the complex with CYP11A1 is lower by a factor of 63 than the $K_d$ for the complex with AdR. The different constants determined in this study are in accordance with studies provided by Ivanov et al. (39) who used the optical biosensor IAsys system for his measurements. In early studies of the group of Lambeth (16) the first order off-rate for the breakdown of the complex between AdR and Adx was shown to be salt independent and calculated to be about 300 s$^{-1}$. The reason for the discrepancy between the data described here and by Lambeth and co-workers (16) remains unclear. According to our stopped flow measurements (see below), the reduction of Adx by AdR is salt dependent. $K_i$ values for the complex between human AdR and human Adx have been reported by Brandt and Vickery (40) to be in the range of 20 nM to 100 nM, which correlates perfectly with our data for the bovine proteins. Taken
together, the SPR experiments clearly prove formation of stable complexes of the oxidized protein components of the CYP11A1 system. The Adx*CYP11A1 complex could be shown to be even more stable than the AdR*Adx complex, which again correlates with the data reported by Ivanov (39).

The reduction of the protein components of the CYP11A1 system was studied using a stopped flow approach. In a first set of measurements, NADPH was mixed with the oxidized protein components and the reaction was followed at 450 nm as described under “Materials and Methods”. In this mixing mode, we observed three distinct phases (Figure 1), and each of them was best described by a mono-exponential equation. These 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) cannot be followed when using pre-reduced Adx (see below). Analysis of the three phases of the reaction traces allowed the determination of the reduction rates for AdR, Adx and CYP11A1 at different ionic strength from one and the same measurement.

The first phase observed in these experiments 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 27 s$^{-1}$ to 50 s$^{-1}$ (Figure 2). The maximal $k_{app}$ for the reduction of AdR is observed at 50 mM potassium chloride. Due to the fast complex formation between AdR and Adx (Table 1) before mixing with NADPH, the reduction of AdR at least partially takes place in the AdR*Adx complex. Therefore the bound Adx might also influence the reduction rate of AdR at different salt concentrations. Early works of Chu and Kimura (41), as well as of the group of Lambeth (13), demonstrate a stable complex formation between oxidized AdR and oxidized Adx. The dissociation constant was estimated to be in the nanomolar range (41). These results are in very good agreement with our
SPR data following the direct binding of oxidized AdR to Adx provided in this study and are comparable with IAsys studies by Ivanov et al. (39).

To verify the data obtained for the reduction rates of all protein components of the CYP11A1 system from one measurement, we examined each step occurring during the reaction alone. Starting with the reaction between two molecules, we studied the reduction of AdR by NADPH (i.e. without Adx and CYP11A1). The measurements were carried out using NADPH and AdR in equimolar amounts as well as in the presence of excess NADPH. The AdR reduction with equimolar NADPH was clearly mono-phasic with an apparent rate constant of 17.4 s$^{-1}$ (without addition of KCl) while the reduction process with an excess of NADPH was bi-phasic with apparent rate constants of 44.9 s$^{-1}$ and 5.9 s$^{-1}$ for the first and second rate, respectively. This corresponds with early studies of Lambeth and Kamin (12) who showed the reduction of AdR by NADPH to be a first order process when using equimolar amounts of AdR and NADPH. The apparent rate constant in these studies was determined to be 28 s$^{-1}$ (without additional salt), which is in good agreement with our measurements (i.e. 17.4 s$^{-1}$). The group of Lambeth could also show that an excess of NADPH leads to a second first order process with a $k_{app}$ of 4.25 s$^{-1}$. They suggested that this second process is the replacement of NADPH for NADP$^+$ and calculated a $K_d$ of 10 nM for the binding of NADP$^+$ to reduced AdR in the absence of Adx.

The reduction process of AdR by equimolar NADPH is comparable to the first phase in the “one shot” mixing mode using NADPH in one syringe and the oxidized proteins in the other syringe. Thus, we accomplished the acquisition of reliable data even at varying salt concentrations from a single experiment, which has not been examined previously. The rate of reduction of AdR (Figure 2) is clearly affected by
the salt concentration which is not surprising taking the polarity of NADPH into account.

Upon mixing the three oxidized proteins with NADPH, which yields three distinct phases (see above), the reduction of Adx by AdR corresponds to the second phase. As visualized in Figure 2, the reduction rates for the Adx reduction are about 10-fold lower than the reduction rates observed for the AdR reduction. The optimal salt concentration is clearly in the range of 100 mM potassium chloride in the buffer, corresponding to the physiological concentration of potassium ions in a mammalian cell. Higher and especially lower salt concentrations lead to a pronounced decrease in the apparent rate constant observed for the Adx reduction process. This points to a well-balanced fine-tuning in the interaction of AdR and Adx with respect to the ionic strength of the surrounding medium that was not shown in literature until now.

Moreover, experiments using NADPH in one syringe and oxidized AdR and Adx (i.e. without CYP11A1) in the other syringe lead to bi-phasic reaction traces with apparent rate constants of \(53.4 \text{ s}^{-1}\) and \(2.2 \text{ s}^{-1}\) for the first and second rate, respectively. These results are in good agreement with the data from the measurement described above, where NADPH was mixed with the three oxidized species. Furthermore, reduction of Adx by pre-reduced AdR was followed at 414 nm (i.e. the main absorbance band of oxidized Adx) and gave bi-phasic reaction traces. The first phase was in the range of \(71.1 \text{ s}^{-1}\), which is typical for the reduction of AdR that can be also followed at 414 nm, suggesting a proceeding of the reduction of AdR after mixing with Adx. This first phase is followed by the reduction of Adx in a second phase that was determined to be \(5.6 \text{ s}^{-1}\), a velocity that is typical for the reduction of Adx (31). Previous works of the group of Lambeth (13,42) showed the existence of an ternary complex between NADP\(^+\), reduced AdR and oxidized Adx with the two
electrons located in a charge transfer complex between the pyridine nucleotide and AdR. In this ternary complex Adx gets reduced only after AdR is completely reduced.

In our “one shot” experiments the third phase, which is characterized by a strong increase of absorbance at 450 nm, is attributed to the reduction and CO complex formation of CYP11A1. This phase was also studied depending on salt concentration and shows the most dramatic differences (Figure 3). A 6-fold decrease in the velocity of reduction of CYP11A1 is observed upon increasing salt concentration, which is consistent with the mostly ionic nature of the complex between CYP11A1 and Adx (for review see (4, 43)). In Figure 7 an schematic overview of the reaction processes taking place is presented. The different velocities of the reactions observed in the “one shot” measurements are included. As shown, the observed velocities decrease significantly from step to step starting with the fastest reaction (i.e. the reduction of AdR).

In a next set of stopped flow experiments, the process of CYP11A1 reduction by pre-reduced Adx was studied. When applying this mixing mode, the reaction traces obtained looked different as compared with the reaction traces seen when Adx was not pre-reduced. We also determined the influence of the ionic strength on the reaction between pre-reduced Adx and CYP11A1. As shown in Figure 3, the velocity of reduction of CYP11A1 by Adx is also strongly dependent on the ionic strength of the reaction buffer with decreasing apparent rate constants upon increasing ionic strength. Surprisingly, the overall reduction rates observed at different salt concentrations in this mode of mixing do not differ significantly for CYP11A1 reduction as compared with the “one shot” measurements (see above). This is consistent with investigations of Beckert and Bernhardt (23). Nevertheless, according to the SPR measurements (Table 1), one would expect that the mixing mode affects the apparent reduction rates observed. This consequence is based on the on-rates
determined for the oxidized Adx*CYP11A1 complex (156-fold higher than for the AdR*Adx complex, see above), and the K_d-values for the two possible complexes (differing by a factor of 63.9 whereby the Adx*CYP11A1 complex is much more stable than the Adx*AdR complex). Therefore, in our “one shot” stopped flow measurements, complexes of the oxidized proteins, with a preference for the Adx*CYP11A1 complex, were already pre-formed before adding NADPH to the mixture of the three oxidized proteins. The effect of such a distribution of pre-formed complexes of the oxidized proteins would be a sequential reduction of Adx in the following manner: First, reduction of AdR-bound Adx could take place followed by dissociation of the reduced Adx from AdR. The reduced Adx could then replace oxidized, CYP11A1-bound Adx so that reduction of CYP11A1 could proceed. Assuming such a scenario, the apparent rate constants for the “one shot” measurements would differ from the measurements carried out with pre-reduced Adx due to the low off-rate of Adx from the complex with CYP11A1 (see Table 1). However, the differences in the velocity of CYP11A1 reduction using the two mixing modes are marginal. This could be explained if the reduced Adx was capable of delivering its reduction equivalent to the CYP11A1-bound oxidized Adx, which then reduces CYP11A1. A second possibility is that CYP11A1 (also in the oxidized Adx*CYP11A1 complex) possesses an even higher affinity for reduced Adx, which would facilitate the dissociation of the oxidized Adx from this complex in favor of the reduced Adx molecule.

To link these experiments with the physiological reaction catalyzed by CYP11A1, we performed substrate conversion assays following the product formation (i.e. of pregnenolone) at different salt concentrations. We observed significant differences in the catalytic efficiency (i.e. k_cat/Km) at varying ionic strength (Figure 6). At increasing salt concentrations the k_cat/Km values decrease by the factor
35.7 which can mainly be attributed to the decrease in CYP11A1 reduction velocity upon increasing salt concentrations. These reduction rates show the same tendency of deceleration than the observed k_cat/Km values. The k_cat/Km values reported here at 100 mM KCl being 0.0034 [s^{-1} \mu M^{-1}] are in excellent agreement with previously published results of our group (19). Due to the fact that it is only possible to measure the first reduction step of the heme iron of CYP11A1 using the stopped flow technique, we cannot directly link the apparent rate constants to the k_cat values of the CYP11A1 dependent cholesterol conversion. Nevertheless, the same tendencies are observed pointing towards a connection between the ionic strength dependency of the CYP11A1 reduction and the one observed for the substrate conversion. In addition, the observed off-rate from the Biacore measurement is in the same order of magnitude as the k_cat value for the substrate conversion (Fig. 7), giving rise to the assumption, that the rate limiting step in the overall reduction cascade of the proteins involved occurs at the stage of the dissociation of the complex between Adx and CYP11A1 after the first reduction of the cytochrome.

Concluding, using a set of different techniques we were able to analyze the reactions and interactions taking place in the mitochondrial CYP11A1 electron transfer in detail. For the first time we could analyze the reduction of all three proteins of the CYP11A1 system within one measurement. The results presented here clearly show that varying amounts of salt tremendously influence the interaction between the compounds of this system. This salt dependency observed for all reduction processes and especially for the CYP11A1 reduction step, clearly reflects the electrostatic nature of the complex formation between the corresponding redox partners. In addition, the on-rate for the Adx•CYP11A1 complex formation suggests this process to be diffusion controlled (39,44) leading to the assumption that the rate-
limiting step in the interaction between these proteins is the dissociation of the CYP11A1-Adx complex (i.e. the off-rate).

The results presented here will help to deepen our knowledge of the kinetics of this first and crucial step in steroid biosynthesis and could be applied for the creation of optimized CYP11A1 electron transfer chains, which are of interest for industries involved in the production of steroids.

**Acknowledgements:** This work was financially supported by a grant of the Deutsche Forschungsgesellschaft Be1343/12/1-2, the Fonds der chemischen Industrie, and the Volkswagen Stiftung.
References:

1. Bernhardt, R. (1996) *Rev.Physiol.Biochem.Pharmacol.* **127**, 137-221

2. Miller, W. L. (1995) *J. Steroid. Biochem. Mol. Biol.* **55**(5-6), 607-616

3. Shikita, M., and Hall, P. F. (1974) *Proc. Natl. Acad. Sci. U S A* **71**(4), 1441-1445

4. Grinberg, A. V., Hannemann, F., Schiffler, B., Muller, J., Heinemann, U., and Bernhardt, R. (2000) *Proteins* **40**(4), 590-612.

5. Kimura, T., and Suzuki, K. (1965) *Biochem Biophys Res Commun* **20**(4), 373-9.

6. Watari, H., and Kimura, T. (1966) *Biochem Biophys Res Commun* **24**(1), 106-12.

7. Kimura, T., and Suzuki, K. (1967) *J.Biol.Chem.* **242**, 485-491

8. Chu, J.-W., and Kimura, T. (1973) *J. Biol. Chem.* **248**, 2089-2094

9. Kimura, T., Parcells, J. H., and Wang, H. P. (1978) *Methods. Enzymol.* **52**, 132-142

10. Katagiri, M., Takikawa, O., Sato, H., and Suhara, K. (1977) *Biochem. Biophys. Res. Commun.* **77**(2), 804-809.

11. Takikawa, O., Gomi, T., Suhara, K., Itagaki, E., Takemori, S., and Katagiri, M. (1978) *Arch. Biochem. Biophys.* **190**(1), 300-306.

12. Lambeth, J. D., and Kamin, H. (1976) *J Biol Chem* **251**(14), 4299-306.

13. Lambeth, J. D., McCaslin, D. R., and Kamin, H. (1976) *J. Biol. Chem.* **251**(23), 7545-7550

14. Lambeth, J. D., and Kamin, H. (1979) *J.Biol.Chem.* **254**, 2766-2774

15. Lambeth, J. D., Seybert, D. W., and Kamin, H. (1979) *J Biol Chem* **254**(15), 7255-64.
16. Lambeth, J. D., Seybert, D. W., and Kamin, H. (1980) *J. Biol. Chem.* **255**(10), 4667-4672

17. Lambeth, J. D., Seybert, D. W., Lancaster, J. R., Jr., Salerno, J. C., and Kamin, H. (1982) *Mol Cell Biochem* **45**(1), 13-31.

18. Hanukoglu, I., and Jefcoate, C. R. (1980) *J. Biol. Chem.* **255**(7), 3057-3061.

19. Schiffler, B., Kiefer, M., Wilken, A., Hannemann, F., Adolph, H. W., and Bernhardt, R. (2001) *J. Biol. Chem.* **276**(39), 36225-36232.

20. Hannemann, F., Rottmann, M., Schiffler, B., Zapp, J., and Bernhardt, R. (2001) *J. Biol. Chem.* **276**, 1369-1375

21. Schiffler, B., and Bernhardt, R. (2003) *Biochemical and Biophysical Research Communications* **312**(1), 223-228

22. Uhlmann, H. (1995), Humboldt Universität, Berlin

23. Beckert, V., and Bernhardt, R. (1997) *J. Biol. Chem.* **272**(8), 4883-4888

24. Porter, T. D., and Larson, J. R. (1991) *Methods Enzymol.* **206**, 108-116.

25. Sagara, Y., Takata, Y., Miyata, T., Hara, T., and Horiuchi, T. (1987) *J. Biochem. (Tokyo)* **102**(6), 1333-1336.

26. Uhlmann, H., Beckert, V., Schwarz, D., and Bernhardt, R. (1992) *Biochem.Biophys.Res.Commun.* **188**, 1131-1138

27. Huang, J. J., and Kimura, T. (1973) *Biochemistry* **12**(3), 406-409.

28. Sagara, Y., Wada, A., Takata, Y., Waterman, M. R., Sekimizu, K., and Horiuchi, T. (1993) *Biol.Pharm.Bull.* **16**, 627-630

29. Omura, T., and Sato, R. (1964) *J. Biol. Chem.* **239**, 2370-2378

30. Estabrook, R. W., Suzuki, K., Mason, J. I., Baron, J., Taylor, W. E., Simpson, J. P., Purvis, J., and McCarthy, J. (1973) in *Iron-Sulfur Proteins* Vol. Vol. 2, pp. 193-223, Academic Press, New York
31. Zollner, A., Hannemann, F., Lisurek, M., and Bernhardt, R. (2002) Journal of Inorganic Biochemistry 91(4), 644-654
32. Sugano, S., Morishima, N., Ikeda, N., and Horie, S. (1989) Anal. Biochem. 182(2), 327-333
33. Hintz, M., and Peterson, J. (1981) J. Biol. Chem. 256(13), 6721-6728
34. Lambeth, J. D., Geren, L. M., and Millett, F. (1984) J. Biol. Chem. 259(16), 10025-10029.
35. Tuls, J., Geren, L., Lambeth, J. D., and Millett, F. (1987) J. Biol. Chem. 262(21), 10020-10025.
36. Tuls, J., Geren, L., and Millett, F. (1989) J. Biol. Chem. 264(28), 16421-16425.
37. Adamovich, T. B., Pikuleva, I. A., Chashchin, V. L., and Usanov, S. A. (1989) Biochim. Biophys. Acta 996(3), 247-253
38. Wada, A., and Waterman, M. R. (1992) J.Biol.Chem. 267(32), 22877-22882
39. Ivanov, Y. D., Usanov, S. A., and Archakov, A. I. (1999) Biochem. Mol. Biol. Int. 47(2), 327-336
40. Brandt, M. E., and Vickery, L. E. (1993) J.Biol.Chem. 268, 17126-17130
41. Chu, J., and Kimura, T. (1973) J. Biol. Chem. 248, 5183-5178
42. Lambeth, J. D., and Kamin, H. (1977) J Biol Chem 252(9), 2908-17.
43. Vickery, L. E. (1997) Steroids 62, 124-127
44. Janin, J. (1997) Proteins 28(2), 153-61.
Table 1:

Binding parameters for the different reaction partners of the bovine CYP11A1 system as determined from measurements with a Biacore 2000 system. Adx was immobilized on a CM5 chip (approx. 200 RU). Analyte concentration (AdR or CYP11A1) were in the range from 10 nm to 500 nm. Binding curves showing the interaction between the corresponding interaction partners, were analyzed using the Biacore evaluation software 3.1. All obtained curves were best described by a 1:1 binding mechanism.

| Protein:protein-interaction | $k_{\text{on}}$ [M$^{-1}$s$^{-1}$] | $k_{\text{off}}$ [s$^{-1}$] | $K_d$ ($k_{\text{off}}/k_{\text{on}}$) [M] |
|-----------------------------|-----------------------------------|-----------------------------|------------------------------------------|
| Adx/AdR                     | 4434                              | 0.0038                      | 0.856 $\times 10^{-6}$                  |
| Adx/CYP11A1                 | 691000                            | 0.00926                     | 0.0134 $\times 10^{-6}$                |
Legends to the Figures:

Figure 1:
Sequential reduction of AdR (2 µM), Adx (4 µM) and CYP11A1 (2 µM) followed within "one shot". NADPH (200 µM) from one syringe was mixed with the oxidized proteins of the CYP11A1 system. The insert shows the first 250 ms of the reaction followed after mixing. Two clearly separated phases can be observed.

Figure 2:
Velocity of reduction of AdR (filled circles) and Adx (open circles) at different ionic strength derived from “one shot” measurements. AdR (2 µM) is reduced by NADPH (200 µM) in the presence of oxidized Adx (4 µM) and CYP11A1 (2 µM). The data correspond to the first phase observed in the “one shot” measurements. In the second phase observed in the “one shot” measurements, reduction of Adx can be followed. The standard deviation of each measurement was below 5% of the plotted values.

Figure 3:
Velocity of reduction of CYP11A1 (2 µM) by Adx (4 µM) at different ionic strength derived from “one shot” measurements (filled circles) in comparison to apparent rate constants of the reduction of CYP11A1 by pre-reduced Adx (open circles). In the latter case, Adx was aged in one syringe together with NADPH (200 µM) and AdR (2 µM) to yield reduced Adx. The standard deviation of each measurement was below 5% of the plotted values.
Figure 4:

(A) Reduction of AdR (10 µM) by a 100-fold excess NADPH. The reaction was followed at 450 nm, the absorbance maximum of oxidized AdR. Residuals are given in the plot below the reaction trace. The correlation coefficient for the fit (dotted grey line) is $R = 0.99$. (B) Reduction of AdR (10 µM) by equimolar NADPH followed at 450 nm. The fit is given as dotted grey line, the correlation coefficient is $R = 0.99$. Residuals are plotted below of the reaction trace.

Figure 5:

Reduction of Adx (4 µM) and AdR (2 µM) by NADPH (400 µM, syringe 1) (A). NADPH was mixed with the oxidized proteins. The reaction was followed at 450 nm. The fit is given as a dotted grey line. The correlation coefficient for the fit is $R = 0.99$ (B) Reduction of Adx (4 µM) by pre-reduced AdR (2 µM). NADPH (200 µM) and AdR (syringe 1) were mixed with Adx (syringe 2). The reaction was followed at 414 nm, as indicated. The dotted grey line indicates the fit. The correlation coefficient for the fit is $R = 0.99$. The residuals are given below of the reaction trace.

Figure 6:

$K_{cat}/K_m$ (i.e. the catalytic efficiency) of the CYP11A1 dependent conversion of cholesterol to pregnenolone. The dependence on the ionic strength is shown. Recombinant AdR (0.5 µM) and Adx (varying amounts from 0.2 µM to 3.2 µM) was used as well as CYP11A1 (0.5 µM) purified from adrenals.

Figure 7:

Schematic overview of the CYP11A1 electron providing chain. The apparent reduction rates measured in the “one shot” measurements at 70 mM salt are given in
seconds. AdR is reduced by NADPH as indicated. Subsequently, the one electron carrier Adx transfers electrons from reduced AdR to CYP11A1. For a successful cholesterol conversion to yield pregnenolone 6 electrons have to be provided (i.e. 3 NADPH molecules are oxidized).
Figure 1:
Figure 2:

\[ k_{\text{app}} \text{ [s}^{-1}\text{]} \]

KCl [mM]
Figure 3:

![Graph showing the relationship between KCl concentration and $K_{app}$](http://www.jbc.org/Downloaded from)
Figure 4A:
Figure 4B:

![Graph showing Time vs. Rel. Absorbance at 450 nm and Time vs. Residuals](image-url)
Figure 5A:
Figure 5B:

[Graph showing time in seconds on the x-axis and residual absorbance at 414 nm on the y-axis. The graph is labeled with time values from 0.0 to 2.5 and residual absorbance values from -0.015 to 0.0015.]
Figure 6:

![Graph showing the relationship between KCl concentration (mM) and $K_{cat}/K_m$]
Figure 7:

\[
\begin{align*}
\text{NADPH} + \text{H}^+ &\rightarrow \text{AdR}_{\text{ox}} (\text{FAD}) \\
&\rightarrow \text{AdR}_{\text{red}} (\text{FADH}_2) \\
&\rightarrow \text{AdR}_{\text{ox}} (\text{FAD}) \\
&\rightarrow \text{Adx}_{\text{ox}} \\
&\rightarrow \text{Adx}_{\text{red}} (\text{FADH}_2) \\
&\rightarrow \text{Adx}_{\text{ox}} \\
&\rightarrow \text{CYP11A1}_{\text{ox}} \\
&\rightarrow \text{CYP11A1}_{\text{red}} \\
\text{Cholesterol} + 3 \text{O}_2 &\rightarrow \text{Pregnenolone} + 3 \text{H}_2\text{O}
\end{align*}
\]
Stripping down the mitochondrial cholesterol hydroxylase system - A kinetic study
Burkhard Schiffler, Andy Zöllner and Rita Bernhardt

J. Biol. Chem. published online June 4, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402798200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts