POSTTRANSLATIONAL MODIFICATION OF THE MITOCHONDRIAL CYTOCHROME *C*

Summary of the Ph.D. Thesis

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Introduction

C-type cytochromes are essential heme-containing proteins found in almost all living organisms. It is their covalently bound heme prosthetic group which distinguishes them from other cytochromes also possessing typical absorption spectra and color. The overwhelming majority of *c*-type cytochromes contain a Cys-Xxx-Xxx-Cys-His heme binding motif to support this covalent binding. The posttranslational modification of *c*-type cytochromes comprising the covalent heme attachment is referred to as maturation, which is assisted under natural conditions by a maturation system. There are three main maturation systems, with different degrees of complexity that are generally responsible for the cytochrome *c* maturation. The covalently bound heme, the large variation of cytochromes *c* and the existence of different cytochrome *c* maturation systems pose numerous, so far unanswered questions in terms of structure, function and evolution.

The mitochondrial *c*-type cytochromes are especially suitable subjects for various experiments among the many *c*-type cytochromes. They are small, soluble and very stable electron transfer proteins in the mitochondrial intermembrane space. These proteins are components of the respiratory electron transfer chain, carrying electrons without H^+ transfer between their two membrane-bound partners.

We have used horse mitochondrial cytochrome *c* in our experiments, a protein which may be suitable to unravel the biochemical conditions necessary for its maturation in both heterologous *in vivo* and *in vitro* studies. Beyond maturation studies, the mitochondrial cytochromes *c* are also convenient molecules in electron transfer investigations. They are not photoactive redox molecules in the sense that they carry out electron release and uptake without the electronic excitation of the heme group. In order to perform fast, good time resolution electron transfer measurements they must be labelled covalently and selectively by a photoactive (i.e. redox active in the excited state) dye. For the selective labelling one has to replace by mutagenesis functionally neutral surface amino acids. The advantage of the external covalent dye is that the protein can be labelled at virtually any position, and thereby the distance between electron donor and acceptor (the dye and the heme, respectively) and the direction of the electron transfer can be varied arbitrarily. Experiments performed on such labelled proteins can answer important questions regarding the nature and mechanism of electron transfer within and between proteins.

In order to produce mitochondrial cytochrome *c* and its mutants one has to assure not only their expression but also their maturation, i.e. the covalent heme attachment and the folding of the protein. To this end the appropriate protein(s) of one of the maturation systems must also be co-expressed in the host cell. The requirement to produce cytochrome *c* mutants has naturally led to the main subject of my thesis, the investigation of the maturation of cytochrome *c* and the structure-function of the maturation enzyme, the cytochrome *c* heme lyase (CCHL).

Aims

Our activity of producing and labelling cytochrome *c* mutants is aimed at clarifying how the protein matrix controls the rate of redox reactions. This is studied by electron transfer experiments carried out on such proteins and protein complexes by varying different physical factors. The main parameter to be varied is the relative position of the electron donor and acceptor, which enables the systematic study of the effects of the distance and the molecular architecture of the protein matrix between donor and acceptor on the electron transfer rate.

The production of cytochrome *c* mutants requires a tightly controllable, high throughput expression construct and a proper bacterial host. Beyond the expression system we need an efficient protein purification procedure. Therefore in this part of my work the goal was to develop a proper expression system and to work out an appropriate purification procedure.

The successful heterologous coexpression of the eukaryotic cytochrome *c* with the maturation enzyme brought up as an independent research project the problem of cytochrome *c* maturation. Successful *in vivo* maturation in the heterologous host cannot answer the question as to what are the minimal experimental conditions necessary for the maturation. In order to find the answer one needs controlled *in vitro* experiments. The prerequisite of such *in vitro* experiments is to have the maturation enzyme purified in its active form. The aim was to develop an ideal expression system and elaborate a purification procedure that provides us the active form of the CCHL. According to the literature neither the purification of the CCHL nor such type of *in vitro* reconstitution of the maturation have ever been done before. Therefore no structural information about the enzyme is available except for its primary structure.

With the purified CCHL at hand we aimed at studying, in addition to the action mechanism, the structure of the enzyme as well. Since the yield of production of the protein in its active form is rather low, we could not think of producing enough for crystallization or

NMR structural determination so far. Therefore, we decided to perform mostly spectroscopic measurements on the purified protein alone and together with its substrates. Using the known sequence of CCHL we also planned to gain information about the protein's expected structure and properties by homology search and *in silico* structure-prediction calculations.

We noticed during the expression experiments that in *E. coli*, following overnight incubation, holocytochrome *c* is produced even when the cells contained only the gene of apocytochrome but not that of CCHL – although at a rather low yield. It was a surprising observation that a eukaryotic cytochrome *c* can mature non-enzymatically in this environment. We decided to purify the protein and to investigate in detail also this non-enzymatic („spontaneous") maturation, since the possibility of non-enzymatic maturation of a eukaryotic cytochrome *c* and the comparison of the so produced holocytochrome with the authentic protein can help us to reveal important details about the mechanism and conditions of the enzymatic maturation as well.

Experimental methods

We have constructed expression plasmids that we used to transform appropriate *E. coli* strains for high level overproduction of horse cytochrome *c*. For the overexpression of yeast CCHL we obtained a plasmid as a kind gift from Dr. Carsten Sanders, Kutztown University of Pennsylvania. The manipulation procedures for cloning, the competent cell preparation, the transformation of strains and the expression of proteins were carried out according to the general practice, as they are described in the laboratory manuals.

The point mutations in the sequence of cytochrome *c* were introduced by two sequential PCR steps. The restriction endonuclease digested DNA fragment was ligated in proper orientation into the similarly digested plasmid that was cloned into the transformed *E. coli* strain. Successful mutations were confirmed by sequencing the region of interest. We have used two different expression systems for the heterologous cytochrome *c* overproduction. In the first case the two genes were placed tandem and transcribed from the same promoter, while in the second case the cytochrome and the CCHL genes were cloned under two different promoters in two genetically competent plasmids.

Succeeding the overproduction the harvested cells were extracted by chemical and mechanical (sonication) treatments. The soluble fraction was separated by ultracentrifugation. Prior to chromatography, the soluble fraction containing cytochrome *c* was subjected to a

50% saturated ammonium sulfate precipitation step, dialysis and concentration of the protein solution. Holocytochrome *c* was separated on a cation exchange matrix. The purity of the proteins was demonstrated by SDS PAGE (sodium dodecyl sulfate poliacrylamide gelelectrophoresis), the protein bands were visualized by staining with Coomassie BB G-250. The Strep-tag II fused cytochrome *c* and the His_6-tag fused CCHL were further purified by affinity chromatography. The chromatography steps were performed on an FPLC (Pharmacia) instrument. Following electrophoresis the Strep-tag II and His_6-tag fused proteins were subjected to a Western-blot analysis and the enhanced chemiluminescence (ECL) signal generated whereupon was recorded by a VersaDoc Imaging System or detected by autoradiography.

The near-UV-visible absorption spectra were used to determine the purity and to calculate the concentration of protein solutions. In addition, the absorption spectrum of cytochrome *c* is sensitive to the orientation of the heme in its pocket, thereby from the spectrum taken in the visible range one can also deduce information on the correct maturation of the protein. Absorption spectra were measured on a UNICAM UV4 spectrophotometer.

UV - Circular Dichroism (CD) spectra were measured on a Jasco J-815 spectropolarimeter to analyze the secondary structural composition of CCHL.

The covalent heme attachment of the non-enzymatically and enzymatically matured cytochromes *c* was confirmed „in-gel" after SDS-PAGE, by a heme peroxidase activity measurement. The heme-dependent enhanced chemiluminescence (ECL) signal was recorded by a VersaDoc Imaging System. To demonstrate the heme attachment to the cysteines of the apoprotein via two thioether bonds we measured the pyridine hemochrome absorption spectrum of the non-enzymatically matured cytochrome *c*. The position of the α peak maximum of a pyridine coordinated heme changes according to the heme substituents. This allows us to differentiate between a heme that is bound via a single thioether linkage and a heme bound by a double linkage.

The reduction potentials of cytochromes *c* were determined by spectrophotometric titration with Na-ferricyanide. The midpoint reduction potentials of different cytochromes *c* were calculated by nonlinear least squares fit of the Nernst equation.

To determine the electron transfer activity of the cytochromes we have done cytochrome *c* oxidase (COX) activity experiments. The activities were monitored by a Model 10 Oxygraph (Rank Brothers Ltd.). We monitored the oxygen consumption that was the

consequence of COX activity. The kinetic parameters (K_m and V_{max}) of the Michaelis-Menten reaction were calculated by fitting the initial rate of oxygen consumption as a function of the substrate (reduced cytochrome *c*) concentration with a nonlinear least squares routine.

Results and Discussion

1. We have elaborated a reliable, tightly controllable, high throughput cytochrome *c* expression system

Based on the antecedents known from the literature and on a plasmid that we got from our partners we succeeded to develop our own reliable, tightly controllable, high yield cytochrome *c* expression system. The system's curiosity is that a eukaryotic enzyme matures perfectly a eukaryotic protein in the cytoplasm of a bacterial host without the need of other external factors. The system includes the cytochrome *c* and the CCHL genes containing cassette or just the cytochrome *c* gene ligated under the tightly controllable arabinose P_{BAD} promoter of the pBAD24 plasmid. The strict control is assured by the AraC protein that is a negative regulator of expression in the absence, and a positive regulator of expression in the presence of arabinose.

2. We have demonstrated that the non-enzymatic assembly of a eukaryotic cytochrome *c* in the cytoplasm of a bacterial host is possible

Our heterologous *in vivo* and *in vitro* maturation experiments could provide some information about the completely unknown molecular mechanism of cytochrome *c* maturation by the CCHL enzyme. We have demonstrated that if the horse cytochrome *c* is expressed in the absence of CCHL, holocytochrome *c* maturation is still possible, albeit with a strongly reduced yield. The non-enzymatically matured cytochrome *c* has native-like heme ligation; it is bound in the right orientation to the polypeptide chain via two thioether linkages. This is the first demonstration of *in vivo* maturation of a eukaryotic cytochrome *c* in a prokaryotic cytoplasm without the assistance by a dedicated enzymatic maturation system. As a control experiment we performed the non-enzymatic maturation in a cytochrome *c* maturation deficient EC06 strain as well. The expression level of apocytochrome *c* in the absence and presence of CCHL is the same, but the attachment of the heme in the absence of CCHL is a much slower process than the catalyzed attachment. The yield of this lyase-free assembly is approximately 2% of the yield obtained in the presence of the yeast CCHL.

The binding of heme was demonstrated by “in-gel” heme peroxidase activity visualized by enhanced chemiluminescence, which is only possible if the heme is covalently attached to the protein and, therefore, retained in SDS gel. The co-migration of heme with the polypeptide chain would be possible even in the case when the heme is bound via one thioether bond only. This circumstance would mean an incorrect orientation of the heme relative to the polypeptide chain (rotation around the α, γ meso-axis). This can be ruled out since the pyridine coordinated hemochrome spectrum demonstrated correct stereo-specific heme attachment to the protein.

3. We have examined the role of the maturation enzyme CCHL in the configuration of the heme’s native environment

The absence of CCHL could be seen not only in the low yield but also in minor spectral and physicochemical differences of the non-enzymatically matured cytochrome *c* relative to the authentic and the enzymatically matured cytochromes *c*.

The spectrum of the non-enzymatically matured cytochrome *c* is typical of a 6-coordinated, strong axial ligand, low spin *c*-type cytochrome with a proper Q band, but exhibits a 1 nm red-shift relative to the authentic cytochrome *c*. Nevertheless, a five-Gaussian decomposition of the Q bands of the reduced authentic, CCHL-matured and non-enzymatically assembled cytochromes *c* yielded consistent results, with similar sub-bands in all three cases. The decomposition of the Q band therefore supports the conclusion about the homogeneity of the non-enzymatically matured cytochrome *c*, and its overall structural similarity to the authentic protein. The 1 nm red-shift of the lowest energy transition reflects a small decrease in the energy gap between the ground and the excited states in the non-enzymatically matured cytochrome *c*. The broadening of a Gaussian component, which is observable as a “tail” of the 551 nm peak, may be the sign of a conformational heterogeneity not present (or present to a lesser extent) in the two enzymatically matured cytochrome *c* species. In addition, it may also indicate a different population of vibrational levels in the ground state of the chromophore, again reflecting a slightly different heme environment and/or geometry in the non-enzymatically matured protein.

The midpoint reduction potential of the non-enzymatically matured cytochrome *c* is slightly lower than that of the authentic or the recombinant, CCHL matured proteins. This relative stabilization of the oxidized (FeIII) form may be the result of a small change in the interaction of the iron with the axial ligands. A slightly altered position of Met80 is also substantiated by the oxidized absorption spectrum (the 695 nm charge transfer band is absent

from the non-enzymatically matured cytochrome *c* spectrum), whereas a complete disruption of this bond would result in a much larger negative shift of the midpoint reduction potential. Alterations of the polarity of the heme pocket and the electric perturbation of the heme may also be the reasons behind the shift of the midpoint reduction potential.

We compared the autoxidation rate of the non-enzymatically matured cytochrome *c* to that of the CCHL-matured and authentic ones and we observed no significant difference. A substantial increase of the autoxidation rate would be expected if major structural changes took place in the heme pocket. Minor changes in the heme geometry, coordination or in the polarity of the heme pocket could explain the Michaelis-Menten parameters that we calculated from COX activity measurements. The value of the parameter K_m showed a deviation relative to the authentic and the enzymatically matured cytochrome, which indicates a slightly lower affinity of cytochrome *c* towards the COX enzyme.

Our results confirm based on the spectral and physicochemical parameters of the non-enzymatically matured cytochrome *c* that i) a lyase-free assembly of functional holocytochrome *c* occurs - although with low efficiency; ii) CCHL is indispensable in assuring a high yield of holocytochrome formation and in the folding of horse holocytochrome *c* to its native conformation during the process of maturation.

4. <u>We have overproduced, purified and partially characterized the maturation enzyme CCHL</u>

We have demonstrated *in vivo* the maturation activity of the yeast CCHL. Co-expressed in the cytoplasm of *E. coli* with the cytochrome *c* it provides a yield approximately equivalent to 4 - 5 mg/1g wet cell. To understand the molecular mechanism of the enzyme catalysis we need to reconstitute the maturation by the purified enzyme in an *in vitro* assay. We succeeded to overexpress the maturation enzyme and to purify it, albeit with a low yield (0.25 mg/1g wet cell). The His_6 peptide fused CCHL was purified by affinity chromatography and its purity was demonstrated by SDS-PAGE.

We have examined the sequence of the enzyme with programs (IUPred, DisProt) that predict disordered protein sequences/structures. According to these examinations the N-terminal part (from amino acid 16 to 80) of the enzyme that contains the two heme regulatory CPV motifs has a disordered structure.

We have measured the UV CD spectrum of the enzyme. From fitting of the spectrum one can calculate the proportion of the regular (like α-helices, β-sheets and turns) and the non-regular secondary structure elements as well as the disordered regions. Accordingly, the

estimated numbers of the secondary structural elements are: 8-α-helices and 12-β-sheets. It is worth noting that some of these structural elements may be short enough to include only non-regularly positioned amino acids.

We have compared the amino acid composition of the disordered sequence and the regions with presumptive secondary structure. We observed that some amino acids show a discernible distribution within these parts of the enzyme, in accordance with the statistical data of the intrinsically disordered proteins. This observation regarding the structure of the N-terminal region is also supported by trypsin digestion experiment known from the literature.

5. We have examined the interaction of CCHL with its substrates and we have reconstituted *in vitro* the maturation of cytochrome *c*

We have studied the interaction of the purified CCHL with heme. The absorption spectra measured during this interaction corroborate the results of the structure prediction by IUPred and the proteolytic digestion experiments in the literature. We could observe two phases of the absorption change. In the first one the heme is likely to interact with the disordered N-terminal region of the enzyme. In the second phase this interaction strengthens by the formation of a six-coordinated heme via its axial ligation by the two CPV motifs, which would be facilitated by the high flexibility of the disordered segment.

We have performed enzymatic assays with the purified CCHL for *in vitro* holocytochrome *c* maturation. We succeeded to reconstitute the maturation in an artificial environment. The heme attachment to the polypeptide chain was demonstrated spectrally and by an “in-gel” heme peroxidase experiment.

Our original goal with the production of recombinant cytochrome *c* mutants was to perform electron transfer experiments. However, the optimization of the production of recombinant mutant cytochromes as well as the elaboration of the heterologous expression and purification of CCHL gives us the opportunity to study the molecular mechanism of cytochrome *c* maturation using site directed mutants of both proteins. After producing sufficient amounts of purified CCHL we may also plan to further explore the structure of CCHL either by X-ray crystallography or by the NMR method.

List of publications and conference proceedings directly related to the Ph.D. Thesis

1. **Tenger K**, Khoroshyy P, Leitgeb B, Rákhely G, Borovok N, Kotlyar A, Dolgikh DA, Zimányi L. **2005**. Complex kinetics of the electron transfer between the photoactive redox label TUPS and the heme of cytochrome *c*. J. Chem. Inf. Mod. 45(6):1520-1526. IF: 2.923
2. **Tenger K**, Khoroshyy P, Kovács KL, Zimányi L, Rákhely G. **2007**. Improved system for heterologous expression of cytochrome *c* mutants in *Escherichia coli*. Acta. Biol. Hung. 58:23-35. IF: 0.688
3. **Tenger K**, Khoroshyy P, Rákhely G, Zimányi L. **2008**. Heterologous overexpression of eukaryotic cytochrome *c* and cytochrome *c* heme lyase to study the mechanism of cytochrome *c* maturation. Biochim. Biophys. Acta 1777:S90.
4. **Tenger K**, Khoroshyy P, Rákhely G, Zimányi L. **2010**. Maturation of a eukaryotic cytochrome *c* in the cytoplasm of *Escherichia coli* without the assistance by a dedicated biogenesis apparatus. J. Bioenerg. Biomembr. 42:125-133. IF: 4.015

Other publications

1. Kotlyar AB, Borovok N, Khoroshyy P, **Tenger K**, Zimányi L. **2004**. Redox photochemistry of thiouredopyrenetrisulfonate. Photochem. Photobiol. 79(6): 489-493. IF: 2.054

Other conference proceedings

1. Khoroshyy P, **Tenger K**, Borovok N, Kotlyar A, Siletsky S, Zimányi L. **2003**. Electron transfer steps in the complex of cytochrome *c* and cytochrome oxidase. 10th European Conference on the Spectroscopy of Biological Molecules, Szeged, Hungary, ISBN 963 482 614 8, p. 153.
2. Zimányi L, Kulcsár Á, **Tenger K**, Borovok N, Kotlyar A. **2003**. Effect of the protein medium and dynamics on electron transfer in cytochrome *c*. 10th European Conference on the Spectroscopy of Biological Molecules, Szeged, Hungary, ISBN 963 482 614 8, p. 154.
3. Zimányi L, **Tenger K**, Khoroshyy P, Dolgikh D, Siletsky N, Borovok N, Kotlyar A. **2004**. Photoinduced electron transfer in cytochrome *c* and cytochrome *c* oxidase. EBEC 2004 Short Reports. Biochim. Biophys. Acta 13:158.
4. **Tenger K**, Khoroshyy P, Leitgeb B, Rákhely G, Borovok N, Kotlyar A, Zimányi L. **2005**. Complex electron transfer kinetics between the photoactive label TUPS and the heme of cytochrome *c*. Eur. Biophys. J. 34(6):665.
5. Khoroshyy P, **Tenger K**, Zimányi L. **2006**. Intra- and interprotein photoinduced electron transfer in respiratory chain redox proteins. Biochim. Biophys. Acta 1757(14):187.
6. Khoroshyy P, **Tenger K**, Zimányi L. **2008**. Tuning the electron transfer rate by the redox potential of cytochrome *c* in complex with cytochrome *c* oxidase. Biochim. Biophys. Acta 1777:S90.