CONCLUSION

Although a large number of investigations were carried out over the last 20-30 years, the mechanism of the electron transfer in the steroid hydroxylase system, consisting of AR, Adx, and $P450_{scc}$, has been not completely explored. In this thesis, further investigations of the electron-transfer mechanism in the P450-dependent system were directed to check the "shuttle" model. It is known as one of the favorable models, discussed in the literature so far, describing the nature of protein-protein interactions. In this model, bovine Adx serves as a shuttle, transferring electrons from AR to $P450_{scc}$. The shuttle model differs from other electron-transfer models in that it invokes only binary complexes (Adx-AR, Adx-P450scc), but no higher-order complexes.

According to the aim and for the first time, a new method of a photo-induced protein reduction is applied to bovine adrenodoxin to show that the ferredoxin can be reduced without presence of AR. The technique describes how one can use the property of the excited transitional ruthenium(II) bipyridyl complex for finding a new solution to the old question: How do the proteins of the steroid hydroxylase system participate in the electron transfer? Here, there are the main results of the present thesis.

Bovine Adx(1-108) has been successfully labeled with a ruthenium(II) bipyridyl complex and characterized by: UV-visible spectroscopy, SDS-PAGE, redox potential measurements, mass spectrometry, X-ray crystallography, and EPR. It was shown that the $Ru(bpy)_2(mbpy)$-modified Adx(1-108) exhibited new spectral properties due to the spectral contribution of the coupled complex. The molecular weight of the modified protein is determined by mass spectrometry and corresponds to the expected value for a 1:1 $Ru(bpy)_2(mbpy)$-Adx(1-108) complex. The more negative redox potential of the ruthenium complex is shown to have no influence on the redox potential of the ferredoxin. The $Ru(bpy)_2(mbpy)$-Adx(1-108) complex was crystallized and structurally characterized. The 1.5-Å resolution structure of the 1:1 $Ru(bpy)_2(mbpy)$-Adx(1-108) complex confirms the expected modification site, Cys95. Structural analysis revealed that two isomers of the ruthenium complex are covalently bound to Adx at Cys95 and tilted toward the iron-sulfur cluster of the protein. The coupled solvent-accessible ruthenium(II) bipyridyl complex did not change the overall fold of the protein. The bound compound is at a distance from the interaction helix F of the ferredoxin, allowing its reduction-dependent movement, which is important for protein-protein interaction of Adx with the redox partners. However, the kinetics of the electron-transfer capability by Adx may be affected by the

ruthenium complex due to steric hindrance, occurring when redox partners interact. Photo-induced electron-transfer rates are calculated using concentration-dependent measurements. Based on the crystal structure of the $Ru(bpy)_2(mbpy)$-Adx(1-108) complex, possible intramolecular electron-transfer pathways were predicted. They lie in the physiological range for electron transfer within and between proteins and could be interpreted with experimentally obtained intramolecular monomolecular rate constant. For the confirmation of the photoreduction of the [2Fe-2S] cluster of Adx(1-108) *via* the ruthenium moiety, EPR measurements were employed. The EPR results clearly indicate that the light-induced reduction of the iron-sulfur cluster of Adx takes place. The EPR signal of the photoreduced cluster does not show new *g*-values as compared to the chemically reduced cluster.

An interesting finding of the current work is the fact that the reduction of Adx, one-electron transfer-protein, may be accompanied by transfer of two electrons from an intermolecular reducing agents. The two-electron reduction mechanism might contribute to a mechanistic understanding of electron transfer in the P450 system. However, additional experiments will be required to clarify this puzzled result.

Altogether, the photoreduction of Adx(1-108) has been successfully established, which encourages the further development of this approach to study electron transfer to $P450_{scc}$ *via* the $Ru(bpy)_2(mbpy)$-Adx(1-108) complex. The first preliminary experimental data on $P450_{scc}$ showed that this is possible in principle, and this might help to reveal the fundamental mechanism of electron transfer in the mitochondrial monooxygenase system.

ZUSAMMENFASSUNG

Elektronentransfer ist ein für Lebensprozesse vitaler Vorgang. Das gilt nicht nur für die Atmungskette, sondern auch für viele andere zelluläre Redoxprozesse. Die Grundlage der Transformation von inaktiven Vorstufen von Steroidhormonen (Sexualhormone, Gluko- und Mineralokortikoide) in die biologisch hochaktive Wirkform beruht ebenfalls auf der Aktivierung von molekularem Sauerstoff zur Übertragung von Elektronen. Zur Aufklärung der dabei ablaufenden Prozesse ist es notwendig, die Struktur der daran beteiligten Enzyme aufzuklären, wobei dem terminalen Schritt des Elektronentransfers – der Übertragung von Elektronen auf den molekularen Sauerstoff als Elektronenakzeptor als notwendige Voraussetzung für dessen Spaltung – besondere Bedeutung zukommt.

Das Adx vom Rind besitzt als aktives Zentrum ein [2Fe-2S]-Zentrum, das vier Cystein-Reste als Liganden trägt. In den Mitochondrien fungiert das 128 Aminosäuren lange mature Adrenodoxin als intermediäres Elektronentransportprotein. Es nimmt Elektronen von der Adrenodoxinreduktase (AR) auf und überträgt diese auf die Cytochrome P450, $P450_{scc}$ und P45011β. Adrenodoxin ist somit entscheidend an der Biosynthese der Steroidhormone beteiligt.

Für den Adrenodoxin-vermittelten Elektronentransfer wurden drei verschiedene Modelle postuliert. Das erste Modell, das so genannte "Shuttle"-Modell von *Lambeth et al.* [Lambeth et al., 1982] geht davon aus, dass Adrenodoxin zuerst an die Adrenodoxinreduktase bindet, reduziert wird und anschließend wieder abdissoziert. Danach tritt es in Kontakt mit dem $P450_{scc}$, wobei dieses reduziert wird. Nach dem Elektronentransfer auf das Cytochrom wird der Komplex aufgelöst und der Zyklus kann von vorne beginnen. Ein anderes Modell [Kido & Kimura, 1979] schlägt die Bildung eines ternären Komplexes zwischen Adrenodoxin, Adrenodoxinreduktase und dem $P450_{scc}$ vor. In dem letzten Modell [Hara & Takeshima, 1994] wird eine Beteiligung von zwei Adredoxin-Molekülen am Elektronentransport und somit die Bildung eines quaternären Komplexes postuliert. Eindeutige Beweise für eines dieser Modelle konnten bis jetzt jedoch noch nicht gefunden werden. Den Mechanismus des Elektronentransfers im Steroidhydroxylasesystem weiter aufzuklären war somit ein wichtiges Anliegen dieser Arbeit.

Zwar sind die einzelnen Komponenten des Steroidhydroxylasesystems sowohl kinetisch als auch thermodynamisch vielfältig untersucht worden, dennoch sind die Protein-Protein-Wechselwirkungen beim Elektronentransport nicht zweifelsfrei geklärt. Zusätzliche Informationen über die Tertiärstruktur des Adrenodoxins könnten mithelfen, diese Fragestellung

aufzuklären. In diesem Zusammenhang haben *Müller et al.* [Müller et al., 1998] mit der Entschlüsselung der Kristalltruktur eines verkürzten bovinen Adrenodoxins, Adx (4-108), sowie *Pikuleva et al.* [Pikuleva et al., 2000] mit der Kristallstrukturanalyse der maturen Form von Adx einen wertvollen Beitrag zum Verständnis der Wechselwirkung zwischen Struktur und Funktion dieses Proteins geliefert. Der Mechanismus des Elektronentransfers im Steroidhydroxylasesystem wurde auch anhand der Kristallstruktur eines kovalent verknüpften Komplexes aus Adrenodoxin und Adrenodoxinreduktase weiter aufzuklären versucht [Müller, J. J. et al., 2001]. Die bekannten Kristallstrukturen zeigen, dass innerhalb der Polypeptidkette des Adrenodoxins eine große, das [2Fe-2S]-Zentrum enthaltende Kerndomäne (gebildet aus den Aminosäuren Asp5-Cys55 sowie Gly91-Pro108) und eine Erkennungsdomäne, die mit Adrenodoxinreduktase und $P450_{scc}$ wechselwirkt, unterschieden werden kann. Die Strukturen deuten auf das oben beschriebene Shuttle-Modell hin. Um dieses Modell weiter zu überprüfen, wurden die Quervernetzungs-Studien durchgeführt. Aus diesen konnte abgeleitet werden, dass ein Komplex zwischen Adrenodoxin und $P450_{scc}$ für den Elektronentransfer nicht aktiv ist. Das darf als weiterer Hinweis darauf verstanden werden, dass Adrenodoxin überlappende Wechselwirkungsbereiche für Adrenodoxinreduktase und $P450_{scc}$ besitzt. Um das Shuttle-Modell noch weiter zu untersuchen, wurde in dieser Arbeit ein neues Verfahren für das Steroidhydroxylasesystem eingesetzt, das auf den Redoxeigenschaften von Ruthenium-Bipyridyl-Komplexen basiert.

Als geeigneter methodischer Ansatz für die Untersuchung des Mechanismus des Elektronentransfers in biologischen Systemen wird die Kopplung von Ruthenium-Bipyridyl-Komplexen an Aminosäurereste auf der Proteinoberfläche betrachtet. Diese Methode ist in der Literatur bereits seit mehr als zehn Jahren eingeführt und an einer Reihe von Hämproteinen erprobt worden [Durham et al., 1989; Karpishin et al., 1994]. In der Literatur ist die kovalente Kopplung an Lysin-, Cystein-, und Histidin-Reste beschrieben. Solche Kopplungen ermöglichen dann photochemisch induzierten Elektronentransfer von einem angeregten Zustand des Ruthenium-Bipyridyl-Komplexes zu einer Redoxgruppe eines Proteins.

Das Hauptziel der vorliegenden Arbeit war, mittels einer Photoreduktion des Adrenodoxins zu zeigen, dass das Eisen-Schwefel-Protein ohne Adrenodoxinreduktase reduziert und damit das Shuttle-Modell für Adrenodoxin überprüft werden kann. Um dieses Hauptziel zu erreichen, wurde eine verkürzte Form des Adrenodoxins, Adx(1-108), mit dem $Ru(bpy)_2(mbpy)$-Komplex kovalent modifiziert. $Ru(bpy)_2(mbpy)$-modifiziertes Adx(1-108) wurde mittels

Röntgenstrukturanalyse, biophysikalischer und biochemischer Methoden untersucht. Anhand der Struktur des 1:1 $Ru(bpy)_2(mbpy)$-Adx(1-108)-Komplexes wurden mögliche Elektronentransferwege innerhalb des Komplexes vorgeschlagen. Photoreduktion des Eisen-Schwefel-Zentrums des Adrenodoxins wurde anhand von EPR-Messungen nachgewiesen. Die Elektronentransferrate ist konzentrationsabhängig und zeigt, dass intermolekulare Elektronenübertragung stattfindet. Die intramolekulare Rate wurde bei unendlicher Verdünnung des $Ru(bpy)_2(mbpy)$-Adx(1-108)-Komplexes berechnet.

Bei der vergleichenden Untersuchung des 1:1 $Ru(bpy)_2(mbpy)$-Adx(1-108)-Komplexes mit dem Wildtyp-Adrenodoxin und freiem Adx(1-108) wurde festgestellt, dass Adrenodoxin als Ein-Elektrontransferprotein eine Tendenz zeigt, bei Übertragung von zwei Elektronen reduziert zu werden, obwohl die Spinquantifizierung deutlich zeigt, dass nur ein Eisenatom den Fe^{2+}-Zustand einnimmt. Die Ursache für das ungewöhnliche Verhalten des Proteins ist unklar und muss weiter untersucht werden.

Die Kombination der theoretischen Kenntnisse mit experimentellen Daten erlaubt einen Einblick in den Mechanismus des Elektronentransports im P450-System. Damit wurde die Grundlage für weiterführende Untersuchungen zum Elektronentransport im Cytochrom P450-System geschaffen.

SUMMARY

One of the most common and fundamental chemical reactions is that of electron transfer. In the realm of biology it has a central role in such processes as respiration and cellular redox reactions. The transformation of inactive forms to active forms of the steroid hormones is based on the activation of molecular oxygen, and this process is accompanied by the electron transfer. To better understand the mechanism of the electron transfer, it is necessary to determine the structures of the proteins that are involved in this process.

Bovine Adx contains the [2Fe-2S] center as a redox-active group, which is coordinated by four cysteine residues of the protein. Adx functions as an electron-carrier protein in mitochondria, where it transfers electrons from AR to cytochromes P450, $P450_{scc}$ and P45011β.

Three alternative models for the mechanism of electron transfer by Adx have been suggested in the literature: (i) a "shuttle" model in which Adx carries electrons from AR to $P450_{scc}$ by sequential binding to these proteins [Lambeth et al., 1982; Hanukoglu et al., 1981], (ii) an electron transfer through an organized ternary complex of AR, Adx, and $P450_{scc}$ [Kido & Kimura, 1979], and (iii) a quaternary complex of AR, Adx dimer, and $P450_{scc}$ [Hara & Takeshima, 1994]. However, it still remains unclear the exact mechanism of the electron transfer. The present work is focused on further investigation of the mechanism.

Despite many kinetic and thermodynamic studies, the protein-protein interactions between the components of the steroid hydroxylase during the electron transfer have been not finally explored. Additional information about the 3D structure of Adx may help to solve this problem. *Müller et al.* [Müller et al., 1998] and *Pikuleva et al.* [Pikuleva et al., 2000] have solved the crystal structures of the truncated and full-length Adx, respectively, which provide a very important evidence for understanding of the structure-function relationship of the protein. The mechanism of the electron transfer in the steroid hydroxylase system was further investigated using the crystal structure of the covalent cross-linked AR-Adx complex [Müller, J. J. et al., 2001]. The crystal structures show that there are two structural domains within the polypeptide chain of Adx. These domains are: (i) core domain (Asp5-Cys55 and Gly91-Pro108); and (ii) interaction domain, which contents the interaction sites for AR and $P450_{scc}$. The resolved crystal structures favor the "shuttle" hypothesis for the electron transfer. In order to check that model, the cross-linking between Adx and $P450_{scc}$ was performed. From those experiments, it was found that the cross-linked complex Adx-$P450_{scc}$ was not active in the reconstituted P450-system. All

mentioned above data provide important information that Adx has the same interaction sites for its redox partners, AR and $P450_{scc}$, what confirms the functionality of the "shuttle" model. In the present doctoral work a new experimental approach was developed to further investigate this model. This approach is based on the redox properties of the ruthenium(II) bipyridyl complexes.

A covalent modification of proteins with the ruthenium(II) bipyridyl complexes is well known method to study the electron transfer between and within the proteins. This technique has already been established over more then ten years, and it has been successfully checked for some heme proteins [Durham et al., 1989; Karpishin et al., 1994]. Covalent modification of the lysine, cysteine, and histidine residues is known. In principle, such labeling allows a photo-induced electron transfer from an excited state of a ruthenium complex to a redox-group of a protein.

The main aim of the present doctoral work was to show that Adx can be photoreduced without presence of AR, and hence, to probe the "shuttle" mechanism. In order to achieve the aim, the truncated form of Adx, Adx(1-108), was covalently modified with $Ru(bpy)_2(mbpy)$. $Ru(bpy)_2(mbpy)$-Adx(1-108) was characterized by X-ray crystallography, biophysical and biochemical methods. Possible electron-transfer pathways within the covalent 1:1 $Ru(bpy)_2(mbpy)$-Adx(1-108) complex were predicted. Photoreduction of the [2Fe-2S] cluster of adrenodoxin was confirmed by EPR measurements. The electron-transfer rate is concentration dependent and indicates that intermolecular electron transfer takes place. The monomolecular intramolecular rate was derived at zero concentration of the $Ru(bpy)_2(mbpy)$-Adx(1-108) complex.

Comparative investigation of the covalent 1:1 $Ru(bpy)_2(mbpy)$-Adx(1-108) complex with the wild-type Adx and unmodified Adx(1-108) showed that, in general, reduction of Adx, one-electron carrier, is accompanied by a transfer of two electrons. However, spin quantification of the reduced [2Fe-2S] cluster indicated that only one of two irons is in Fe^{2+} state. At present, there is no clear explanation for this unusual feature and it needs further investigation.

In general, the combination of theory and present experimental data provides a basis for further investigations of the mechanism of the electron transfer in the P450-system.

APPENDIX

Appendix A

Bacterial Media, and Antibiotics

LB medium (1 L)

10 g of Bacto-Tryptone, 5 g of Bacto-Yeast extract (both from Becton, Dickinson and Company, Sparks, MD, USA) and 10 g of NaCl (Carl Roth, GmbH + Co. KG, Karlsruhe, Germany) in 900 ml of water were dissolved. The pH of the medium was adjusted to 7.0-7.4 with 1 M NaOH (Carl Roth, GmbH + Co. KG, Karlsruhe, Germany). Water was added to the final volume of 1 L, and the medium either in bottles or flasks was sterilized by autoclaving; stored at 4 °C.

LB agar plates (1 L)

Liquid LB medium was prepared as described above. 15 g of Bacto-Agar (Becton, Dickinson and Company, Sparks, MD USA) was added to the medium and sterilized by autoclaving. When the medium had cooled down to 50 °C the appropriate amount of antibiotics was added and gently mixed. The medium was solidified and stored at 4 °C. Before use, a plate was incubated at 37 °C in the inverted position for 1 hour to remove condensation within the plate.

Table A.1. Antibiotics solutions

Antibiotic[a]	Working concentration	Stock solution[b]
Ampicillin (Amp)	20-100 μg/ml	100 mg/ml in H_2O[c]
Chloramphenicol (Cm)	25-170 μg/ml	34 mg/ml in ethanol
Kanamycin (Kan)	10-50 μg/ml	30 mg/ml in H_2O

[a]Carl Roth, GmbH + Co. KG, Karlsruhe, Germany. [b]Stored at -20 °C. [c]Stock solutions in H_2O should be sterilized by filtration and stored protected from light. Stock solutions in ethanol should not be sterilized.

Appendix B

Preparation of Buffers

All buffers were prepared by dissolving of a weighed quantity of analytical grade reagents with bi-distillated water and sterilized by filtering through a 0.2 μm membrane filter (cellulose acetate) (Schleicher & Schuell, Dassel, Germany) or autoclaving. Lower-molarities buffers were prepared using the stock buffers by their dilution with H_2O. If buffers contained salts, detergents, etc. pH was checked after all components were dissolved.

1 M Tris (1 L)

Dissolve 121.14 g tris(hydroxymethyl)aminomethane (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) in 800 ml H_2O. Adjust pH to the desired value by adding concentrated HCl, filter, and store at room temperature.

1 M potassium phosphate buffer (1 L)

To prepare 1 M potassium phosphate buffer, 1 M potassium phosphate (mono-potassium salt and di-potassium salt) (both from Merck KGaA, Darmstadt, Germany) stocks were prepared by dissolving of 136.09 g and 174.18 g in water, respectively. These stocks were further mixed to get 1 M potassium phosphate buffer with desired pH value. Store at room temperature.

Table B.1. Preparation of TFBI buffer (per 100 ml)

Compound[a]	Amount	Final molarity
potassium acetate	1 ml[b]	30 mM
rubidium chloride	1.21 g	100 mM
calcium chloride	1 ml[c]	10 mM
manganese chloride	1.02 g	50 mM
glycerol[d]	17.4 ml	15% v/v

[a]All compounds are from Carl Roth, GmbH + Co. KG, Karlsruhe, Germany. [b]3 M stock solution of potassium acetate was used. [c]1 M stock solution of $CaCl_2$ was used. [d]86% glycerol.

Table B.2. Preparation of TFBII buffer (per 100ml)

Compound[a]	Amount	Final molarity
MOPS	1 ml[b]	10 mM
rubidium chloride	0.12 g	10 mM
calcium chloride	7.5 ml[c]	75 mM
glycerol[d]	17.4 ml	15% v/v

[a]All compounds are from Carl Roth, GmbH + Co. KG, Karlsruhe, Germany. [b]1 M MOPS stock solution, pH 6.8 was used. [c]1 M stock solution was used. [d]86% glycerol was used.

Table B.3. Resolving gel for denaturing SDS-PAGE[27]

% gel	Components	5 ml[a]	50 ml[b]
12%	H_2O	1.6 ml	16.5 ml
	30% acryl-bisacrylamide mix[c]	2.0 ml	20.0 ml
	1.5 M Tris (pH 8.8)	1.3 ml	12.5 ml
	10% SDS	0.05 ml	0.5 ml
	10% ammonium persulfate	0.05 ml	
	TEMED	0.002 ml	
18%	H_2O	0.675 ml	6.75 ml
	30% acryl-bisacrylamide mix	3 ml	30 ml
	1.5 M Tris (pH 8.8)	1.25 ml	12.5 ml
	10% SDS	0.05 ml	0.5 ml
	10% ammonium persulfate	0.025 ml	
	TEMED	0.0025 ml	

[a]Working solution (5 ml) was used for one gel run. [b]Stock solution, where water, 30% acryl-bisacrylamide mix, 1.5 M Tris, and 10% SDS (SERVA Feinbiochemica GmbH & Co. KG, Heidelberg, Germany) were mixed and stored at 4 °C. For preparing a working solution, 5 ml of the stock were taken and the appropriate amount of 10% ammonium persulfate (SERVA Feinbiochemica GmbH & Co. KG, Heidelberg, Germany) and TEMED (Carl Roth, GmbH + Co. KG, Karlsruhe, Germany) were added to drive and accelerate the polymerization of acrylamide and bisacrylamide, respectively. Alternatively, to prepare a working solution, one mixes all components as listed in the table. [c]Rotiphorese® Gel 30 (37.5:1) (Carl Roth, GmbH + Co. KG, Karlsruhe, Germany). The molecular weight marker was used from (SERVA Feinbiochemica GmbH & Co. KG, Heidelberg, Germany).

[27] SDS-PAGE was carried out according to *Laemmli* [Laemmli 1970].

Table B.4. 5% stacking gel for denaturing SDS-PAGE

Components	2 ml[a]	10 ml[b]
H_2O	1.4 ml	6.8 ml
30% acryl-bisacrylamide mix	0.33 ml	1.7 ml
1.5 M Tris (pH 8.8)	0.25 ml	1.25 ml
10% SDS	0.02 ml	0.1 ml
10% ammonium persulfate	0.02 ml	
TEMED	0.002 ml	

[a, b] Preparation of the solutions was done in the same manner as described for resolving gel.

10% Ammonium persulfate (10 ml)

1 g of ammonium persulfate was dissolved in 8 ml of H_2O. Then the volume was adjusted to 10 ml with water and the stock was stored at 4 °C. It was stable for two weeks.

10% SDS (1 L)

100 g of sodium dodecyl sulfate crystals were dissolved in 900 ml of water. Heating of the solution to 68 °C is necessary to dissolve the crystals. Then adjust pH to 7.2 with HCl (Carl Roth, GmbH + Co. KG, Karlsruhe, Germany) and finally to volume of 1 L. Store at room temperature.

2×SDS-PAGE sample buffer (100 ml)

10 ml of 1.5 M Tris (pH 6.8), 6 ml of 20% SDS, 30 ml of glycerol, 15 ml of β-mercaptoethanol, and 1.8 mg of bromophenol blue (Fluka Chemie AG, Buchs, Switzerland) were mixed together. The volume was adjusted to 100 ml with water. Aliquots in 10 ml of stock solution were stored at -20 °C. Working solution was stored at 4 °C.

10×SDS-PAGE running buffer (1 L)

10 g of SDS, 30.3 g of Tris, and 144.1 g of glycine (MP Biomedicals, LLC, Eschwege, Germany) were dissolved in 800 ml of water. The pH of the buffer was measured and adjusted to 8.25 with 5 N KOH (Merck KGaA, Darmstadt, Germany). The volume of the buffer was then adjusted to 1 L. Store at room temperature.

Coomassie Blue staining solution (1 L)

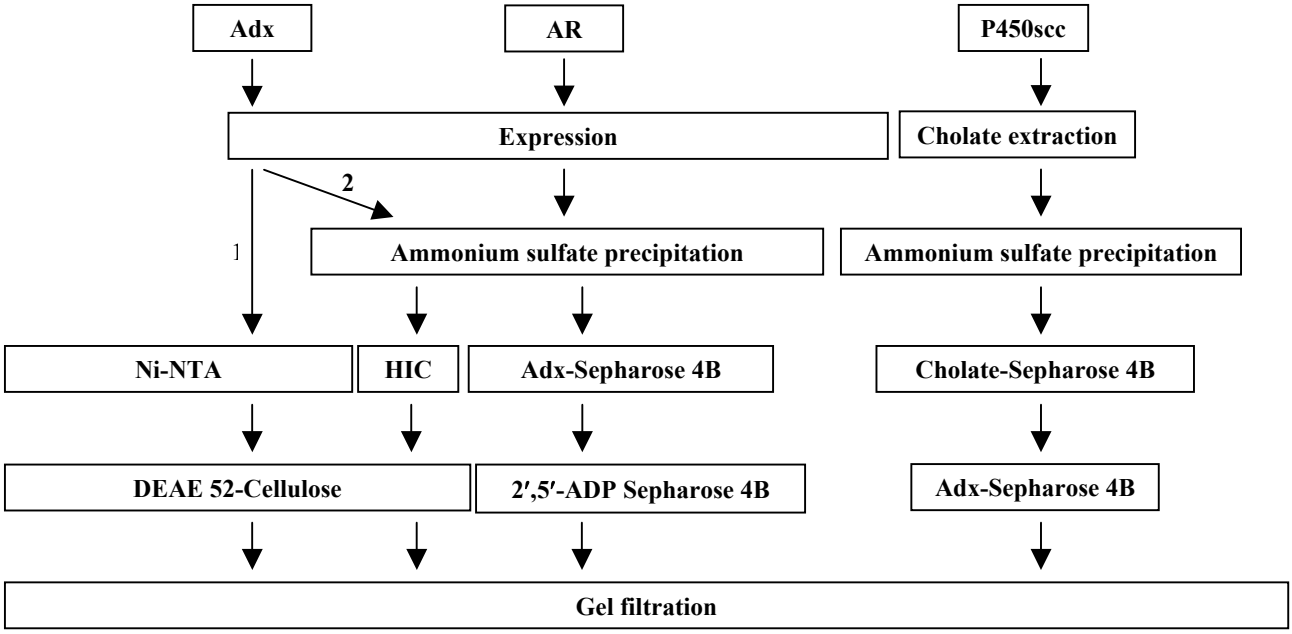
2.5 g "Coomassie Brilliant Blue R-250" (Carl Roth, GmbH + Co. KG, Karlsruhe, Germany) were dissolved in a mixture of 450 ml of methanol (Carl Roth, GmbH + Co. KG, Karlsruhe, Germany), 100 ml of acetic acid, and 400 ml water. The volume was then adjusted to 1 L with water. Store at room temperature.

Coomassie Blue destaining solution (1 L)

450 ml of methanol, 100 ml of acetic acid (Carl Roth, GmbH + Co. KG, Karlsruhe, Germany), and 400 ml water were mixed, and the volume was adjusted to 1 L. Stored at room temperature.

Coupling of Adx to CNBr-activated Sepharose 4

20 μmol of Adx were coupled to 17 g of CNBr-activated Sepharose 4 (Pharmacia, Uppsala, Sweden) in accordance with manufacturer's recommendations. The coupling yield was 95%.



Scheme B.1. The protein purification strategy. 1. Strategy for Adx(N-6×His-tag/Xa/1-108) purification. 2. Purification of Adx(WT). Hydrophobic Interaction Chromatography (HIC).