2. EXPERIMENTAL PART

2.1 Materials and Methods

2.1.1 Preparation of chemically competent *E. coli* cells

Chemically competent cells of a desired *E. coli* strain were prepared by the RbCl method[12]. 5 ml of LB (Luria Bertani) medium was inoculated with the appropriate *E. coli* strain and incubated overnight at 37 °C, 160 rpm. Next morning, 1 ml of the overnight culture was added to 500 ml freshly prepared LB medium and incubated at 37 °C, 160 rpm, until the absorbance at 600 nm reached O.D. of ~0.6-0.7. Cells were then chilled for at least 10 min on ice before they were spun. Centrifugation was performed for 10 min at 5300 rpm and 4 °C. All next steps were performed on ice. The pellet was gently resuspended in 30 ml ice-cold TFBI buffer (Appendix B, Table B.1) and incubated on ice for 10 min. The cells were again centrifuged (as above), and the pellet was resuspended in 9 ml of TFBII buffer (Appendix B, Table B.2). The cell suspension was aliquoted in 100 μl per tube and each aliquot was immediately frozen in liquid nitrogen. Competent cells were stored at -70 °C before use. They remained competent for at least 6 months.

2.1.2 Transformation

For initial cloning, plasmid DNA was transformed to One Shot® TOP10 Electrocomp™ Cells (Invitrogen GmbH, Karlsruhe, Germany) for maximum efficiency[13]. RbCl-competent cells were used with a plasmid, isolated using standard mini- or maxi-prep procedures (QIAGEN GmbH, Hilden, Germany), after successful initial cloning. Frozen (-70 °C) competent cells were thawed on ice for 5 min. 1-2 μl of a ligation reaction or purified plasmid DNA was added to the competent cells and incubated on ice for 10 min. Then, the tube was heated at 42 °C for 1-2 min, and placed back on ice for 10 min. Further, 1 ml of pre-chilled LB medium was directly added to the sample and incubated in a thermoshaker at 37 °C for 0.5-1 h. During this time, agar plates were removed from 4 °C storage and placed in the 37 °C incubator to prevent possible condensation in the Petri-plates prior to plating. In order to increase the transformation yield, cells were spun to reduce the volume by decanting the supernatant. Finally, 50-100 μl of cells

[12] Competent cells have a transformation frequency of 10^6-10^7 colonies per microgram of DNA.

[13] TOP10 *E. coli* are provided at a transformation efficiency of 1×10^9 cfu/μg supercoiled DNA and are ideal for high-efficiency cloning and plasmid propagation. They allow stable replication of high-copy number plasmids.

were spread on plates with appropriate antibiotics and placed in an incubator overnight at 37 °C. Next morning, a single colony was selected for sequence verification (plasmid DNA from miniprep was subjected to sequencing). If the expected sequence was confirmed, analyzed plasmid DNA was further used for transformation in chemically competent cells.

2.1.3 Cloning and expression of recombinant bovine Adx(N-6×His tag/Xa/1-108)

The plasmid pET3d-AdxZNT108 encoding the Adx(1-108) fragment, with an N-terminal-6×His tag followed by a Factor Xa (Xa) cleavage site (Adx(N-6×His tag/Xa/1-108)) was kindly provided by Dr. F. Hannemann. A single colony of BL21(DE3)pLysE CodonPlus *E. coli* strain with pET3d-AdxZNT108 from the LB agar plate or *E. coli* from the glycerol stock was used for inoculation of an overnight culture, which was then incubated at 37 °C, 160 rpm in 10 ml of LB medium. The 8 L main LB culture (1 L×8) was inoculated using 1 ml of the overnight culture and then incubated again at 37 °C, 140 rpm, for the next 24 h. Always, ampicillin and chloramphenicol were added to the LB medium at their final concentrations of 100 μg/ml and 50 μg/ml, respectively. Further, an overnight induction with 0.001 M isopropyl-β-D-thiogalactopyranoside (IPTG) was carried out at 30 °C. Before adding IPTG, the medium was cooled down to 30 °C. After induction, cells were placed in the cold room for 20 min and then harvested at 4 °C, 5000 rpm, for 10 min. Pellet was frozen and stored at –70 °C before use.

2.1.4 Cloning and expression of recombinant bovine wild-type adrenodoxin

The plasmid pKKAdx, which encodes the sequence of 128 amino acids of wild-type Adx with a Ser1-to-Gly1 exchange was a kind gift of Dr. A. Lapko. Competent BL21 cells were transformed with the plasmid according to the standard transformation protocol and spread on agar plates with ampicillin (100 μg/ml, final concentration). Next morning, a single colony was picked with a sterile loop or a tip to prepare a 10 ml overnight culture. The culture was incubated at 37 °C with shaking at 180 rpm. 1 ml of the overnight culture was inoculated in a 1 L flask with LB medium containing ampicillin (100 μg/ml, final concentration). 8×1 L LB cultures were incubated at 37 °C and 150 rpm. Expression was started adding 1 ml of 1 M IPTG (0.001 M final concentration) per each flask. Prior to addition of the inductor, the medium was cooled to 30 °C.

Induction of expression followed 12-24 h. Finally, cells were first prechilled on ice and then harvested at 4 °C, 5000 rpm, for 10 min. Pellet was frozen and stored at –70 °C before use.

2.1.5 Cloning and expression of recombinant bovine adrenodoxin reductase

E. coli shows a tendency, especially for eukaryotic proteins like AR, to express the synthesized peptide in inclusion bodies [Hartl 1996]. The amount of correctly folded protein, however, can be increased by coexpression of chaperones [Cole 1996; Dionisi et al., 1998]. A high-yield expression system that allowed purification of AR from which X-ray-grade crystals could be grown has been recently developed [Vonrhein et al., 1999]. Recombinant bovine AR was co-expressed with the hsp60-chaperone system. The plasmid pBAR1067 encoding AR [Sagara et al., 1993] and pREP4-groESL encoding the hsp60-chaperone system were kindly provided by Dr. A. Lapko.

E. coli BL21(DE3) harboring both plasmids, pBAR1067 and pREP4-groESL, were grown overnight at 37 °C in 10 ml of LB medium with 100 μg/ml of ampicillin and 25 μg/ml kanamycin. 8×1 L flasks with LB medium were inoculated with 1 ml of the overnight culture, and antibiotics were added. Flasks were incubated at 37 °C overnight with constant shaking at 160 rpm. Before induction with 0.001 M IPTG, cells were cooled to 30 °C. After addition of IPTG, cells were cultivated for 12-16 h and then harvested by centrifugation at 4 °C. If the pellet was not used, it was stored at -70 °C.

2.1.6 Protein purification

AR, Adx(WT), Adx(N-6×His tag/Xa/1-108), and $P450_{scc}$ were purified according to their specific purification strategies (Scheme B.1 in Appendix B, for buffer recipes also see Appendix A and Appendix B). All proteins were purified at 4 °C to minimize degradation. Recombinant proteins, AR, Adx(WT), Adx(N-6×His tag/Xa/1-108) were purified after induction of their expression in *E. coli*. Cells were chilled on ice for 20 min and then collected by centrifugation at 4° C, 5000 rpm for 10 min. Usually, the pellet was used immediately for protein purification. Alternatively, the pellet was washed with suitable buffer twice and frozen at -70 °C until use.

Purification of recombinant proteins was started by disrupting of *E. coli* cells in a FRENCH® Press (FP). 1 ml of 10 mg/ml phenylmethylsulfonyl fluoride (PMSF) (Merck KGaA,

Darmstadt, Germany) or 1 ml of Protease Inhibitor Cocktail Set III (without ethylenediaminetetra acetic acid (EDTA)) (Calbiochem-Novabiochem GmbH, Schwalbach/Tc., Germany) per 20 g of *E. coli* cells were added before the FP procedure to inhibit host proteases. Further, *E. coli* lysate was incubated with some crystals of deoxyribonuclease I (Roche Diagnostics GmbH, Mannheim, Germany) to prevent unwanted cell clumping, which is known to occur during tissue disaggregation procedures [Carlsen 1981; Hornsby 1980], for 30 min at room temperature or, alternatively, for 2 h at 4 °C. Cells debris and soluble fraction were separated by centrifugation at 4 °C, 20000 rpm for 30 min. Supernatant was further used for protein purification.

5 kg of fresh bovine adrenal glands were used for preparation of native $P450_{scc}$ [Suhara et al., 1978]. The glands were homogenized in 0.05 M potassium phosphate buffer, pH 7.2 with 20% glycerol (Carl Roth, GmbH + Co. KG, Karlsruhe, Germany) (buffer A) using BLENDOR (Dynamic Corporation of America, USA). Then, the homogenized suspension was centrifuged at 4 °C, 3000 rpm for 15 min to get the mitochondrial fraction into the supernatant. The pellet (destroyed glands) was re-homogenized to increase the yield of mitochondria. All collected supernatant was again re-centrifuged at 4 °C, 10000 rpm for 30 min to isolate mitochondria. Organelles were frozen in buffer A adding 10% glycerol at –70 °C until use.

2.1.6.1 Purification of native side-chain cleavage cytochrome P450

Mitochondria were thawed at 4 °C and mixed with buffer A in a 1:1 ratio. This homogeneous solution was then centrifuged at 4 °C, 10000 rpm for 40 min. Pellet (mitochondria) was solubilized with 0.4% sodium cholate (final concentration) (Fluka Chemie GmbH, Buchs, Swiss) in buffer A for 2 h at 4 °C. The cholate extract was further fractionated between 27 and 43% saturation of ammonium sulfate ($AmSO_4$) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) with overnight incubation at 4 °C [Suhara et al., 1978]. The precipitant was added in small portions to the protein solution by constant mixing and pH checking. Every new portion of solid $AmSO_4$ was used when the previous was dissolved and no changes in pH were observed. The pH was always maintained as a constant value of 7.2 adjusting with 5 N KOH (Merck, KGaA, Darmstadt, Germany). The resulting precipitate of the first salting-out was dissolved in buffer A and reprecipitated with $AmSO_4$ to 40% saturation. The precipitate was collected by centrifugation, dissolved in buffer A, and applied to a Cholate-Sepharose 4B column pre-equilibrated with buffer A. The column was washed with equilibrating buffer and

then with the same buffer, containing 0.05% sodium cholate and 0.3 M KCl (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). $P450_{scc}$ was eluted with buffer A, containing 0.3% sodium cholate and 0.3 M KCl. The fractions were pooled and applied to an adrenodoxin-Sepharose 4B (coupling of Adx to CNBr-activated Sepharose 4B, see in Appendix B) column also pre-equilibrated with buffer A. To promote better binding to the Adx-Sepharose, where an immobilized Adx functions as the natural ligand for the side-chain cleavage cytochrome, the whole fraction of $P450_{scc}$ after Cholate-Sepharose 4B was diluted 6 times with buffer A. Cytochrome $P450_{scc}$ was eluted with buffer A, containing 0.3% sodium cholate and 1 M KCl. Finally, $P450_{scc}$ homogeneity was analyzed by gel filtration on a HiLoad™ 26/60 Superdex™ 200 (Amersham Pharmacia Biotech, Freiburg, Germany). The purity of the cytochrome was estimated according to the A_{393}/A_{276}[14] ratio and 12% polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE). $P450_{scc}$ concentration was determined using the carbon monoxide (CO) difference spectrum assuming $\varepsilon_{450\text{-}490} = 91$ $(\text{mM cm})^{-1}$ [Omura & Sato, 1964]. The protein was concentrated in the last elution buffer, aliquoted in 200 µl samples, flash-frozen in liquid nitrogen, and then stored at -70 °C.

2.1.6.2 Purification of recombinant bovine wild-type adrenodoxin

The first purification step involved two ammonium sulfate precipitations (ASP) in 0.05 M Tris/HCl buffer pH 7.4 (buffer B) at 4 °C: (i) 0-30% of $AmSO_4$; (ii) 30-60% of $AmSO_4$ [Uhlmann et al., 1992], which were separated by centrifugation. The precipitant was added in the same manner as for $P450_{scc}$. The protein solution was finally saturated with 2.2 M $AmSO_4$. As next step, Fractogel TSK Butyl-650 (S) (Merck KGaA, Darmstadt, Germany) pre-equilibrated with 0.02 M Tris/HCl, pH 9.0 containing 2.2 M $AmSO_4$, was used to concentrate Adx. The protein was eluted from the column with a descending gradient of $AmSO_4$ (2.2-1.2 M) in 0.02 M Tris/HCl, pH 9.0. The Adx-containing fractions were collected, diluted, and applied to DEAE 52-Cellulose SERVACEL® (SERVA Electrophoresis GmbH, Heidelberg, Germany). Adx was chromatographed with a linear gradient (0-50%) of 1 M KCl in buffer B. Finally, Adx homogeneity was analyzed by Fast Protein Liquid Chromatography (FPLC) on a HiLoad™ 26/60 Superdex™ 200 in buffer B with 0.3 M KCl and 20% glycerol. The purity of Adx was

[14]Spectrophotometrical control of the purity of all proteins, studied in this thesis was done with the UV-vis scanning spectrophotometer UV-2102 (Kyoto, Japan).

estimated according to the A_{414}/A_{276}[15] ratio and on 18% SDS-PAGE. The concentration was determined using $\varepsilon_{414} = 9.8$ (mM cm)$^{-1}$ [Huang & Kimura, 1973]. The protein was concentrated in the last elution buffer and flash-frozen in liquid nitrogen in 200 µl samples. They were stored at -70 °C.

2.1.6.3 Purification of recombinant bovine adrenodoxin reductase

At the beginning, the same gradient of ASP as for Adx(WT) was used in purification of AR. The pellet of the second salting-out step obtained after centrifugation at 4 °C, 19950 rpm was dissolved in buffer A, and the solution was dialyzed against buffer A to decrease $AmSO_4$ concentration. Serva VISKING® dialysis tubing with 12-14 kDa molecular-weight cutoff was used. The buffer was changed every 1 h to speed up the dialysis. After dialysis, the solution was centrifuged at 4 °C, 10000 rpm, for 20 min. Supernatant was diluted 10 times with dialysis buffer and applied to Adx-Sepharose 4B. Then, the resin was washed with the same buffer to minimize unspecific binding. Elution was carried out with 0.7 M KCl in buffer A. AR was further purified on 2',5'-ADP-Sepharose 4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) using the same elution buffer (prior to loading, AR was diluted 10 times for better binding). The purity of AR was estimated according to the A_{280}/A_{450} ratio and 12% SDS-PAGE. The concentration was determined using $\varepsilon_{450} = 10.9$ (mM cm)$^{-1}$ [Chu & Kimura, 1973]. The protein was concentrated in the last elution buffer, aliquoted in 200 µl samples, then flash-frozen in liquid nitrogen, and stored at -70 °C.

2.1.6.4 Purification of recombinant bovine Adx(N-6×His tag/Xa/1-108)

Immobilized-metal affinity chromatography (IMAC) with Ni-NTA agarose (Qiagen GmbH, Hilden, Germany) was the first purification step, instead of ASP. The 6×His affinity tag of the recombinant Adx(N-6×His tag/Xa/1-108) facilitates its binding to Ni-NTA[16]. Purification was carried out under native conditions.

Since there is a higher potential for binding background contaminants under native conditions than under denaturing conditions, low concentrations of imidazole (0.02 M) in the

[15] The resolution of the Adx spectra was improved at the temperature of liquid nitrogen. The sample after flash-freezing was placed in DN1704 Variable Temperature Liquid Nitrogen Cryostat (Osney Mead, OXFORD). The temperature was controlled using a DTC2 controller (OXFORD). The spectra were measured with the UV-vis scanning spectrophotometer UV-2102 (Kyoto, Japan).

[16] Nitrilotriacetic acid (NTA) is a tetradentate chelating agent which occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6×His tag.

lysis and wash buffers are recommended. Adx(N-6×His tag/Xa/1-108) was eluted with 0.25 M imidazole (Merck KGaA, Darmstadt, Germany) in buffer B. Further, Adx was purified using DEAE 52-cellulose SERVACEL® and gel filtration (FPLC, HiLoad™ 26/60 Superdex™ 200S) with the same buffer composition, as described for wild-type form. The purity of Adx(N-6×His tag/Xa/1-108) was estimated according to the A_{414}/A_{276} ratio and 18% SDS-PAGE. The concentration was determined using $\varepsilon_{414} = 9.8$ $(mM\ cm)^{-1}$ [Huang & Kimura, 1973]. The protein was concentrated in the last elution buffer adding 20% glycerol, aliquoted in 200 µl samples, flash-frozen in liquid nitrogen, and then stored at -70 °C.

2.1.6.5 Cleavage of the N-terminal 6×His tag of Adx(N-6×His tag/Xa/1-108)

The Factor Xa Cleavage Capture Kit is designed for highly specific cleavage of fusion proteins followed by convenient affinity-based capture and removal of Xa. The preferred cleavage site of Xa is IEGR [Nagai et al., 1985]. After cleavage, Xa can be removed from the reaction by affinity capture on Xarrest™ agarose.

For enzymatic cleavage of the fusion 6×His tag, frozen stock solution of Adx(N-6×His tag/Xa/1-108) or the protein after gel filtration was used. Adx was first concentrated to a final volume of ≤ 0.5 ml and final concentration ≤ 0.001 M in 0.05 M Tris/HCl (cleavage buffer), pH 8.0, 0.1 M NaCl, 0.005 M $CaCl_2$. The reaction was carried out with 10:1 (Adx:Xa) molar ratio at 4 °C for 20 h to prevent possible protein degradation at room temperature. After incubation time, Xa was inhibited with 0.001 M Pefablock® SC (PENTAPHARM Ltd., Basel, Switzerland) at room temperature (15 min), before the reaction mixture was applied to the affinity capture Xarrest™ agarose. Then, the sample was loaded, gently mixed with the agarose, and incubated for 5 min to increase the binding of Xa. Adx was recovered by spin-filtration at room temperature, 2000 rpm for 5 min. Collected protein was applied to a small Ni-NTA test-column to check the completeness of the reaction. 18% SDS-PAGE and electro-spray time-of-flight mass spectrometry (ES-TOF-MS) were performed to determine the molecular weight of the modified protein.

2.1.6.6 Covalent modification of Adx(1-108) with ruthenium(II) bipyridyl complex

FPLC- and ES-TOF-MS-analyzed Adx(1-108) was used for covalent modification with solid (4-bromomethyl-4′-methyl-2,2′-bipyridine)bis(2,2′-bipyridine)ruthenium(II) bishexafluorophosphate[17] as described for *Pseudomonas putida* P450 ($P450_{cam}$) (Figure 2.1.6.6.1) [Contzen et al., 2002].

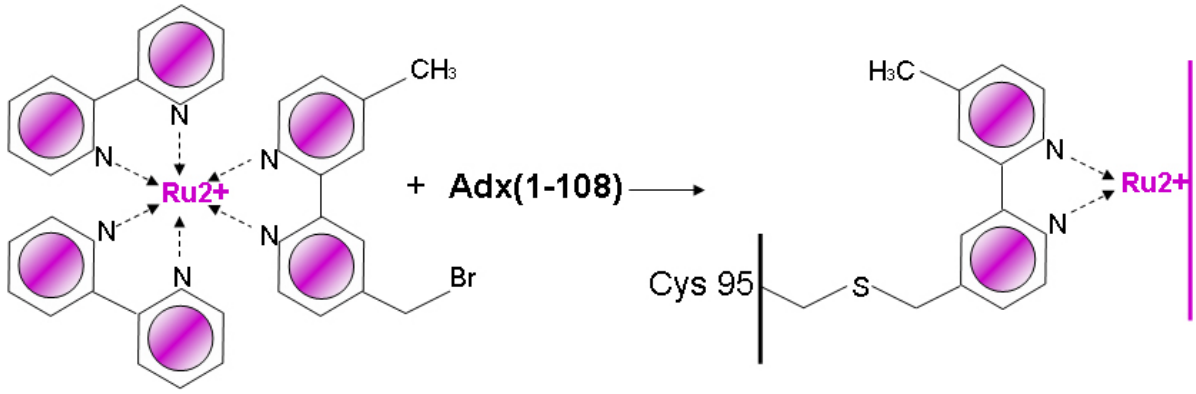

Figure 2.1.6.6.1 Covalent modification of Adx(1-108). Coupling of $Ru(bpy)_2(Br\text{-}mbpy)(PF_6)_2$ on Adx(1-108) is followed by the nucleophilic substitution of the 4-bromomethyl group of the bipyridyl ligand by the thiol group of Cys95 with a formation of a thioether.

Adx(1-108) was concentrated to a final volume 0.5 ml and concentration of ≤ 0.001 M in 0.1 M potassium phosphate buffer, pH 8.0 and then pre-incubated (4 °C, 30 min) with threefold molar excess of 1,4-dithiothreitol (DTT) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) to avoid formation of disulfide bonds. After that time, a tenfold molar excess of $Ru(bpy)_2(Br\text{-}mbpy)(PF6)_2$ was dissolved in dimethyl sulfoxide (DMSO) (Merck KGaA, Darmstadt, Germany) and added to an aliquot of protein, and then the reaction mixture was incubated with slight shaking for 2 h in the dark. The reaction was stopped by removing excess of reagents on Sephadex™ G-25 M (Amersham Pharmacia Biotech, Freiburg, Germany), pre-equilibrated with buffer B at 4 °C. The crude mixture was further purified on an anion-exchange Bioscale Q2 column using a BioLogic high pressure chromatography system (Bio-Rad Laboratories, Inc., München, Germany) with a linear gradient (0-50%) of 1 M KCl in buffer B.

The stoichiometry of $Ru(bpy)_2(mbpy)$ bound to Adx(1-108) was determined by fitting a spectrum (365-700 nm) of each colored fraction with a linear combination of spectra of pure

[17] The compound was provided by Jörg Contzen.

oxidized Adx(1-108) (ε_{414} = 9.8 (mM cm)$^{-1}$) and pure ruthenium complex (ε_{456} = 14.6 (mM cm)$^{-1}$) [Contzen et al., 2002]. All colored fractions were also subjected to 18% SDS-PAGE.

In order to prove that Cys95 is the modification site, the Adx(C95S) mutant was labeled with the ruthenium complex and further purified as described for Adx(1-108). This Adx mutant was kindly supplied by Dr. F. Hannemann.

2.1.7 Electro-spray time-of-flight mass spectrometry

ES-TOF-MS was performed by Dr. E.-C. Müller to check the molecular weights of Adx(1-108) and Ru(bpy)$_2$(mbpy)-Adx(1-108). Mass spectra were obtained using electrospray ionization (ESI) mass spectrometry employing a Q-TOF1 mass spectrometer (Micromass, Manchester, UK) with an accuracy of 0.01%. The protein solutions were desalted on Poros R1 material (Applied Biosystems, Darmstadt, Germany), eluted with 60% acetonitrile, 39% water, 1% formic acid and introduced *via* a Harvard syringe pump at a flow rate of 200 nl/min.

2.1.8 Electron-transfer capability of Adx

Adx(WT), Adx(1-108), and Ru(bpy)$_2$(mbpy)-Adx(1-108) were investigated using the cytochrome *c* reduction [Lapko et al., 1997] and NADPH-induced reduction of P450$_{scc}$ assays [Schwarz et al., 1994].

2.1.8.1 Cytochrome c reduction assay

The kinetics of the cytochrome *c* reduction was investigated in 0.033 M potassium phosphate buffer, pH 7.4 (buffer C), at room temperature. The reaction mixtures (1 ml) contained 60 μM cytochrome *c* (Boehringer Mannheim GmbH, Germany), 1 unit/ml catalase (SERVA Feinbiochemica GmbH & Co. KG, Heidelberg, Germany), 2 μM AR, and variable amount (0.05 μM, 0.1 μM, 0.15 μM, 0.75 μM, 1.25 μM, and 1.5 μM) of the investigated adrenodoxins. The reaction was started adding NADPH (SERVA Feinbiochemica GmbH & Co. KG, Heidelberg, Germany) at final concentration of 0.14 mM. Reduction of cytochrome *c* was monitored by measurement of the increase of absorbance at 550 nm. The activity was calculated using ε_{550} = 20 (mM cm)$^{-1}$ for cytochrome *c*.

2.1.8.2 NADPH-induced reduction of cytochrome $P450_{scc}$

This assay was used to check whether the covalent modification of Adx(1-108) with $Ru(bpy)_2(mbpy)$ may switch off the ferredoxin function as an electron carrier. Enzymatic reduction of $P450_{scc}$ was studied in the reconstituted steroid hydroxylase system in buffer C at room temperature. The reaction mixture (1 ml) containing 0.67 μM $P450_{scc}$, 3.34 nM AR, and 0.33-1.67 μM $Ru(bpy)_2(mbpy)$-Adx(1-108) was treated with CO for 2 min avoiding foam formation in the sample. Samples with Adx(WT) and Adx(1-108) were also prepared in the same manner as described for coupled Adx(1-108). The reaction was initiated adding NADPH-regenerating system (final concentrations were 0.14 mM NADPH, 0.002 M glucose-6-phosphate (SERVA Feinbiochemica GmbH & Co. KG, Heidelberg, Germany), and 1 unit/ml glucose-6-phosphate dehydrogenase (SERVA Feinbiochemica GmbH & Co. KG, Heidelberg, Germany). The formation of the CO-heme complex was detected spectrophotometrically by an appearance of the peak at 450 nm.

2.1.9 Redox potential measurements

Redox potentials of Adx(WT), Adx(1-108), and $Ru(bpy)_2(mbpy)$-Adx(1-108) were determined at 298 K using the dye photoreduction method with safranin T (Sigma-Aldrich, Steinheim, Germany) as an indicator and mediator [Sligar & Gunsalus, 1976]. $P450_{cam}$ was used as well studied as a control one-electron acceptor protein instead of $P450_{scc}$, because the latter has been less investigated. The concentration changes of Adx(WT), Adx(1-108), $Ru(bpy)_2(mbpy)$-Adx(1-108), and $P450_{cam}$ during illumination were followed by difference spectroscopy.

The reaction conditions (2.6 ml) for each protein were 0.1 M potassium phosphate buffer pH 7.3, containing 0.01 M EDTA (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), freshly prepared glucose (8 mg/ml; final concentration), 18 μl of 0.001 M safranin T, and oxygen-scavenger system (4 mg/ml glucose oxidase and 2 mg/ml catalase). First, a protein solution was stirred with N_2 for 30 min in a black precision cell of quartz (HELLMA® GmbH & Co. KG, Müllheim, Germany) and finally incubated with the oxygen-scavenger system in the dark for the next 30 min. All oxidized and reduced spectra were obtained anaerobically. Reduction was started by illumination (3-5 s) of a sample with a 500 W Xenon lamp and then a spectrum was immediately measured. 10-15 illuminations were carried out recording the spectra. Then, the

reduction was completed adding some crystals of sodium dithionite to reach 100% reduction level. The redox potentials were calculated from the Nernst equation (2.1.9.1):

$$E = E_o + \frac{RT}{nF} \cdot 2.303 \cdot \log \frac{[C_{ox}]}{[C_{red}]} \qquad (2.1.9.1)$$

where *R, T, F* and *n* are the gas constant (8.314 J mol^{-1} K^{-1}), temperature (K), the Faraday constant (96485 C), and the number of transferred electrons, when the reaction is completed, respectively. E_o is the standard redox potential given in mV. $[C_{ox}]$ and $[C_{red}]$ represent the concentrations of the oxidized and reduced components, respectively. At the equilibrium conditions, where the potentials of the dye and an enzyme are equal ($E_{dye} = E_{enzyme}$), the Nernst equation may be reformulated according to Eqn. 2.1.9.2:

$$E_{0(dye)} + 2.303 \cdot \frac{R \cdot T}{n_{(dye)} \cdot F} \cdot \log \frac{[C_{ox(dye)}]}{[C_{red(dye)}]} = E_{0(protein)} + 2.303 \cdot \frac{R \cdot T}{n_{(protein)} \cdot F} \cdot \log \frac{[C_{ox(protein)}]}{[C_{red(protein)}]} \qquad (2.1.9.2)$$

where *R, T,* and *F* are as in Eqn.2.1.9.1, and $n_{(protein)}$ and $n_{(dye)}$ are the number of electrons transferred in the reaction for each redox pair of a protein (Adx or $P450_{cam}$) and the dye, respectively. $E_{0(dye)}$ and $E_{0(protein)}$ are the standard redox potentials given in mV. For safranin T, $n_{(dye)}$ is 2, giving a pre-logarithmic factor of ~30 in Eqn. 2.1.9.2.

2.1.10 Photoreduction of the [2Fe-2S] cluster of Adx(1-108)

Adx(1-108) in buffer B containing 0.1 M EDTA and 10% glycerol was checked for the effect of irradiation on its stability. The sample (0.1 ml) was gently stirred with nitrogen for 1 h before illumination in a black precision quartz cell with a small optical window (2 mm × 2 mm). The sample was then irradiated for 60 min (in 10 min intervals) at 25 °C using the argon ion laser INNOVA® 90 (Coherent, Inc., Palo Alto, USA) at 457.9 nm with the final output of 60 mW. The stability was controlled spectrophotometrically.

The 1:1 $Ru(bpy)_2(mbpy)$-Adx(1-108) sample was prepared in the same manner and exposed for 20 to 40 min in an EPR tube and 75 min for spectroscopic studies in a precision cell. Photoreduction was monitored by the decrease of the absorbance at 414 nm.

The observed reduction rates (k_{obs}) were derived from the slope of the plot *ln(1-α) versus* illumination time *t*, where α is the fraction of the photoreduced Adx(1-108). Rates k_{et} at three

different $Ru(bpy)_2(mbpy)$-Adx(1-108) concentrations (0.01, 0.05, and 0.17 mM) were calculated using the equation (2.1.10.1) according to *Contzen et al.* [Contzen et al., 2002]:

$$k_{et} = \frac{k_d}{\frac{I_0 \cdot \kappa \cdot d}{k_{obs}} - 1} \qquad (2.1.10.1)$$

where k_d summarizes all decay processes of the excited ruthenium complex [Contzen et al., 2002]. Its value of $(7\pm1)\times10^6$ s^{-1} was determined for $Ru(bpy)_3$-cytochrome *c* [Geren et al., 1991; Durham et al., 1989] and assumed to be also an approximate value for the ruthenium complex used here. I_0, κ, and d are the intensity of the laser, extinction coefficient of the ruthenium complex absorption at 457.9 nm, and optical path length, respectively. The error of k_{et} is calculated taking into account the errors, arising from fluctuations of the illuminating laser power (±0.5%), and the errors of k_d, and k_{obs}. The rate constant k_{et} consists of two contributions (Eqn. 2.1.10.2). The intermolecular (k_{et2}) rate constant was determined from the slope in the plot k_{et} *versus* the concentration of $Ru(bpy)_2(mbpy)$-Adx(1-108). The intramolecular rate (k_{et1}) is obtained from the extrapolation of the $Ru(bpy)_2(mbpy)$-Adx(1-108) concentration to zero using Eqn. 2.1.10.2:

$$k_{et} = k_{et1} + k_{et2} \cdot [Ru(bpy)_2(mbpy) - Adx(1-108)] \qquad (2.1.10.2)$$

After photoreduction all samples were reoxidized at 4 °C to check the reversibility of the process.

2.1.11 EPR measurements

All EPR measurements were performed by Dr. Marcus Galadner at the Technical University of Berlin. "Negative" control samples (Adx(1-108); $Ru(bpy)_3$ (Sigma-Aldrich, Steinheim, Germany) plus Adx(1-108) in a 1:1 ratio; and just water-soluble ruthenium complex itself) and $Ru(bpy)_2(mbpy)$-Adx(1-108) were thoroughly deoxygenated with nitrogen for 1 h in buffer B, containing also 0.1 M EDTA and 10% glycerol [Shimada et al., 1999]. Then, each EPR tube was placed in the laser beam so that tube length and laser beam were parallel aligned. This ensures an optimal irradiation of the sample. Additionally, the sample with unmodified Adx(1-108) ("positive" control) was reduced in an EPR tube with fresh solid sodium dithionite ($Na_2S_2O_4$). After illumination and chemical reduction, samples were flash-frozen by immersing

the EPR tubes into liquid nitrogen. EPR measurements were performed on an X-band (9.5 GHz) EPR spectrometer (Bruker 300E, Germany) equipped with an Oxford ESR 9 helium flow cryostat at 80 K with modulation frequency, 12.5 kHz; microwave power, 5 mW; modulation amplitude 5 G (10 G for the illuminated sample of $Ru(bpy)_2(mbpy)$-Adx(1-108)). The spin quantification of Adx(1-108) was carried out using $CuSO_4$ as a standard [Watari & Kimura, 1966].

2.1.12 X-ray crystallography

2.1.12.1 Crystallization of the $Ru(bpy)_2(mbpy)$-Adx(1-108) complex

The complex was crystallized by the hanging-drop vapor-diffusion method, as described for Adx(4-108) [Müller et al., 1998] (0.1 M Tris, 30% PEG 4000, 100 mM $MgCl_2$, 200 mM glucose, 0.02% NaN_3, 10% glycerol). The Linbro 24-well crystallization plates and circular siliconized cover slides (Jena Bioscience GmbH, Jena, Germany) were used for setting up the crystallization conditions. First small crystals were obtained after one day at 10 °C. These crystals were used for data collection at beamline BL1 (PSF, BESSY, Berlin)[18]. After screening of protein concentration (~20-25 mg/ml), bigger crystals were obtained within one week at 4 °C. Data was also collected from a big crystal at 90 K on a MAR-165 CCD at X06SA-PX (Swiss Light Source, Villigen, Switzerland). Processing, scaling and conversion to structure factor amplitudes were done with DENZO/SCALEPACK (small crystal) [Otwinowski & Minor, 1997] and with XDS (by Dr. J. J. Müller, big crystal) [Kabsch 2001]. The section 2.1.12.2 describes determination and refinement of crystallographic data, which was collected from the big crystal.

2.1.12.2 Structure determination and refinement

An initial structure of the 1:1 $Ru(bpy)_2(mbpy)$-Adx(1-108) complex was determined by molecular replacement using AMoRe [Navaza, J., 1994] and Adx(4-108) [Müller et al., 1998] (PDB entry 1AYF) as a search model. The structure was refined with REFMAC [Murshudov et al., 1997] at 20–1.5 Å resolution, including: (i) isotropic refinement of Adx(1-108) without the ruthenium complex; (ii) placement of water molecules; (iii) positioning of the ruthenium moiety into the remaining difference density and subsequent refinement. The coordinates of the ruthenium complex isomers were taken from the PDB entry 1K2O [Dunn et al., 2001].

[18] This data set was used for crystal structure determination and refinement, using AMoRe [Navaza, J., 1994] and REFMAC [Murshudov et al., 1997], but it was not completed. More information is written in the section 2.2.9.

Refinement results and fitting of the ruthenium complex were analyzed using the graphical display program O [Jones et al., 1991]. Grouped TLS refinement produced the final model. The structure was validated with PROCHECK [Laskowski et al., 1993; Morris et al., 1992] and WHAT_CHECK [Hooft et al., 1996] at 1.5 Å resolution.

The density of the ruthenium complex and the side chain of Cys95 was improved by Dr. J. J. Müller using the program EDEN [Szoke 1993]. Possible electron transfer pathways within the $Ru(bpy)_2(mbpy)$-Adx(1-108) complex were calculated using the program HARLEM [Curry et al., 1995], which was provided by Dr. C. Jung. The two crystal structures, $Ru(bpy)_2(mbpy)$-Adx(1-108) and Adx(4-108) (1AYF), were compared by least-squares fitting of their C^{α} positions using the program LSQKAB [Kabsch 1976]. This program was also used to fit C^{α} positions of $Ru(bpy)_2(mbpy)$-Adx(1-108) with those of the unmodified Adx, while the latter was in the modeled complexes with cytochrome *c*, $P450_{scc}$, and in the crystal structure of the cross-linked complex AR-Adx. However, such fitting does not represent direct modeling of $Ru(bpy)_2(mbpy)$-Adx(1-108)-cytochrome *c*, $Ru(bpy)_2(mbpy)$-Adx(1-108)-$P450_{scc}$, and AR-$Ru(bpy)_2(mbpy)$-Adx(1-108) complexes. It only shows that if Adx would interact with its redox partners as it will do being unmodified by the ruthenium complex, then assuming ruthenation of Adx one can show where the ruthenium complex would be situated during protein-protein interaction.

Using the program XSAE_1.6.1, the subunit interface between two chains of Adx in the asymmetric unit, obtained from the crystal structures of full-length Adx (PDB: 1CJE; chainA-chainB pair and chainC-chainD pair), Adx(4-108) (PDB: 1AYF; chainA-chainB), and $Ru(bpy)_2(mbpy)$-Adx(1-108) (PDB: 2BT6; chainA-chainB) was estimated as $B/2$, where B was:

$$B = ASA_{monomer1} + ASA_{monomer2} - ASA_{dimer} \qquad (2.1.12.2.1)$$

where ASA is solvent accessible surface area.