Diplomarbeit
zur Erlangung des akademischen Grades
Diplom-Ingenieur

CYTOCHROM C OXIDASES IN NITROGEN-FIXING CYANOBACTERIA

Durchgeführt am Department für Chemie
Abteilung für Biochemie
Metalloprotein Research Group
der Universität für Bodenkultur Wien

Vorstand: Univ. Prof. Mag. Dr. Paul Kosma
Betreuer: ao. Univ. Prof. Mag. Dr. Christian Obinger

Eingereicht von
Martin Pairer

Oktober 2009

Table of Contents

Acknowledgements

First I want to thank Prof. Christian Obinger for giving me the opportunity to work in his research group and his support and assistance during my diploma thesis. Also I want to thank him for great inspiration and motivation far beyond study topics.

Special thanks goes to Margit Bernroitner, who was always there to guide me through the practical work, to give me helpful advice and to answer all my questions, even though I had plenty of them.

Many thanks to all the members of the Metalloprotein Research Group, to Markus Auer, Srijib Banerjee, Farnaz Faridi, Paul Furtmüller, Tanja Kriebernig, Chantal Lucini, Monika Soudi, Johanna Stampler, Jutta Vlasits and Marcel Zamocky for the unique pleasant working atmosphere. Their willingness to support me (especially Jutta Vlastis, who fought her way through endless imperfectly pages of my diploma thesis) with their knowledge and experience at any time was a great help and made many bad days look good.

I owe thanks to my family, in particular to my parents, who never stopped believing in me, for their patience and support throughout my studies.

Abstract

Cyanobacteria were the first organisms on earth to produce O_2 by photosynthesis. Gradual modification of the preexisting photosynthetic electron transport and enzyme systems could have changed a photosynthetic into a respiratory chain and today cyanobacteria are capable of both, photophosphorylation and oxidative phosphorylation.

Nostoc sp. PCC 7120 is a filamentous cyanobacterium which can fix nitrogen in terminally differentiated cells called heterocysts. Heterocysts and vegetative cells express two different cytochrome *c* oxidases, the key enzyme of respiration: in vegetative cells the product of the *coxBACI* (cox1) gene is found, whereas heterocysts express *coxBACII* (cox2).

It is well known that in oxygenic photosynthesis the transport of electrons from the cytochrome b_6f complex to PSI is performed by either plastocyanin (PC) or cytochrome c_6 (Cyt c_6), which are both small soluble metalloproteins located on the P-side of membranes containing photosynthetic electron transport. Plastocyanin (encoded by the *petE* gene) consists of a single polypeptide chain forming a β-barrel with eight β-strands and a small α-helix, along with a type-1 blue copper center.

In order to understand more about cyanobacterial respiration and stress mechanism, a truncated form of the subunit II of cox2, SUIIj3 cox2 (carrying the electron binding site and electron entry site) and plastocyanin were cloned, recombinantly expressed in *E. coli* and purified with chromatography.

Recombinant SUIIj3 cox2 lacks copper in its active site and had to be reconstituted. Reconstitution by dialysis with urea was successful but the reconstitution efficiency was not satisfying. Reconstitution by ultrafiltration with urea led to higher amount of pure, functional holoprotein, but still not sufficient to perform kinetic measurements. Additionally the stability of the pure, functional holoprotein was not guaranteed due to loss of the attached copper during reconstitution.

The amount of produced plastocyanin was adequate and the protein solution held the necessary pureness to perform further kinetic measurements.

Zusammenfassung

Cyanobakterien waren die ersten Organismen auf der Erde, die durch Photosynthese Sauerstoff bilden konnten. Derzeitige Thesen belegen, dass Cyanobakterien durch sukzessive Veränderung des Elektronentansport- und Enzymsystems der Photosynthese die Atmungskette entwickelt haben, was dazu führte, dass diese Organismen heutzutage sowohl Photophosphorylierung als auch oxidative Phosphorylierung betreiben können. Das filamentöse Cyanobakterium *Nostoc sp*. PCC 7120, welches Stickstoff in terminal differenzierten Zellen, den sogenannten Heterocysten, fixiert, besitzt neben diesen auch vegetative Zellen. Diese exprimieren zwei verschiedene Cytochrom *c* Oxidasen, die Schlüsselenzyme der Atmung: in vegetativen Zellen wird das Produkt des *coxBACI* Gen exprimiert; in Heterocysten findet man hingegen das Produkt des *coxBACII* Gen.

In Organismen, welche oxygene Photosynthese betreiben wird der Elektronentransport vom Cytochrom b_6f Komplex zum Photosystem I entweder von Plastocyanin oder Cytochrom c_6 durchgeführt. Beides sind kleine, lösliche Metalloproteine, die sich an der P-Seite von Membranen befinden, welche photosynthetische Elektronentransportketten enthalten.

Plastocyanin (kodiert durch das *petE* Gen) besteht aus einer einzelnen Polypeptidkette, die sich aus einem β-Barrel mit 8 β-Faltblättern und einer kleinen α-Helix nebst eines Typ1-Kupfer Zentrums zusammensetzt.

Um cyanobakterielle Respiration besser zu verstehen und den Weg der Elektronen innerhalb dieses Systems nachvollziehen zu können, wurde eine verkürzte Form der Untereinheit II von cox2, SUIIj3 cox2 (enthält die Elektronen Bindungsstelle und die Stelle des Elektroneneingangs) und der Elektronencarrier Plastocyanin in *E. coli* kloniert, rekombinant exprimiert und durch Chromatographie gereinigt.

Rekombinant hergestellte SUII besitzt kein Kupfer in seinem aktiven Zentrum, wodurch dieses durch Rekonstitution zugeführt werden muss. Die Rekonstituierung durch Dialyse mit Harnstoff war zwar erfolgreich, aber sowohl Effizienz, als auch Reproduzierbarkeit waren zu gering. Durch Rekonstitution mittels Ultrafiltration konnte die Ausbeute an reinem, funktionellem Holoprotein gesteigert und ein etabliertes Protokoll erstellt werden. Allerdings erreichte die Ausbeute noch keinen

akzeptablen Wert und die Stabilität des Produktes war mangelhaft, weshalb es nicht möglich war, kinetische Messungen durchzuführen.
Die Menge an produziertem Plastocyanin war ausreichend für weitere kinetische Analysen und die Reinheit gleichermassen sehr hoch.

1. Introduction

1.1. Origin of Cyanobacteria

Cyanobacteria (blue-green algae) form the largest, most diversified, evolutionary most significant and ecologically most successful micro-organisms on our earth. They are found in many different environments such as subzero lakes in Antarctica, hotsprings, oceans, fresh-water lakes, nutrient-excessive sewage waters and in the colonization of bare rock. Colonies appear in a range of colours from dark green to black, yellow-green to red-brown and become both abundant and visually conspicuous **[1, 2]**.

Cyanobacteria were the first and are up to now the only oxygenic phototrophic prokaryotes. Using light as the most ubiquitous energy source, liquid water as the most ubiquitous electron source ("reductant"), and CO_2 as the most ubiquitous carbon source, together with the capability of N_2-fixation of many cyanobacteria, these oxygenic phototrophic organisms were indeed, and still are, the nonplus-ultra of bioenergetics **[3]**.

They are true pace makers of evolution in a geological and a biological sense. They set the pace for an oxygen-containing biosphere and atmosphere about 3.2 billion years ago. Since they were the first organisms to produce O_2, they also may have been among the first to sense and utilise it, for example in elaborating enzymes for the detoxification of partially reduced, reactive oxygen species. In the course of evolution gradual modification of the preexisting photosynthetic electron transport and enzyme systems could have changed a photosynthetic into a respiratory chain (conversion hypothesis) and therefore initiated a change in the earth's atmosphere from anaerobic to aerobic **[3-5]**.

1.2. Photosynthesis

Photosynthesis is very essential for our biosphere as it allows aerobic metabolism by producing the major organic molecules and O_2. The mechanism of photosynthesis is quite complicated by the fact that many proteins and small molecules are involved. In plants photosynthesis takes place in the chloroplasts, which have their origin in cyanobacteria (endosymbiont hypothesis) **[6]**. Photosynthesis consists of two sequential phases: the light reaction, which produces NADPH (see Eq.1) and ATP (see Eq.1), and the dark reaction or Calvin cycle, where CO_2 is reduced by NADPH and ATP to form organic molecules (see Eq.2). An estimated 10^{11} tons of carbon (in the form of CO_2) per year is converted into biomass by plant-type photosynthesis and the equivalent amount of O_2 is thereby released from water according to Eq.3. Recent estimates assign between 20% and 30% of this worldwide primary productivity to cyanobacteria, in particular to small unicellular marine *Synechococcus* species **[7]** and likewise unicellular planktonic Prochlorophytes **[8, 9]**.

$$2\ H_2O + 2\ NADP^+\ 10\ H^+_{stroma} \leftrightarrow O_2 + 2\ NADPH + 12\ H^+_{lumen}$$

Equation 1

$$3\ ADP^{3-} + 3\ P_i^{2-} + H^+ \leftrightarrow 3\ ATP^{4-} + H_2O$$

Equation 2

$$6\ CO_2 + 6\ H_2O \leftrightarrow C_6H_{12}O_6 + 6\ O_2 \quad \Delta G^\circ = \pm 2821.5\ kJ/mol$$

Equation 3

During the reactions in oxygenic phosphorylation, the flow of electrons in an electron transfer chain produces a proton motive force that is used by ATP-synthase for ATP-production according to the chemiosmotic hypothesis **[10]**. The fundamental difference between oxygenic phosphorylation and photosynthesis is the source of high potential electrons. In oxidative phosphorylation, they come from the oxidation of fuels; in photosynthesis, they are produced by photoexcitation.

The photosynthetic electron transport chain of chloroplasts is composed of three integral membrane-embedded structures: Photosystem I, Photosystem II and the cytochrome *b*$_6$*f* complex. They are located in the thylakoid membrane and connected by mobile redox carriers. The two photosystems, containing the light-energy transducer chlorophyll, cooperate to produce NADPH and ATP. The electron flow is referred to as the Z-scheme of photosynthesis **[11]**. Photosystem II is a big transmembrane complex composed of more than 20 subunits and catalyses the light induced transfer of electrons from H_2O to the lipid soluble carrier plastochinon (Eq.4), which is similar to ubichinon of the mitochondrial electron transport chain. Plastochinon changes between its oxidized (Q) and reduced form (QH_2) named plastochinol. The role of water is to provide electrons for these redox reactions. However, as the water molecule is a weak reducing agent, the electrons have to be further energized by photons in a rather complex process driven by chlorophyll.

$$2\ Q + 2\ H_2O \leftrightarrow O_2 + 2\ QH_2$$

Equation 4

The electron transfer between the two photosystems is mediated by the cytochrome *b*$_6$*f* complex, a homolog to complex III of oxygenic phosphorylation, which catalyses the transfer of electrons from plastochinol to plastocyanin (PC), a small copper protein in the thylakoid lumen (Eq.5). The cytochrome *b*$_6$*f* complex generates an electrochemical proton gradient across the thylakoid membrane, which is the driving power of ATP-synthase (the water photolysis mediated in photosystem II also releases protons inside the lumen, thus contributing the proton gradient **[12]**).

$$QH_2 + 2\ PC(Cu^{2+}) \leftrightarrow Q + 2\ PC(Cu^{+}) + 2\ H^{+}_{\text{thylakoid lumen}}$$

Equation 5

The last step of the light reaction is catalysed by Photosystem I, a transmembrane complex out of 14 polypeptides and many associated proteins and cofactors. It mediates the electron transfer from plastocyanin to ferredoxin (Eq.6). Finally ferredoxin reduces $NADP^+$ to NADPH **[13]**.

$$PC(Cu^{+}) + Fd_{ox} \leftrightarrow PC(Cu^{2+}) + Fd_{red}$$

Equation 6

Basically, the same transmembrane structures are found in cyanobacteria, located in the intracytoplasmatic membranes **[14]**. The subsequent photosynthetic electron transport chain is essentially the same as in chloroplasts, apart from the fact that cyanobacteria can use either cytochrome c_6 or plastocyanin as watersoluble electron carriers from cytochrome b_6f to Photosystem I, depending on the availability of copper **[15-19]**.

1.3. Respiration

Respiration comprises the electron transfer from NADH to O_2, mediated by three protein complexes: NADH-Q-Oxidoreductase (complex I), Q-cytochrome-*c*-oxidoreductase (complex III) and cytochrome-*c*-oxidase (complex IV). Plastochinon and cytochrome c_6, whose pool is shared between photosynthesis and respiration, manage the transport from one complex to another. Electron transport causes the translocation of protons across the thylakoid membrane, thus generating a proton gradient important for ATP synthesis. Electrons from $FADH_2$ [produced by succinat-Q-reductase (complex II) of the citrat cycle] enter the transport chain at the stage of complex III. Complex I catalyses the electron transfer from NADH to Q, which changes into its reduced form QH_2 (Eq.7).

$$NADH + Q + 5\ H^{+}_{matrix} \leftrightarrow NAD^{+} + QH_2 + 4\ H^{+}_{cytoplasm}$$

Equation 7

Complex III mediates the reduction of cytochrome *c* by plastochinol (Eq.8).

$$QH_2 + 2\ Cyt\ c_{ox} + 2\ H^{+}_{matrix} \leftrightarrow Q + 2\ Cyt\ c_{red} + 4\ H^{+}_{cytoplasm}$$

Equation 8

Finally, cytochrome *c* oxidase catalyses the electron transfer from reduced cytochrome *c* to molecular O_2 (Eq. 9) **[13]**.

$$4\ \text{Cyt}\ c_{red} + 8\ H^+_{matrix} + O_2 \leftrightarrow 4\ \text{Cyt}\ c_{ox} + 2\ H_2O + 4\ H^+_{cytoplasm}$$

Equation 9

1.4. Cyanobacterial photosynthesis and respiration

Cyanobacteria have uniquely accommodated both oxygenic photosynthesis and aerobic respiration within a single prokaryotic cell. A typical cyanobacterium comprises two types of bioenergetically competent membrane systems, the thylakoids or intracytoplasmic membranes (ICM) and the cytoplasmatic membrane (CM). While the CM only contains a respiratory electron transport chain but no photosynthetic reaction centers, the ICM is utilised for both photosynthetic and respiratory electron transport. Photosynthesis and respiration share the same location and several proteins (cytochrome c_6, plastoquinol, plastocyanin, cytochrome b_6f-complex), but they occur in different time and conditions, as light inhibits respiration and oxygen inhibits photosynthesis. The ICM represents a dual function RET-PET system (see Figure 1.1.) with a strong functional interaction of respiration and photosynthesis **[20, 21]**.

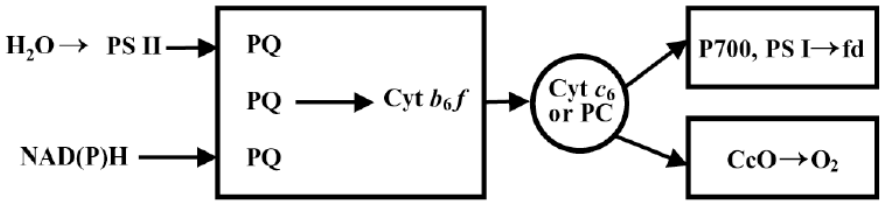

*Figure 1.1. The dual-function RET-PET system in cyanobacterial ICM. Either cytochrome c_6 or plastocyanin is absolutly indispensable for integral electron transport in both photosynthesis and respiration **[22]**. PS I: photosystem I, PS II: photosystem II, PQ: plastoquinone, Cyt c_6: cytochrome c_6, PC: plastocyanin, CcO: cytochrome c oxidase, fd: ferredoxin*

Cyanobacteria serve as an excellent model for studying oxygenic photosynthesis. The structure and function of most components of the photosynthetic electron transport chain are similar in cyanobacteria and higher plants **[23, 24]**.

The rates of cyanobacterial (endogenous) respiration are very low (between 1 and 10% of maximum photosynthetic rates) and most cyanobacteria are, as obligate photo(auto)trophs, unable to sustain efficient growth and proliferation in the dark at the expense of respiration. Respiration is needed for energy generation in the dark and whenever photosynthesis does not work. Growth in stress conditions, as diazotrophic growth, initiates a significant increase of activity and concentration of respiratory components on the non-photosynthetic CM and/or on PSII-inhibited ICM, while photosynthesis slows down. The rate of respiratory electron transport is increased up to ten-fold and temporarily substitutes for the lack of ATP, thus helping the cyanobacteria at least to survive **[3, 25, 26]**. A summary of the bioenergetic membrane functions in cyanobacterias's CM and CM is shown in Figure 1.2.

Given the presence of a photosynthetic electron transport chain in cyanobacterial CM, inherited from anoxygenic ancestors, part of this chain can also serve respiratory purposes in an aerobic environment in accordance with the conversion hypothesis which, in view of the striking similarity of all biological electron transport chains, emphasizes the monophyletic origin of biological electron transport **[5]**.

That is the main reason for a dual photosynthetic and respiratory electron transport chain in all respiring phototrophic prokaryotes, which in cyanobacteria, is necessarily confined to ICM. A consequence of the identity of respiratory and photosynthetic electron transport components (in ICM) is the strong functional interaction of the two processes; that is, light inhibits respiration and oxygen inhibits photosythesis under appropriate conditions **[27]**.

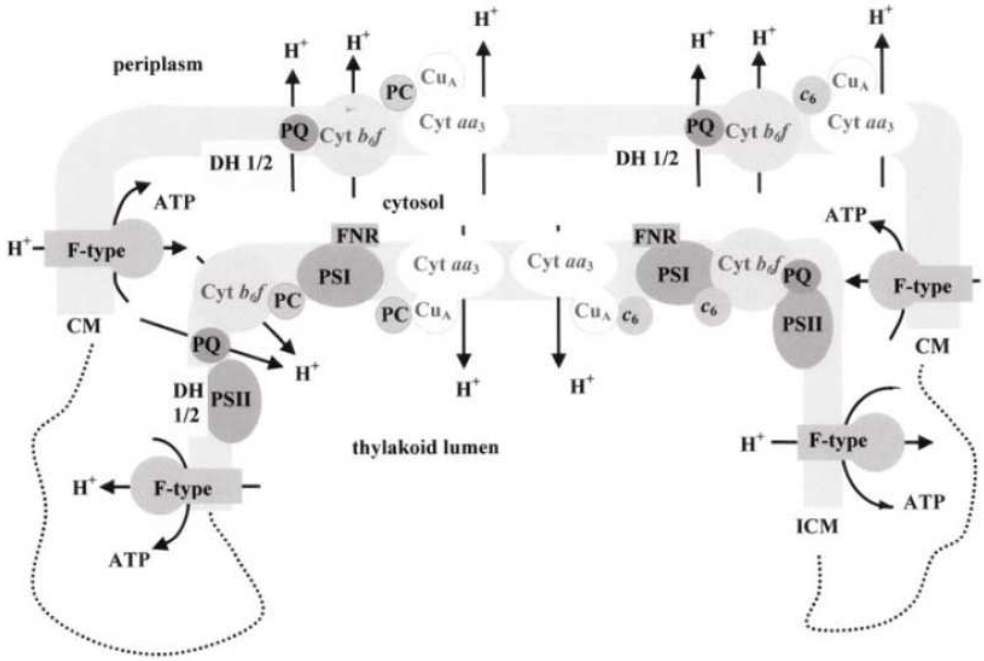

Figure 1.2. Bioenergetic membrane functions in cyanobacteria's CM and ICM PSI and PSII: photosystems I and II, PQ: plastoquinone, FNR: ferredoxin-NADP reductase, c_6: cytochrome c_6, PC: plastocyanin, $DH_{1/2}$: either bacteria-like nonproton-translocating one-subunit NADH dehydrogenase, or mitochondria-like multisubunit NADH dehydrogenase, Cyt b_6f: cytochrome b_6f complex, Cyt aa_3: cytochrome c oxidase, CuA: binuclear copper center of subunit II of cytochrome c oxidase, F-type: F-type H^+-translocating ATPase

1.5. *Nostoc sp*. PCC 7120

Nostoc sp. PCC 7120 is a filamentous cyanobacterium capable of both nitrogen fixation and oxygenic photosynthesis. These two processes are carried out in two different types of cells, vegetative cells and heterocysts. In absence of combined nitrogen, approximately every tenth vegetative cell along the filament differentiates into a heterocyst, which provides the anaerobic environment that is required for nitrogenase (reduces N_2 to ammonia) to function. Heterocysts are uniquely effective in protecting this highly O_2-sensitive enzyme from molecular oxygen produced in the neighbouring vegetative cells during oxygenic photosynthesis. The nitrogen-fixing

activities of *Nostoc* sp. contribute to the quality of nutrient poor soils, especially in karst regions **[2, 28-32]**.

It is thought that the capacity for heterocyst differentiation has arisen only once in cyanobacterial evolution. Oxygen evolution is regarded as the selective pressure that led to heterocyst-development. Based on sulphur isotope studies it was suggested that between 0.64 and 1.05 billion years ago the atmospheric oxygen levels rose from 5% to 18% of the present 100% level and based on this heterocyst forming species arose before the oxygen content of the atmosphere was especially high**[2]**.

Nostoc sp. PCC 7120, like other diazotrophs and N_2- fixing cyanobacteria, prefers fixed nitrogen sources. It expresses the system for nitrogen fixation only in the absence of combined nitrogen, since it requires high levels of energy and reductant. Ammonium can independently inhibit the development of both heterocysts and nitrogenase synthesis in heterocystous and also in non heterocystous nitrogen fixing cyanobacteria **[2, 49]**.

1.5.1. Heterocysts

Heterocysts are differentiated, O_2 depleted cells, which harbour the enzyme complex nitrogenase for nitrogen fixation. They are morphologically distinguished from vegetative cells through an extensive cell wall thickening, called the envelope, and the occurrence of distinct cyanophycin inclusion bodies (polar nodules) located intracellularly in the area of contact to the always adjacent vegetative cells. While vegetative cells of *Nostoc sp*. PCC 7120 are spherical, barrel-shaped or oval, heterocysts tend to be broader and longer. Representing a unique terminally differentiated cell stage, heterocysts have lost the ability to divide and redifferentiate into vegetative cells **[2, 34]**.

They are metabolically highly active cells and particularly well adapted for diazotrophic growth as heterocysts fix nitrogen in the light while the vegetative cells continue to carry out oxygenic photosynthesis. Nitrogen fixation and oxygen evolving photosynthesis are nominally incompatible processes. In order to prevent irreversible inactivation of the nitrogenase complex in the presence of oxygen, nitrogen fixing

cyanobacteria had to evolve protection strategies. The most complex of these was the development of heterocysts **[33, 35-39]**.

The protection of nitrogenase from inactivation by oxygen is virtually perfect and appears to depend on several characteristics of the heterocyst. A semipermeable barrier for penetration of gases is provided by the heterocyst envelope. It consists of an inner glycolipid layer localised just outside of the cell walls' peptidoglycan layer and an outer polysaccharide layer. Although the van der Waal's radii of nitrogen and oxygen are similar (1.5 Å and 1.4 Å) the barrier by the envelope reduces the permeability of both gases to the extent that the nitrogen that enters still suffices for the needs of the organism for nitrogen, while the oxygen that enters can be reduced to water by respiration **[1]**. The oxygen-evolving photosystem II complex is inactivated in heterocysts. Therefore they don't carry out oxygenic photosynthesis although they retain the capacity for photosystem-I-dependent photosynthetic reactions. Nitrogen fixation is associated with an increased respiratory activity, reflecting the high energetic costs of the process and also the need to minimize the oxygen level within the heterocyst. Other processes, including peroxidative activities and posttranslational modification of dinitrogenase reductase that limit the damage by oxygen, seem to play a role in the protection mechanism **[1, 30, 35, 40-48]**.

The relationship between heterocysts and vegetative cells is mutualistic. For nitrogen fixation heterocysts require the products of *nif* genes, reductant and ATP. Nitrogen is provided by heterocysts, reductant by vegetative cells. Thus some channels must remain open between the two cell-types. Heterocysts exhibit one to three pores, which provide a route unblocked by the envelope **[1]**.

Many non-heterocystous cyanobacteria (heterocysts are unknown in unicellular cyanobacteria) are able to fix nitrogen efficiently under ambient oxygen concentrations, without the recourse of heterocyst differentiation. They have to separate photosynthesis from nitrogen fixation either spatial or temporal (in the dark or transferred to anaerobic conditions). As shown under alternating light/dark cycles in cultures of *Anabaena*, photosynthesis occurs during the day, while nitrogen fixation can proceed at night, when the cellular concentration of oxygen is below the level at which nitrogenase is inactivated **[2]**.

1.5.2. Heterocyst development

The filamentous cyanobacterium *Nostoc sp*. PCC 7120 undergoes a developmentally programmed cell differentiation when it is deprived of a source of fixed nitrogen. Genes encoding components of the heterocyst differentiation machinery are scattered throughout the cyanobacterial genome. The process of differentiation requires a complex pattern of changes in gene expression **[2, 31]**.

The initial response to nitrogen step-down is the activation of genes required for the uptake and assimilation of nitrate. The next significant change is the activation of genes involved in the regulation of heterocyst development, perhaps the most important of which is *hetR*.

Additionally the generation of a pattern of single heterocysts requires the activation of genes responsible for regulation of the position at which heterocysts develop. At present only three genes are known to be involved in the control of heterocyst spacing: *patA*, *patB* and *patS*.

The creation of the microanaerobic interior to the heterocyst requires a series of major modifications to the original vegetative cell, including the adoption of the envelope, loss of photosystem II and loss of CO_2-fixation. The innermost of the new wall layers is the laminated layer, which consists of glycolipid; the next is the homogeneous layer, which consists of polysaccharide; and the outermost is the fibrous layer, which probably consists of uncompacted strands of the same polysaccharide.

The expression of the nitrogenase structural genes, *nifHDK*, is under developmental regulation. It requires completion, or at least partial completion, of heterocyst development in addition to nitrogen starvation and anaerobiosis. Under aerobic growth conditions, the latter is usually only available in heterocysts as they complete their morphological development. In general, nitrogen starvation and anaerobiosis do not induce *nif* gene expression in vegetative cells.

The *nif* operons in the vegetative cells of *Nostoc sp*. 7120 are interrupted by two excision elements **[1]**. During the late stages of heterocyst differentiation in *Nostoc sp*. PCC 7120, three developmentally regulated DNA rearrangements occur, involving the excision of the 11 kbp element within the coding sequence of *nifD* (encoding the α-subunit of dinitrogenase), the excision of *fdxN* (encoding a bacterial-

type ferredoxin) and *hupL* (encoding the large subunit of an uptake hydrogenase). These excisions seem to play no role in heterocyst development or spacing **[39]**.
The large 11 kbp insertion in the coding region of *nifD* is one of the most striking features in vegetative cells of *Nostoc. sp*. PCC 7120*.* The insertion is spliced from the genome by exicase encoded by *xisA*, creating a functional *nifHDK* operon. In non heterocystous nitrogen fixing cyanobacteria *nifHDK* genes have a contigous disposition, the 11 kbp insertion is not present **[1, 2]**.

1.6. Cytochrome *c* oxidase *(CcO)*

All eukaryotic cells, which grow under heterotrophic growth, synthesize ATP in two ways: by degrading glucose to lactic acid or alcohol via glycolysis, or by oxidative phosphorylation in mitochondria. The latter pathway yields about 15 times more ATP from glucose and is essential for all multicellular organisms **[36]**.
The mitochondrial respiratory chain transfers reducing equivalents from nutrients successively to molecular oxygen, accompanied by storage of the released energy in a proton gradient across the inner mitochondrial membrane via three proton pumping enzyme complexes: (i) NADH-ubiquinone oxidoreductase (complex I), (ii) cytochrome *c* reductase (complex III) and (iii) an aa_3-type cytochrome *c* oxidase, (complex IV). This either proton or electrochemical gradient is used by the enzyme ATP synthase for the endergonic synthesis of ATP from ADP and inorganic phosphate **[51 – 53]**. The terminal enzyme of the respiratory chain, C*c*O, reduces molecular oxygen to water without the formation of reactive oxygen species (ROS).
Terminal respiratory oxidases can best be functionally classified as a large superfamiliy of heme-copper oxidases **[54, 55]** and non-copper or *bd*-type and (certain) *o*-type oxidases **[56, 57]**. A unifying property of the former is electrogenic proton translocation through the membrane-bound oxidase **[58]**. Based on amino acid sequence similarities and the specific electron donor, cytochrome *c* or plastoquinol, respectively, cyanobacterial heme-copper cytochrome *c* or quinol oxidases have been separated into three groups: (i) aa_3-type cytochrome *c* oxidases, (ii) heme-copper quinol oxidases, and (iii) putative cytochrome *bd* quinol oxidases **[59]**, the latter do not contain copper but two heme *b* and one heme *d*, do not act as

proton pumps, and do not show any sequence similarity with either of the two groups of heme-copper oxidases.

Mammalian C*c*O consists of 13 subunits: In addition to the three mitochondrially encoded subunits I-III, which contain the four catalytic redox centers (CuA, heme *a*, heme a_3 and CuB) **[60]**, ten smaller subunits are nuclear-coded and partly expressed in tissue-specific isoforms **[61 - 64]**.

The yeast enzyme contains eleven **[65]** and the enzyme from the bacterium *Paracoccus denitrificans* (*P. den*.) consists of four subunits, three of which correspond to the mitochondria-encoded subunits of the eukaryotic enzyme **[66]**. It was postulated, that the increasing number of subunits during evolution reflects an increasing regulatory complexity of the enzyme **[61 - 63, 67]**. But only within the last few years regulatory functions could be identified for some nuclear-coded subunits of the mammalian enzyme.

The aa_3-type C*c*O is also the terminal respiratory oxidase of many aerobic bacteria with subunit I-III showing strong homology to their mitochondrial counterparts.

Bacterial C*c*Os have simpler structures than mammalian C*c*O, resulting in a lower complexity of physiological and genetic structure. Therefore bacterial C*c*Os can be easily manipulated with molecular genetic techniques and are very useful model enzymes. For this reason the structure of C*c*O from *Paracoccus denitrificans* was chosen as a model to analyse structure and function.

1.6.1. C*c*O from *Paracoccus denitrificans* serves as a model for structure-function analysis

The arrangement of the four subunits (SU) of *Paracoccus denitrificans* (*P. den*.) C*c*O in the membrane and the location of the redox centers are schematically illustrated in Figure 1.3. (A) **[66, 68]**.

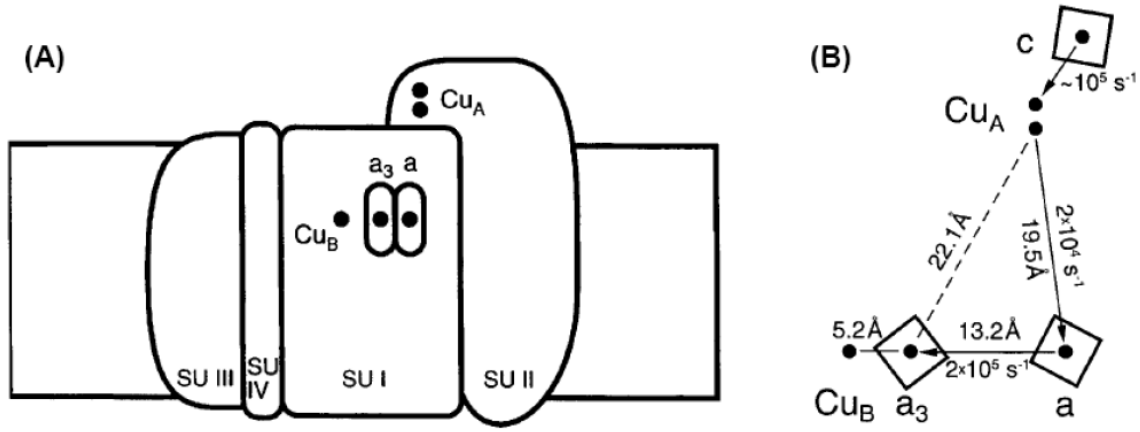

Figure 1.3: ***(A)*** *Arrangement of the four protein subunits of CcO from* Paracoccus denitrificans *in the membrane and placement of the redox centers.* ***(B)*** *Distances and electron transfer rates between the redox centers in P. den. CcO.*

The CuA center, composed of two electronically coupled, mixed-valence copper ions, is located at the hydrophilic domain on the cytosolic side of subunit II. An analysis of site-directed mutants **[69]** has confirmed the classical view that this center is indeed the first, and only, acceptor site in the complex. The electron is subsequently transferred from the CuA site to heme *a* in subunit I. This low-spin heme is closer to the Cu_A center than heme a_3, and therefore heme *a* is the kinetically preferred electron acceptor. Both heme planes are oriented perpendicular to the membrane, at an angle of 108° to each other. Their iron centers are 13 Å apart, but their closest edge-to-edge distance amount to only 5 Å. The high-spin heme a_3 and an electronically coupled CuB ion form the binuclear center of the enzyme **[50]**.

The rate of electron transfer from Cyt *c* in the enzyme-substrate complex is as high as 1 x 10^5 s^{-1} **[70, 71]**, as illustrated in Figure 1.3. (B). From CuA the electron is transferred to cytochrome *a* (cyt *a*) (2 x 10^4 s^{-1}) and it then equilibrates rapidly (2 x 10^5 s^{-1}) with cytochrome a_3 (cyt a_3) **[72, 73]**. These rates have been determined in experiments with the mixed-valence oxidase-CO complex, in which CO dissociation by flash photolysis induces a backflow of electrons from the reduced cyt a_3-CuB site to the oxidized cyt *a* and CuA site **[71-74]**. It has been suggested, that the rate of the normal forward ET from cyt *a* to cyt a_3 is limited by proton uptake **[72, 75, 76]**.

1.6.2. Cyanobacterial cytochrome *c* oxidase

In cyanobacteria a mitochondria-like aa_3-type C*c*O appears to be the major terminal respiratory oxidase. Its genes are always clustered in a single operon: *coxBAC*, encoding subunit II, subunit I and subunit III **[47]**. Subunit I harbours the heme-copper active site, subunit II contains a copper center that functions as the primary electron acceptor from cytochrome *c* and subunit III is a membrane protein without redox centers **[77 - 80]**.

Subunit I

The membrane-embedded subunit I (see Figure 1.4 A) is the largest and most conserved subunit of cytochrome *c* oxidase and contains the active site where oxygen is reduced. It binds the cofactors of the catalytic centers, the heme-copper binuclear site (high spin heme a_3 and CuB) and the low spin heme *a*. In some oxidases subunit I is merged with subunit III or parts of it. It usually contains 12 transmembrane helices with a short loop between helices I and II. Thus the 12 transmembrane helices of cyanobacterial subunit I seem to form an unusually compact confinement (scaffold) for the redox centers. The C-terminal endings of cyanobacterial subunit I is normally about 10 to 20 amino acid residues longer than that of *P. denitrificans*. The transmembrane helices form three arcs within the membrane. Each arc is shaped by four transmembrane helices and together with the last segment of the previous arc pore-like arrangements termed pore A, B, C are formed. The first pore A is filled with mostly conserved aromatic residues, whereas pore B holds the heme a_3-CuB binuclear center and pore C contains the low spin heme *a* **[81-85]**.

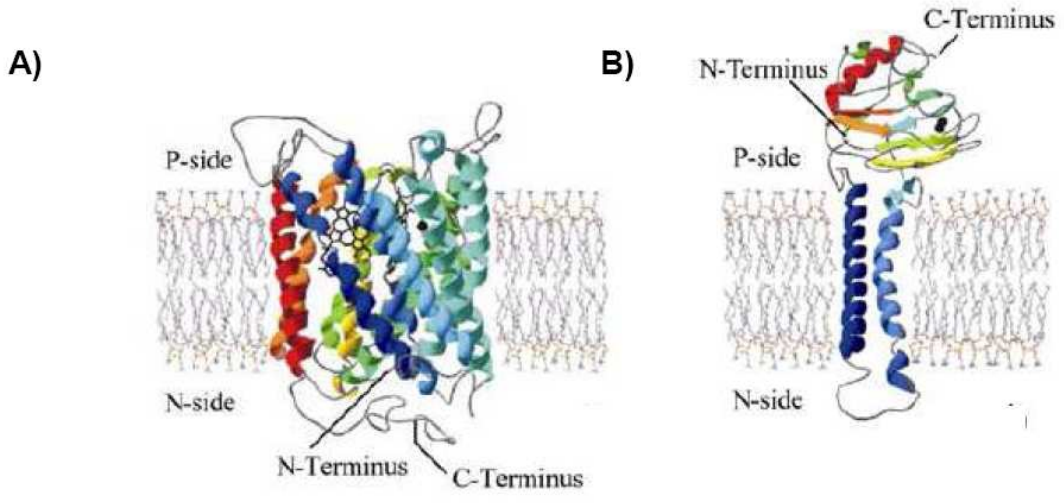

Figure 1.4: Subunit I A) and II B) of the cytochrome c oxidase in Synechocystis sp. *PCC 6803. Model building was based on the crystal structure of cytochrome c oxidase from P. denitrificans by using SWISS-Model and the SWISS-PdbViewer. P-side: periplasmic side, N-side: cytosolic side, copper ions and hemes a and a_3 are depicted in black.*

Subunit II

Subunit II is associated with the CuA center (see Figure 1.4.b.). It is composed of an N-terminal transmembrane helical hairpin followed by a soluble globular domain exposed on the outer membrane surface, whereas latter can be either CM or ICM. The soluble domain of subunit II provides the inner sphere ligands for the binuclear CuA center. Although the copper site of CuA is quite distinct, this domain is related in its protein fold to the cupredoxin fold of simple blue copper proteins **[86, 87]**.

The core of the soluble domain of cyanobacterial heme-copper oxidases compared to *P. denitrificans* cytochrome *c* oxidases shows similar structural elements as a β-barrel formed by 10 β-sheets, but there are significant structural deviations at the N- and C-terminal ends. The fold of the periplasmic domain from *P. denitrificans* is a 10 stranded β-barrel with an overall greek key topology. The loops between strands β3/β4 and β5/β6 contain carboxylic amino acid residues that are conserved among cytochrome *c* oxidases but not among quinol oxidases. These residues have been proposed to form the cytochrome c binding site **[24, 83]**.

Cyanobacterial heme-copper-oxidase subunit II displays the typical loop located between the second transmembrane helix and the cupredoxin-like CuA-domain. Both cyanobacterial cytochrome *c* oxidases and quinol oxidases have this unusual insertion, which is not found in *P. denitrificans*, nevertheless its length and sequence

varies. Sequences of cyanobacterial subunits II include a putative N-terminal signal peptide, which is most likely cleaved off after translocation **[24, 87, 88]**.

CuA-domain

The CuA site is a redox center in cytochrome *c* oxidase and acts as a single electron acceptor and donor. Spectroscopic studies established that the CuA-domain is a binuclear, highly delocalized, mixed valence, class IV center, often denoted as [Cu(1.5)-Cu(1.5)]. The single unpaired electron is shared by the two copper ions and is found with equal probability at either metal. Spin delocalization appears to be a fundamental property of CuA centers that distinguishes it from the Fe/S clusters of ferredoxins **[79, 89 - 92]**.

The key amino acids of the conserved CuA site are found near the C-terminal end of cytochrome *c* oxidase and are defined by a specific motif, containing one methionine, two histidine and 2 cysteine residues. This part of the protein projects into the periplasmic space and apparently represents an independent folding domain **[89, 93]**.

The CuA site is formed by residues from strand β6 and the loop connecting strands β9 and β10 and is basically defined by a Cu_2S_2 core. The two copper atoms, located within bonding distance of each other, are bridged by S_γ atoms of Cys-II-281/249 and Cys-II-285/253 (*Nostoc sp*. PCC 7120 coxBACI/coxBACII numbering) **[86, 91]**. These four atoms are arranged almost in a single plane. Each copper ion is further coordinated by a histidine ligand (Nδ1 atoms of His-II-246/214 and His-II-289/257, *Nostoc sp*. PCC 7120 *coxBACI*/*coxBACII* numbering).

Axial ligands are the Sδ atom of Met-II-292/260 and the backbone carbonyl atom of Glu-II-283/251 (*Nostoc sp.* PCC 7120 *coxBACI*/*coxBACII* numbering). The result is an almost symmetric structure, although the weak axial ligands are different for the individual copper ions. The identity of the ligands is conserved among cytochrome *c* oxidases and also among nitrous oxide reductases that contain a similar CuA center, with the exception of the residue donating the backbone carbonyl oxygen atom **[59, 63, 78, 83, 90]**.

1.6.3. Cytochrome *c* oxidase from *Nostoc sp.* PCC 7120

The genomic sequence of *Nostoc sp.* PCC contains four respiratory operons encoding terminal oxidases: *coxBACI, coxBACII, coxBACIII*, their gene clusters are homologous to *coxBAC* of *Synechocystis sp*. PCC 6803, and all4023-all4024. The *coxBACI* (cox1) operon is essential for chemoheterotrophic growth whereas *coxBACII* (cox2) is the dominating cytochrome *c* oxidase under diazotrophic conditions **[29]**. *coxBACIII* (cox3) is designated as a cytochrome *c* oxidase, although its subunit II lacks the CuA site. It is similar to *Synechocystis* ARTO **[41]**. The all4023-all4024 sequences encode a two subunit operon defined as a cytochrome D ubiquinol oxidase **[28, 94, 95]**.
Analysis of *coxBACI* and *coxBACII* and the comparison of the amino acid sequence of *Nostoc sp*. PCC 7120 with *P. denitrificans* showed that both encode for proteins containing conserved residues known to be required for cytochrome *c* oxidase function. Subunit II of both operons possesses the conserved CuA motif, a distinctive feature of cytochrome *c* oxidases and subunit I from *coxAII* and *coxAI* contains three conserved motifs: binding sites for the heme *a* and a_3, and a CuB site **[28]**.

coxBACI, which appears to be the ortholog of *coxBACI* operon of *Anabaena variabilis* **[95]**, is constitutively expressed in all vegetative cells with regard to the nitrogen source. No indication of expression in mature heterocysts was obtained so far. Under conditions of nitrogen deprivation every tenth cell differentiates into a heterocyst. During the process of heterocyst differentiation the expression of *coxBACII* operon is specifically upregulated in heterocysts and proheterocysts. The pattern of expression of the *coxBACIII* gene cluster is parallel with that observed for *coxBACII*. Both *coxBACII* and *coxBACIII* are bioenergetically active as they contribute to respiration in heterocysts, thus they are essential for nitrogenese activity and diazotrophic growth. Mutants of *Nostoc sp*. PCC 7120 in which either *coxBACII* or *coxBACIII* was deleted were still able to grow diazotrophically, whereas deletion of both resulted in almost zero growth under nitrogen-fixing conditions **[28, 36, 42]**.

1.7. The small soluble electron donors in photosynthesis and respiration: cytochrome c_6 and plastocyanin

Cytochrome c_6 (Cyt c_6) functions as an electron carrier between the cytochrome b_6f complex and either photosystem I **[96]** or C*c*O **[97]** in cyanobacteria and in several algae. In the presence of copper plastocyanin (PC) serves as a functionally equivalent electron carrier **[98, 99]**. In species which can synthesize both Cyt c_6 and PC the expression levels of the corresponding genes are controlled by the copper concentration in the growth medium **[100, 101]**.

During the course of evolution, due to a change in the environment and by this the availability of soluble copper and iron, PC seems to have been able to replace Cyt c_6 as PC is the only electron donor in the photosynthesis of higher plants **[96]**. Nevertheless, database analysis has revealed a gene for Cyt c_6 in higher plants, but significant differences to cyanobacterial or algal Cyts c_6 suggest a modified function **[102, 103]**.

Despite inherent differences Cyt c_6 and the copper protein PC have to share common physicochemical and structural features in order to accomplish equivalent redox reactions with the same reaction partners.

The total genomic sequence of *Nostoc sp*. PCC 7120 has revealed the presence of three copies of genes for Cyt c_6 **[94]**. No data are available about the function of these genes and the protein they may encode, but one might speculate that the multitude of genes is related to the fact that this strain develops heterocysts upon removal of combined nitrogen **[104]**. In absence of information about plastocyanin from *Nostoc sp*. PCC 7120 and to elucidate the structure and function of both proteins as shown in Figure 1.5, Cyt c_6 and PC from *Synechocystis sp*. PCC 6803 are briefly described in the following chapters.

1.7.1. Cytochrome c_6 from *Synechocystis sp*. PCC 6803

Cyt c_6 from *Synechocystis sp*. PCC 6803, encoded by the single copy *petJ* gene is a typical class-I *c*-type cytochrome containing four *α*-helices and a covalently-linked heme group (Cys14 and Cys17, *Arthrospira maxima* numbering) in which the Fe-

atom is axially coordinated by a histidine (His18) and a methionine (Met62) as shown in Figure 1.5. The edge of pyrrole ring C and D propionic groups are solvent-accessible, thus establishing a tentative electron-transfer pathway to and from the heme Fe-atom **[105]**.

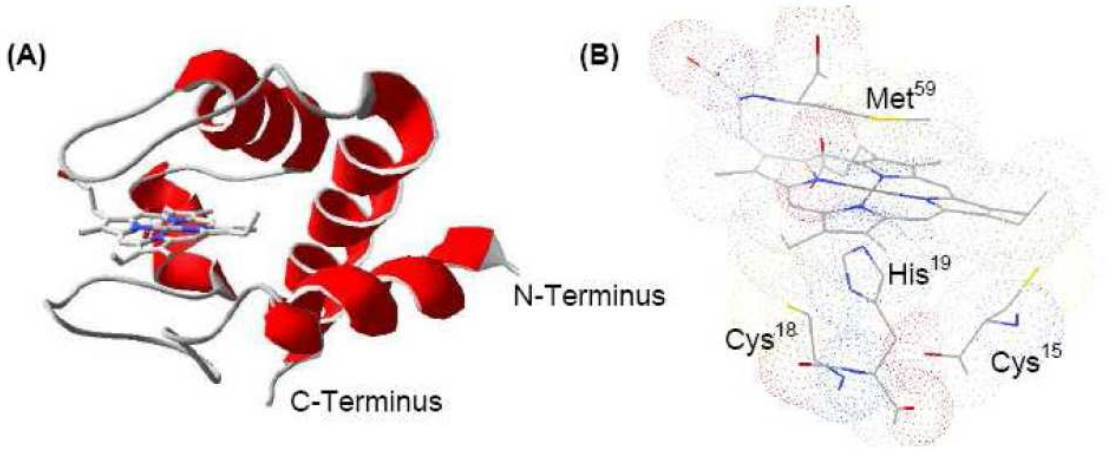

Figure 1.5: ***(A)*** *3D model of Cyt c_6 from Syn. using SWISS-Model and the SWISSPdbViewer. The model building was based on the crystal structure of Cyt c_6 from* Porphyra yezoensis *(PDB code 1GDV). The four typical α-helical segments are shown in red, coils in grey. The heme group is shown.* ***(B)*** *Close-up of the heme group with ligands of the* Synechocystis *Cyt c_6 model.*

Maturation of *c*-type cytochromes involves posttranslational events. These include the targeting of the protein and heme to the correct subcellular compartment, the processing of the targeting sequence, heme attachment and the formation of the covalent linkage. The molecular mechanisms involved are still not quite clear and in discussion.

1.7.2. Plastocyanin from *Synechocystis* PCC 6803

Plastocyanin (PC), classified as a “small blue” or type I copper protein and in recognition of its redox-function as a cupredoxin (encoded by the *petE* gene) consists of a single polypeptide chain forming a *β*-barrel with eight *β*-strands and a small *α*-

helix, along with a type-1 blue copper center (Fig. 1.6). The metal is coordinated by two histidines (His39 and His86, *Synechocystis* numbering), one methionine (Met91), and one cysteine (Cys83) and the Cu-site geometry is best described as “distorted trigonal-pyramidal”. His86 is the only solvent-exposed Cu-ligand, thus making this residue the most probable electron-entry site of PC. The shortest distance from the Cu atom to the molecular surface, i.e. to the imidazole ring edge of His87, is about 6 Å **[106]**.

The surface of the protein in the vicinity of the copper site includes a hydrophobic patch. It has been proposed that this patch is the likely site of interaction between plastocyanin and its redox-partner proteins **[107]**.

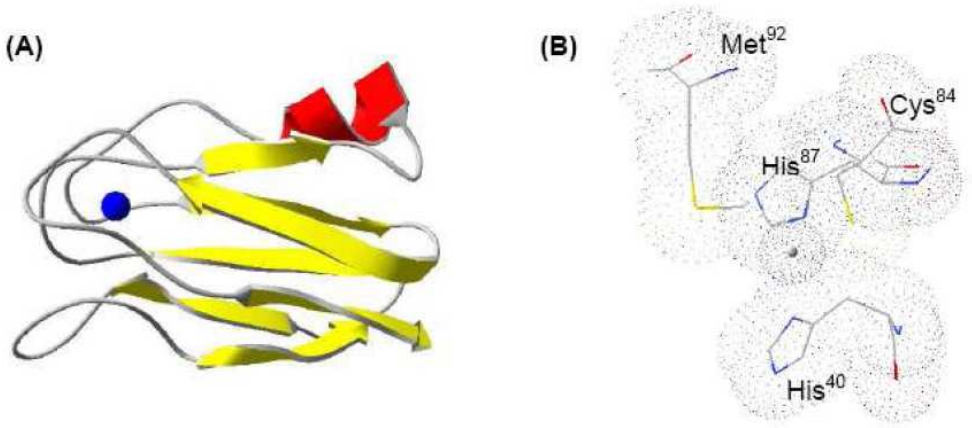

Figure 1.6: **(A)** *3D structure of PC from* Synechocystis*. (PDB code 1J5C).* α*-helical segments are shown in red,* β*-strands are shown in yellow and coils in grey. The copper ion is depicted in blue****. (B)*** *Close-up of the PC active site with ligands. The copper ion is drawn as grey sphere.*

2. Aims of work

The Metalloprotein Research Group (Biochemistry B) at the Department of Chemistry, BOKU Vienna investigates the structure-function relationship of cyanobacterial aa_3-type cytochrome *c* oxidase. C*c*O is the key enzyme of cell respiration and cyanobacterial C*c*O is particularly interesting to study since cyanobacteria were the first organisms carrying out oxygenic photosynthesis, producing molecular oxygen in a previously anoxic, O_2-free biosphere. Thus, it seems likely that cyanobacteria were among the first to elaborate a mechanism for aerobic respiration essentially by modifying and adapting pre-existing photosynthetic electron transport systems **[3]**.

The soluble part of the CuA-domain of subunit II of C*c*O from *Synechocystis sp*. PCC 6803 was successfully expressed recombinantly in high yield and electron transfer kinetics with the soluble electron donors cytochrome c_6 and plastocyanin were determined **[25, 108]**. This research revealed that both, the heme- as well as the copper protein can serve as electron donors for the C*c*O. Highly pure recombinant cytochrome c_M was also obtained even if not in high yeld. The bimolecular rate constants for the forward reaction were determined and showed to be comparable with rates from cytochrome c_6 and plastocyanin by Tangl et al. **[109]** but the results were not very significant because of the instability of the reduced cytochrome c_M.

The genomic sequence of *Nostoc sp*. PCC contains four respiratory operons. Of particularly interest are the *coxBACI* (cox1) operon essential for chemoheterotrophic growth and *coxBACII* (cox2) which is the dominating cytochrome *c* oxidase under diazotrophic conditions **[110]**. Judith Schachinger successfully overexpressed and purified the soluble CuA-domain containing part of *coxBII* (SUIIj3 cox2, amino acid residues 137-327), but could not reconstitute it **[111]**. A recombinant, purified apoprotein of part of *coxBI* was also produced by Doris Gusenbauer **[112]**, setting the cutting site to the amino acid residue 113 (the clone was named SUIId2 cox1, fragment length: amino acid residues 113-355). Chantal Lucini continued the work on expression and reconstitution of SUII cox2, producing a new clone of recombinant SUII cox2 named SUIIc3 cox 2 (fragment length: amino acid residues 113-327) with

the same cutting site as SUIId2 cox1, comprising both the electron binding site and the electron entry site but without the two transmembrane helices at the N-terminus. In order to investigate the mode of interaction and kinetics of electron transfer between soluble CuA-domain and the potential electron donors plastocyanin, cytochrome c_6 and cytochrome c_M SUIIc3 cox2 had to be reconstituted. Even tough, the reconstitution of SUIIc3 cox2 had been successful, the yield of total protein amount was not enough for kinetic measurements and the protein solution failed being pure enough **[113]**.

Aim of this work was to apply and improve the existing reconstitution protocol for SUIIc3 cox2 to the new clone SUIIj3 cox2 (fragment length: amino acid residues 137-327) to achieve higher reconstitution efficiency, which should lead to more available holoprotein for kinetic measurements. With an appropriate and reproducible method of reconstitution this protocol could also be used for reconstitution of SUIIc3 cox2 as well as SUIId2 cox1.

Further, it was tried to recombinantly produce and purify the electron transporter plastocyanin (fragment length: amino acid residues 29-126) in amounts sufficient to perform preliminary kinetic measurements with the CuA-domain of subunit II of both C*c*Os. These analyses should shed light on whether plastocyanin could react with the CuA-domain of subunit II of both C*c*Os. Furthermore, kinetic measurements with C*c*O and all available potential electron donors plastocyanin, cytochrome c_6 and cytochrome c_M should be performed to investigate respiratory electron transport in *Nostoc sp*. PCC 7120.

3. Material and Methods

3.1. Cloning of recombinant proteins

The soluble part of cytochrome *c* oxidase 2 subunit II (SUIIj3 cox2) and plastocyanin of *Nostoc sp.* PCC 7120 was cloned in the same *E. coli* strain to allow recombinant expression of the proteins. The sequences to be inserted were amplified by polymerase chain reaction (PCR) and transferred into an expression vector. The plasmid containing the desired protein was then transformed in electrocompetent *E. coli* BL21(DE3)pLysS cells by electroporation. The used expression system is listed in Table 3.1:

Table 3.1: Recombinant proteins and the used expression systems

Protein	**Number**	**Amino acid residues**	**Molecular Weight**	***E. coli* strain**	**Expression vector**
Cytochrome c Oxidase 2 Subunit II	j3-clone	137-327	22.5 kDa	BL21(DE3)pLysS	pET-3a
Plastocyanin		29-126	11 kDa	BL21(DE3)pLysS	pET-3a

3.1.1. Cloning of SUIIj3 cox2

The insert SUIIj3 cox2 was amplified by PCR and ligated into a pET-3a expression vector as shown in Figure 3.1. The pET-3a vector carries the IPTG-inducible T7-promoter sequence, various restriction sites and an ampicillin resistance gene for selection purposes. The plasmid encoding for SUIIj3 cox2 was transformed into electrocompetent *E. coli* BL21(DE3)pLysS cells carrying a pLysS-plasmid coding for T7-lysozym (natural inhibitor of T7-RNA-polymerase) and additionally, a chloramphenicol resistance gene.

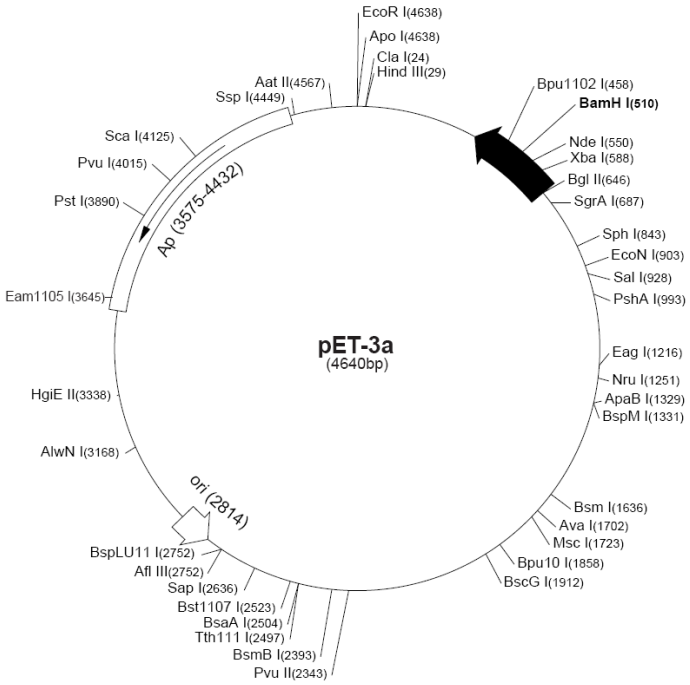

Figure 3.1: pET-3a Vector used for expression

3.1.1.1. Amplification of SUIIj3 cox2 by PCR

- **Principles**

PCR is used to amplify target DNA enzymatically. Therefore a specific oligonucleotide called primer, flanking the target DNA-sequence were designed. The reaction mix used for PCR consisted of template DNA, DNA-polymerase, oligonucleotide-primers, dNTPs, buffer and $MgCl_2$. Several cycles were performed, each cycle beginning with a brief heat treatment to separate the DNA double strands (denaturation step), followed by a cooling step where the two primers, present in large excess, hybridize to the complementary sequence in the two DNA strands and finally elongate. Because of the high annealing temperature required for the primers to hybridize properly, the annealing gradient extends up to the defined temperature (twostep PCR). Hot-start-PCR is used to avoid pre-PCR-mismatching.

- **Protocol**

- 1.5 mL culture pellet of *Nostoc sp*. PCC 7120 was centrifuged 10 minutes at 14000 rpm and the supernatant discarded.
- The sample and the negative control were prepared as follows:

Table 3.2: Components of the mastermix

	Sample	**Negative control**
upstream primer suIIj3_cox2_p1 [10 pmol/µl]	2.5 µL	2.5 µL
downstream primer suIIj3_cox2_p2 [10 pmol/µl]	2.5 µL	2.5 µL
dNTPs [10 mM]	5 µL	5 µL
10x Gold buffer	10 µL	10 µL
$MgCl_2$ [25 mM]	10 µL	10 µL
Template DNA (*Nostoc sp*. PCC 7120)	5 µL	---
Sterile water	63 µL	68 µL

- 99 µL of the mastermix and of the negative were transferred into a PCR-tube.
- The PCR was started using the thermocycler program shown in Table 3.3.
- After 6 minutes 1 µL AmpliTaq Gold polymerase 5.0 U/µl was added to the mastermix and the negative control.

Table 3.3: Thermocycler program

Step	**Action**	**Temperature**	**Time**	**Number of cycles**
1	Heating	95℃	10 min	
2	Denaturation	94℃	40 sec	x30
3	Annealing	51℃	30 sec	
4	Synthesis	72℃	70 sec	
5	Synthesis extension	72℃	10 min	

- **Chemicals and equipment**

Primer: genXpress

N-Terminal: suIlj3_cox2_p1 [10 pmol/µl]:

5’ GGGAATTCCATATGAACCAAATGGGAGGATTAGAACCTGGGACTCATCCCC 3’
Nde I Start Insert

C-Terminal: suIlj3_cox2_p2 [10 pmol/µl]

5’ CGCGGATCCCTAATTTACAGACGTTGTATGCAATGTCTCTAAAGTGGCTGC 3’
BamHI Stop Insert

AmpliTaq Gold: Applied Biosystems AmpliTaq Gold 5.0 U/µl
Gene Amp 10x PCR Gold Buffer
Template: *Nostoc sp. PCC 7120*
10 mM dNTP Mix: Fermentas
25 mM $MgCl_2$: Fermentas

Centrifuge: Sigma 1-15 (Sigma, Germany)
Thermocycler: Techne TC-312

3.1.1.2. Agarose gel electrophoresis

- **Principles**

The successful amplificiation of SUIlj3 cox2 after PCR was controlled by agarose gel electrophoresis performed with E-Gel® stained with SYBR Safe™.
E-Gel® pre-cast agarose gels are self-contained gels that include electrodes packaged inside a dry, disposable, UV-transparent cassette. The E-Gel® agarose gels run in a specially designed device that is a base and power supply combined

into one device. The gel contain SYBR Safe™ DNA gel stain. Agarose gel electrophoresis is used as an analytical or preparative method. In solution DNA is negatively charged and migrates to the cathode, when exposed to an electric field. Due to the sieve-effect of the gel, shorter DNA-fragments migrate faster than longer, hence DNA is separated according to its size. SYBR Safe™ DNA gel stain has been specifically developed for reduced mutagenicity, making it safer than ethidium bromide for staining DNA in agarose gels. The detection sensitivity of SYBR Safe™ stain is similar to that of ethidium bromide. DNA bands stained with SYBR Safe™ DNA gel stain can be detected using a standard UV transilluminator.

- **Protocol**

- The E-Gel® PowerBase™ v.4 was plugged into an electrical outlet using the adaptor plug on the base.
- The gel was removed from the package and inserted with the comb in place into the base right edge first.
- The gel was pre-run for 2 minutes and the comb removed from the E-Gel® cassette.
- 10 µL of PCR sample & negative control were loaded per sample well.
- 10 µL of prepared DNA molecular weight marker (5 µL marker + 5 µL sterile water) were loaded.
- Remaining empty wells were filled with 10 µL of sterile water
- Electrophoresis was performed for 15 minutes and the DNA bands were visualised using an UV- transilluminator.

- **Equipment**

E-Gel® with SYBR-Safe™: Invitrogen
E-Gel® PowerBase™ v.4: Invitrogen
UV-Transilluminator: Gene Flash
Syngene Bioimaging
GeneRuler™ 1 kb DNA Ladder: Fermentas

3.1.1.3. DNA Purification after PCR

- **Principles**

The successfully amplified insert of SUIIc3 cox2 was purified using the Gene JET™ Plasmid Mini Prep-Kit. Chaotropic salts promote the binding of DNA to the silicabased membrane of the column and denature protein contaminants. Contaminants and salts are removed by subsequent washing with an ethanolic buffer. Purified DNA was eluted from the glass fibre matrix by sterile water.

- **Protocol**

- The GeneJET™ spin column was prepared by placing the column in a collection tube.
- 500 µL Neutralisation solution and 100 µL PCR sample were applied to the column and mixed gently with a pipette. The solution was centrifuged at 14000 rpm for 60 sec. The flow-through was discarded.
- 500 µL Washing solution (diluted with ethanol prior to first use) were added and centrifuged at 14000 rpm for 30 sec. The flow-through was discarded.
- The wash procedure was repeated using 500 µL Washing solution.
- The flow-through was discarded and the column centrifuged for an additional minute to remove residual ethanol.
- The GeneJET™ spin column was transferred into a fresh 1.5 mL eppendorf tube. 85 µL sterile water were added to the center of GeneJET™ spin column membrane to elute the plasmid DNA, incubated for 2 minutes at room temperature and centrifuged at 14000 rpm for 2 minutes to recover the purified DNA.

- **Equipment**

Gene JET™ Plasmid Mini Prep-Kit: Fermentas
Centrifuge: Sigma 1-15 (Sigma, Germany)

3.1.1.4. Preparation of the pET- 3a vector

- **Principles**

The pET-3a vector, used for expression of SUIIc3 cox2, carries an IPTG-inducible T7-promoter sequence, various restriction sites and an ampicillin resistance gene for selection purposes.
The Gene JET^{TM} Plasmid Mini Prep-Kit was used for the preparation of the pET-3a vector. Previously cultivated Top F10' cells were lysed by an alkaline lysis procedure. The resulting lysate is neutralized to create appropriate conditions for binding of plasmid DNA on the silica membrane in the spin column. Cell debris was pelleted by centrifugation, and the supernatant containing the plasmid DNA was loaded onto the spin column membrane. The adsorbed DNA was washed to remove contaminants, and was then eluted with a small volume of sterile water.

- **Protocol**

- 15 mL of LB_{Amp}-medium were inoculated with Top F10' cells containing pET-3a from a cryo-culture and incubated overnight at 37℃ and 180 rpm.
- 10 x 1 mL of the culture were transferred into eppendorf tubes and centrifuged 10 minutes at 14 000 rpm. The supernatant was discared.
- The pelleted cells were resuspended in 250 µL of the Resuspension Solution by vortexing.
- 250 µL of the Lysis Solution were added and mixed thoroughly by inverting the tube 4-6 times until the solution became viscous and slightly clear.
- 350 µL of the Neutralization Solution were added, mixed immediately by inverting the tube 4-6 times and centrifuged for 5 minutes at 14000 rpm.
- The supernatant was transferred to the supplied GeneJET™ spin column by carefully pipetting and centrifuged for 1 minute at 14000 rpm. The flow-through was discared.
- 500 µL Washing solution (diluted with ethanol prior to first use) were added and centrifuged at 14000 rpm for 30 sec. The flow-through was discarded.

- The wash procedure was repeated using 500 µL Washing solution
- The flow-through was discarded and the column centrifuged for an additional minute to remove residual ethanol.
- The GeneJET™ spin column was transferred into a fresh 1.5 mL eppendorf tube.
- 50 µL sterile water were added to the center of GeneJET™ spin column membrane to elute the plasmid DNA, incubated for 2 minutes at room temperature and centrifuged at 14 000 rpm for 2 minutes to recover the purified DNA.
- To concentrate the plasmid DNA the eluate of two columns was put together and purified with the DNA purification protocol (see chap. 3.1.1.3) until 100 µL concentrated vector-solution were obtained.

- **Chemicals and equipment**

Top F10' cells containing pET-3a: Novagene

LB-Medium:	**LB-MediumAmp**:
10 g Peptone	LB-Medium
10 g NaCl	100 µg/mL Ampicillin
5 g Yeast extract	**Ampicillin stock solution**:
RO-water added to 1000 mL	100 mg/mL RO-water
autoclaved 20 min at 121℃	filter-sterilised and st ored ad –20℃

Gene JET^{TM} Plasmid Mini Prep-Kit: Fermentas
Centrifuge: Sigma 1-15 (Sigma, Germany)

Orbital shaker: Infors HT, Ecotron, Infors AG
Autoclave: Fritz Gössner, Hamburg

3.1.1.5. Digestion of pET- 3a and insert with restriction enzymes

3.1.1.5.1. Digestion of the pET- 3a vector with *Nde*I and *BamH*I

- **Principles**

For insertion of the amplified DNA-fragment into the vector DNA, both the insert and the vector have to be digested by the same specific restriction enzymes. The pET-3a vector possesses a multiple cloning site with various restriction sites. The digestion of DNA with the chosen restriction enzymes *BamH*I and *Nde*I results in linear DNA fragments with so called sticky ends, which can be ligated in a further step (see Chapter 3.1.1.6). Calf intestine alkaline phosphatise (CIP), which is added to the digestion assay of vector DNA, dephosphorylates the sticky ends of plasmid DNA and therefore prevents the ligation of the vector-DNA without an insert.

- **Protocol**

Digestion with *Nde*I:

- The components shown in Table 3.4 were transferred to an eppendorf tube and incubated at 37℃ for 2 hours.

Table 3.4: Components for digestion with NdeI

pET-3a	84 µl
10x buffer 0	10 µl
*Nde*I	6 µl

The restriction enzyme was added step by step:
1.) 3 µL at the beginning
2.) 2 µL after 1 hour
3.) 1 µL after 1.5 hour

DNA-purification:

- The same protocol was used as described in Chapter 3.1.1.3, with the exception of the following point:

82 µL sterile water were used to recover the purified DNA.

Digestion with *BamH*I:

- The components shown in Table 3.5 were transferred to an eppendorf tube and incubated at 37℃ for 2 hours.

Table 3.5: Components for digestion with BamHI

pET3a digested with NdeI	80 µL
10x *BamH*I-buffer	10 µL
*BamH*I	6 µL
CIP	1 µL after 1 h
CIP	1 µL after 1,5 h

DNA-purification:

- The same protocol was used as described in Chapter 3.1.1.3, with the exception of the following point:

50 µL sterile water were used to recover the purified DNA.

3.1.1.5.2. Digestion of insert-DNA with *Nde*I and *BamH*I

- **Protocol**

Digestion with *Nde*I:

- The components shown in Table 3.6 were transferred to an eppendorf tube and incubated at 37℃ for 2 hours.

Table 3.6: Components for digestion with NdeI

amplified insert of SUIIc3 cox2	85 µL
10x buffer 0	10 µL
*Nde*I	5 µL
sterile water	4 µL

The restriction enzyme was added step by step:

1.) 3 µL at the beginning

2.) 2 µL after 1 hour

DNA-purification:

- The same protocol was used as described in Chapter 3.1.1.3, with the exception of the following point:

86 µL sterile water were used to recover the purified DNA.

Digestion with *BamH*I:

- The components shown in Table 3.7 were transferred to an eppendorf tube and incubated at 37℃ for 2 hours.

Table 3.7: Components for digestion with BamHI

PCR fragment digested with *Nde*I	86 µL
10x *BamH*I-buffer	10 µL
*BamH*I	4 µL

DNA-purification:

- The same protocol was used as described in Chapter 3.1.1.3, with the exception of the following point:

50 µL sterile water were used to recover the purified DNA.

Agarose gel electrophoresis:

- The digested insert-DNA and the pET-3a vector were analysed by agarose gel electrophoresis as shown in Chapter 3.1.1.2.

- **Chemicals and equipment**

***Nde*I**: Fermentas (10 U/µL)
10x buffer 0: Fermentas
Calf intestine alkaline phosphatase: Fermentas
***BamH*I**: Fermentas (10 U/µL)
10x *BamH*I-Buffer: Fermentas

3.1.1.6. Ligation

Principles

The amplified and digested insert-DNA was inserted into the vector DNA by ligation. The digestion of double-stranded DNA with the chosen restriction enzymes *BamH*I and *Nde*I resulted in linear DNA fragments with so called sticky ends, which allow a specific insertion by complementary base pair matching. The enzyme DNA-ligase, which catalyses the formation of a phosphodiester bridge between the 3' – hydroxyl group of one DNA strand and the 5' – phosphate group of another strand ws used for ligation. The molecular concentration ratio of vector and insert DNA should be about 1:10 in order to prevent self-ligation of the vector and make sure that the insert is included into the vector.

- **Protocol**

- To verify the molecular concentration ratio 5 µL vector and 5 µL insert were analysed with agarose gel electrophoresis.
- Vector and insert were mixed with T4-DNA-ligase, Ligase buffer and sterile water and incubated overnight at 16℃.

- **Chemicals and Equipment**

T4-DNA-Ligase: Fermentas
10x Ligase buffer: Fermentas

3.1.1.7. Production of competent E. coli BL21(DE3)pLysS cells

- **Principles**

Electrocompetent cells are used for electroporation to achieve high transformation efficiency. They are prepared for electroporation under low temperature conditions by transferring them into a medium of low conductivity.

- **Protocol**

- 20 mL LB_{Cm}-medium were inoculated with *E. coli* BL21(DE3)pLysS cryo-culture and incubated overnight at 37℃ and 180 rpm .
- 1 L LB_{Cm}-medium was inoculated with 10 mL of overnight-culture and incubated for 3 hours at 37℃ and 180 rpm until an OD_{600} of 0.6 was reached. The optical density OD_{600} was measured photometrically at 600 nm using a 1 cm cuvette.
- The culture was incubated in four sterile centrifuge beakers on ice for 30 min and subsequently centrifuged for 6 min at 4℃ and 6 000 rpm. The supernatant was discarded.
- The pellet was resuspended in 500 mL ice cold 1 mM HEPES and centrifuged for 6 min at 4℃ and 6000 rpm. The supernatant was discarded.
- The pellet was resuspended in 250 mL ice cold 1 mM HEPES and centrifuged for 6 min at 4℃ and 6000 rpm. The supernatant was discarded.
- The pellet was resuspended in 100 mL ice cold 1 mM HEPES and centrifuged for 6 min at 4℃ and 6000 rpm. The supernatant was discarded.
- The pellet was resuspended in 30 mL ice cold 10% glycerol and centrifuged for 6 min at 4℃ and 6000 rpm. The supernatant was discarded.

- The pellet was resuspended in 5 mL ice cold 10% glycerol.
- 100 µL aliquots of the suspension were transferred into eppendorf tubes and shock freezed in liquid nitrogen and stored at –80°C.
- It is of great importance that not only 1 mM HEPES and 10% glycerol are cooled on ice, but also the pellet and collection tubes.
- The quality of the electrocompetent cells was controlled by transferring 1 mL SOC-medium into a collection tube with an aliquot of electrocompetent cells.

Glutinousness would be a sign for bad quality.

- **Chemicals and equipment**

BL21(DE3)pLysS: Novagene

LB_{Cm}-Medium:
LB-medium
25 µg/mL Chloramphenicol

LB-Medium:
See Chapter 3.1.1.4

Chloramphenicol stock solution:
25 mg/mL 96% Ethanol
stored at –20℃

1 mM HEPES buffer pH 7.0:
autoclaved 20 min at 121℃

10% Glycerol:
autoclaved 20 min at 121℃

Liquid nitrogen

SOC-medium:
5 g NaCl
2.5 g Yeast extract
7.5 g Bacto-agar
RO-water added to 500 mL
autoclaved 20 min at 121℃

Autoclave: Fritz Gössner, Hamburg
Orbital Shaker: Infors HT, Ecotron, Infors AG
UV-VIS Spectrophotometer: U-1100 Spectrophotometer, Hitachi
RC-5C Centrifuge: Sorvall Instruments, Du Pont
SLA-Rotor 1500
PHM92 pH meter: Radiometer, Copenhagen

3.1.1.8. Transformation by electroporation

- **Principles**

Transformation is the uptake of foreign DNA in a bacterium, thus changing the properties of the organism. Electroporation is an efficient and easy transformation method. Cells are transformed by applying a short high voltage pulse, which creates transient pores and therefore makes the cell membranes permeable for DNA molecules. Length and intensity of the pulse have to be optimised for the different cell types in order to allow pore formation but to prevent lethal cell damage.

- **Protocol**

- Aliquots of 1 mL SOC-medium were incubated at 37℃ .
- Meanwhile 15 µL of pET-3a/suIIj3 cox2 from ligation assay 1 and 2 and the negative control (vector DNA only) of the ligation were each transferred to ice cold electroporation cuvettes.
- As negative control 15 µL sterile water and as positive control 3 µL of undigested pET-3a vector after preparation (see Chapter 3.1.1.4) diluted with 12 µL sterile water were used and treated the same way.
- The aliquoted electrocompetent cells were cooled on ice.
- 100 µL of electrocompetent cells were added to the samples and mixed thoroughly.
- Electroporation was performed using a Biorad Gene Pulser.
- After electroporation the cells were transferred into the pre-warmed SOC-medium and incubated for 30 min and 37℃.
- 200 µL aliquots of the cells were plated on selective LB_{AmpCm}-agar and incubated overnight at 37℃.

- **Chemicals and equipment**

LB-Agar:
5 g Peptone
5 g NaCl
2.5 g Yeast extract
7.5 g Bacto-agar
RO-water added to 500 mL
autoclaved 20 min at 121℃

LB_{AmpCm}-Agar:
LB-agar
100 µg/mL Ampicillin
25 µg/mL Chloramphenicol

SOC-medium:
See Chapter 3.1.1.7

Ampicillin Stock Solution:
See Chapter 3.1.1.4

Chloramphenicol stock solution:
See Chapter 3.1.1.7

Electroporation Cuvettes: 1 mL
Biorad Gene Pulser
Pulse controller: Biorad

3.1.1.9. Screening for a positive clone

3.1.1.9.1. PCR-Screening

- **Principles**

PCR is used to screen for successfully transformed clones carrying the recombinant protein, which can be amplified with the specific suIIj3 cox2 primers. An additional heating step previous to the denaturation was performed in order to break down the bacterial cell wall.

- **Protocol**

- A mastermix for 20 assays was prepared (see Table 3.9).

Table 3.9: Components of Mastermix for PCR screening

Upstream primer T7 [5 pmol/µL]	9 µL
Downstream primer T7 [5 pmol/µL]	9 µL
dNTPs [25mM]	2.5 µL
10x Buffer + $(NH_4)_2SO_4$	45 µL
Taq DNA Polymerase	4.5 µL
$MgCl_2$	36 µL
Sterile water	36 µL

- Aliquots of 20 µL mastermix were transferred into PCR-tubes.
- In eppendorf cups 50 µL units of sterile water were prepared.
- 12 single colonies were picked with pipette tips and each suspended in 50 µL sterile water. 10 µL of the suspension were transferred into a PCR tube and a drop was plated on selective LB_{AmpCm}-agar.
- This procedure was repeated with 12 other colonies. The LB_{AmpCm}-Agarplate was used as masterplate.
- The masterplate was incubated overnight at 37℃ and then stored at 4℃.
- 10 µL sterile water were used as negative control and PCR-product (insert after amplification) was used as positive control and was added to the mastermix.
- SUIIj3 cox2 was amplified using the thermocycler program shown in Table 3.10.

Table 3.10: Thermocycler program

Step	Heat	Description	Time	Cycle
1	95℃	Pre-Heating	2 min	
2	94℃	Denaturation	40 sec	x30
3	53℃	Annealing	30 sec	
4	72℃	Synthesis	50 sec	
5	72℃	Extension	10 min	

- 10 µL of the amplified samples were analysed with agarose gel lectrophoresis.

- **Chemicals and equipments**

Primer: Novagene
T7-upstream primer
T7-downstream primer
Taq DNA Polymerase : Fermentas
10x buffer + 200 mM $(NH_4)_2SO_4$: Fermentas
25 mM $MgCl_2$: Fermentas
10 mM dNTP Mix: Fermentas
LB_{AmpCm}-Agar: See Chapter 3.1.1.8
Template: single colonies from the transformation LB-plate

Thermocycler: Techne TC-312

3.1.1.9.2. Expression-Screening

- **Principles**

Small-scale expression experiments were performed to investigate the expression abilities of successfully transformed cells. The molecular weight of the expressed proteins were checked by SDS-PAGE and compared to the theoretical molecular weight of the SUIIj3 cox2 fragment. Clones performing well in these experiments were preserved by setting up cryo-cultures.

- **Protocol**

- 3 mL LB_{AmpCm}-medium were inoculated with a successfully transformed cell colony and incubated overnight at 37℃ and 180 rpm. As positive control 3 mL LB_{AmpCm}-medium were inoculated with a pre-existing clone of SUIIj3 cox2 from the work of JS.
- 20 mL $M9ZB_{AmpCm}$-Medium were inoculated with 1 mL of overnight culture and incubated for 4 hours at 37℃ and 180 rpm.

- The optical density OD_{600} was measured photometrically at 600 nm using a 1 cm cuvette and should be about 1.4.
- The expression was induced with 1 mM IPTG and the suspension was further incubated for 4 hours at 37℃ and 180 rpm.
- The culture was transferred in a falcon tube and centrifuged for 15 min at 5000 rpm. The supernatant was discared and the pellet stored overnight at –20℃ in order to facilitate cell lysis.
- The pellet was resuspended in 4 mL lysis buffer and sonicated on ice (3 x 40 sec of short pulses). 1 mL was transferred to an eppendorf cup and centrifuged at 14000 rpm for 3 min.
- The supernatant and the pellet, resuspended in 400 µL RO-water, were analysed by SDS-PAGE (see Chapter 3.3.3).

- **Chemicals and equipment**

LB_{AmpCm}-Medium:
LB-Medium
100 µg/mL Ampicillin
25 µg/mL Chloramphenicol

LB-Medium:
See Chapter 3.1.1.4

$M9ZB_{AmpCm}$-Medium:
800 mL Solution A
100 mL Solution B
100 mL Solution C
1 mL Solution D
1 mL Chloramphenicol [25 mg/mL]
1 mL Ampicillin [100 mg/mL]

Solution A:
10 g Peptone
10 Yeast extract
5 g NaCl (Mr = 58.44)

Solution B:
12 g KH_2PO_4
24 g Na_2HPO_4 * $7H_2O$
RO-water added to 400 mL
autoclaved 20 min at 121℃

Solution C:
16 g Glucose
RO-water added to 400 mL
autoclaved 20 min at 121℃

Solution D:
12.3 g $MgSO_4$.$7H_2O$
RO-water added to 50 mL
autoclaved 20 min at 121℃

1 M IPTG stock solution:
238.3 mg IPTG/mL RO-water
filter sterilised
stored at –20℃

1 g NH_4Cl (Mr = 53.49)
RO-water added to 800 m
autoclaved 20 min at 121℃

200 mM PMSF:
34.8 mg PMSF in 1 mL Isopropanole

0.5 M DTT:
0.0771 g in 1 mL RO-water

Chloramphenicol stock solution:
See Chapter 3.1.1.7

Lysis buffer:
50 mM Tris / HCl pH 8.0
2 mM EDTA pH 8.0
0.1 % Triton X-100
RO-water added to 80 mL
1 mM PMSF
0.5 mM DTT

Ampicillin stock solution:
See Chapter 3.1.1.4

Autoclave: Fritz Gössner, Hamburg
Orbital Shaker: Infors HT, Ecotron, Infors AG
UV-VIS Spectrophotometer: U-1100 Spectrophotometer, Hitachi
RC-5C Centrifuge: Sorvall Instruments, Du Pont
SLA-Rotor 1500
Centrifuge: Sigma 1-15 (Sigma, Germany)
Ultrasonic probe: Sonics & Materials Inc., Vibra-Cell, type CV17

3.1.1.10. Set-up of a cryo-culture

- **Principles**

Cell cultures of clones tested positive in PCR- and expression-screening, were preserved by setting up cryo-cultures using cryo protective agents like glycerol.

- **Protocol**

- 3 mL of LB_{AmpCm}-medium were inoculated with a SUIIj3 cox2 colony of the masterplate and incubated overnight at 37℃ and 180 rpm.
- 900 µL overnight culture and 900 µL 30% glycerol were combined in a cryo vial and mixed gently with a pipette.
- The cryo-culture was stored at –80℃.

- **Chemicals and equipment**

LB_{AmpCm}-Medium:
See Chapter 3.1.1.9
30% Glycerol:
autoclaved 20 min at 121℃

Autoclave: Fritz Gössner, Hamburg
Orbital Shaker: Infors HT, Ecotron, Infors AG
2 mL Cryo Vials: Nalgene

3.1.1.11. Sequencing of plasmid DNA

- **Principles**

The sequence of purified, using Gene JET™ Plasmid Mini Prep-Kit, plasmid DNA was determined to assure that the plasmid DNA and as a consequence the expressed protein in transformed cells does not contain any mutations. Comparison of the cloned sequence with the original sequence was performed by a software tool called DNAStar SeqMan 4.0. Changes in the nucleotide sequence can cause not only ‘silent mutations’, which do not change the amino acid sequence of the protein but also mutations that alter the amino acid sequence of the encoded protein.

- **Protocol**

The DNA sequencing was conducted by I.B.L., Dennisgasse 23, 1200 Vienna. Purified plasmid DNA (for purification protocol see chapter 3.1.1.2) and primers were provided in the following concentrations:

- 50 µL template (plasmid DNA): 0.2 - 0.5 µg/µL,
- T7-Primers: 5 pmol/µL each

T7-Promoter-Primer: Novagene

T7-Terminator-Primer: Novagene

Two primers were used to receive a representative result. Therefore the insert was sequenced starting at the N-terminal- as well as at the C-terminal-end.

3.1.2. Cloning of plastocyanin from *Nostoc sp*. PCC 7120

The insert, the sequence encoding for plastocyanin was amplified by PCR and transferred into a pET-3a expression vector. The pET-3a vector carries an IPTG-inducible T7-promoter sequence, various restriction sites and an ampicillin resistance gene for selection purposes. The prepared plasmid was then transformed into electro-competent *E. coli* BL21(DE3)pLysS cells carrying a pLysS-plasmid coding for T7-lysozym (natural inhibitor of T7-RNA-polymerase). Additionally, pLysS is bearing a chloramphenicol resistance gene. Screening for positive clones was performed by PCR-screening and analysing the expression patterns.

3.1.2.1. Amplification of plastocyanin by PCR

- **Principles**

See Chapter 3.1.1.1 with the exception that a three-step PCR, which includes an extended elongation step, was performed because the used primers do not anneal at high temperature.

- **Protocol**

- 1.5 mL culture Pellet of *Nostoc sp*. PCC 7120 was centrifuged 10 minutes at 14000 rpm and the supernatant discarded.
- 2PC_N7120_cterm_MP was used as upstream primer and 2PC_N7120_nterm_MP was used as downstream primer

- The components of the Mastermix are shown in Table 3.11, Table 3.12 shows the components for the PCR samples
- The sample and the negative control were prepared as follows:

Table 3.11: Components of the sample

	Sample
Primer upstream 2PC_N7120_cterm_MP [10 pmol/µL]	15 µL
Primer downstream 2PC_N7120_nterm_MP [10 pmol/µL]	15 µL
dNTPs [25 mM]	3 µL
Sterile water	189 µL

- Two different buffers were tested. HF-Buffer, the attached buffer for Hot Phusion Polymerase and GC-Buffer, which works especially for GC-rich sequences such as plastocyanin.

Table 3.12: Components for the PCR Tubes

	PC GC	**PC HF**	**Negative control**
Mastermix	74 µL	74 µL	74 µL
5x Phusion HF Buffer	---	20 µL	20 µL
5x Phusion GC Buffer	20 µL	---	---
Template DNA (*Nostoc sp. PCC 7120*)	5 µL	5 µL	---
Sterile water		63 µL	68 µL

- After 9 minutes 1 µL Phusion DNA Polymerase Hot Start (2U/µL) was added to the tubes and the negative control.
- The steps and temperatures used for the PCR are listed in Table 3.13.

Table 3.13: Thermocycler program

Step	Action	Heat	Time	Cycle
1	Heating	98℃	10 min	
2	Denaturation	98℃	10 sec	x35
3	Annealing	65℃	10 sec	
4	Synthesis	72℃	10 sec	
5	Extension	72℃	10 min	

- **Chemicals and equipment**

See Chapter 3.1.1.1 with the exception of the primers:

Primer: genXpress

N-terminal: 2PC_N7120_nterm_MP

5’ GGGAATTCCATATGGAAACATACACAGTAAAAC 3’

NdeI Start Insert

C-terminal: 2PC_N7120_cterm_MP

5’ CGGGATCCCTAGCCGGCGACAGTGATTTTACC 3’

BamHI Stop Insert

Agarose gel electrophoresis and Purification after PCR were peformed as described in Chapter 3.1.1.2.

- **Chemicals and equipment**

See Chapter 3.1.1.4 except 5x Phusion GC Buffer

5 x Phusion™ GC Buffer: Finnzyme

3.1.2.2. Digestion of pET- 3a and insert with restriction enzymes

The same vector prepared for cloning SUIIj3 cox 2 (see Chapter 3.1.1.5.1) was used for this approach.

3.1.2.2.1. Digestion of insert with *Nde*I and *BamH*I

- **Protocol**

Digestion with *Nde*I:

- The components shown in Table 3.14 were transferred to an eppendorf tube and incubated at 37℃ for 2 hours.

Table 3.14: Components for digestion with NdeI

amplified insert of plastocyanin	60 µL
*Nde*I	2 µL
10x NEB2 buffer	10 µL
sterile water	28 µL

DNA was purified as described in Chapter 3.1.1.3 and recoverd in 88 µL sterile water.

Digestion with *BamH*I:

- The components shown in Table 3.15 were transferred to an eppendorf tube and incubated at 37℃ for 2 hours.

Table 3.15: Components for digestion with BamHI

PCR fragment digested with *Nde*I	60 µL
*BamH*I	2 µL
10x NEB3 buffer	10 µL

DNA-purification:

- The same protocol was used as described in Chapter 3.1.1.3, with the exception of the following point:

40 µL sterile water were used to recover the purified DNA.

3.1.2.3. Ligation

- **Protocol**

To verify the molecular concentration ratio 5 µL vector and 5 µL insert were analysed with agarose gel electrophoresis as written in Chapter 3.1.1.2.
The components shown in Table 3.16 were merged and incubated overnight at 25℃.
The ligated plasmid was transformed into competent *E. coli* BL21(DE3)pLysS cells as described in Chapter 3.1.1.7.

3.1.2.4. Screening for a positive clone

3.1.2.4.1. PCR-Screening

- **Protocol**

A mastermix for 23 assays was prepared (see Table 3.17).

Table 3.17: Components of Mastermix for PCR screening

Upstream primer T7 [5 pmol/µL]	13.8 µL
Downstream primer T7 [5 pmol/µL]	13.8 µL
dNTPs [25mM]	3.5 µL
10x Buffer + $(NH4)_2SO_4$	69 µL
Taq DNA Polymerase	6.9 µL
$MgCl_2$	55 µL
Sterile water	298 µL

- Aliquots of 20 µL mastermix were transferred into PCR-tubes.
- In eppendorf cups 50 µL units of sterile water were prepared.
- 19 single colonies were picked with pipette tips and each suspended in 50 µL sterile water. 10 µL of the suspension were transferred into a PCR tube and a drop was plated on selective LB_{AmpCm}-agar.
- The masterplate was incubated overnight at 37℃ and then stored at 4℃.
- 10 µL sterile water were used as negative control and PCR-product (insert after amplification) was used as positive control and was added to the mastermix.
- The steps and temperatures used for the PCR are listed in Table 3.18.

Table 3.18: Thermocycler program

Step	**Temperature**	**Description**	**Time**	
1	94℃	Heating	4min	
2	94℃	Denaturation	40 sec	x33
3	60℃	Annealing	30 sec	
4	72℃	Synthesis	20 sec	
5	72℃	Extension	10 min	

- 10 µL of the amplified samples were analysed with agarose gel electrophoresis.

3.1.2.4.2. Expression-Screening

- **Protocol**

- 20 mL LB_{AmpCm}-medium were inoculated with a successfully transformed cell colony and incubated overnight at 37℃ and 180 rpm. As positive control 3 mL LB_{AmpCm}-medium were inoculated with a positive clone of PC from *Synechocystis*.
- 20 mL $M9ZB_{AmpCm}$-medium were inoculated with 1 mL of overnight culture and incubated for 4 hours at 37℃ and 180 rpm.
- The optical density OD_{600} was measured photometrically at 600 nm using a 1 cm cuvette and should be about 1.4.
- The expression was induced with 1 mM IPTG and the suspension was further incubated for 20 hours at 16℃ and 180 rpm.
- The culture was transferred in a falcon tube and centrifuged for 15 min at 5000 rpm. The supernatant was discared.
- The pellet was resuspended in 4 mL lysis buffer and sonicated on ice (3 x 40 sec of short pulses). 1 mL was transferred to an eppendorf cup and centrifuged at 14000 rpm for 3 min.
- The supernatant and the pellet, resuspended in 400 µL RO-water, were analysed by SDS-PAGE (see Chapter 3.3.3).

3.1.2.4.3. Expression-Screening at different conditions

- **Protocol**

- 20 mL LB_{AmpCm}-medium were inoculated with a successfully transformed cell colony and incubated overnight at 37℃ and 180 rpm.
- 20 mL $M9ZB_{AmpCm}$-medium were inoculated with 1 mL of overnight culture and incubated for 4 hours at 37℃ and 180 rpm.

- The optical density OD_{600} was measured photometrically at 600 nm using a 1 cm cuvette and should be about 1.4.
- The expression was induced with various concentrations of IPTG and the suspension was further incubated at different conditions considering time and temperature as shown in Table 3.19.

Table 3.19: Pre-Test Culture Conditions for plastocyanin Expression (the standard protocol is coloured in red)

Medium	Temperature	Duration	IPTG Addition
$M9ZB_{AmpCm}$	16℃	20h	1 mM
$M9ZB_{AmpCm}$	20℃	16h	1 mM
$M9ZB_{AmpCm}$	RT	20h	1 mM
$M9ZB_{AmpCm}$	30℃	4h	1 mM
$M9ZB_{AmpCm}$	37℃	4h	1 mM
$M9ZB_{AmpCm}$	37℃	4h	0.5 mM
$M9ZB_{AmpCm}$	37℃	4h	0.2 mM

- Each culture was transferred in a falcon tube and centrifuged for 15 min at 5000 rpm. The supernatant was discarded.
- The pellet was resuspended in 4 mL lysis buffer and sonicated on ice (3 x 40 sec of short pulses). 1 mL was transferred to an eppendorf cup and centrifuged at 14 000 rpm for 3 min.
- The supernatant and the pellet, resuspended in 400 µL RO-water, were analysed by SDS-PAGE (see Chapter 3.3.3).

3.1.2.4.4. Set-up of a cryo-culture

- **Protocol**

- 3 mL of LB_{AmpCm}-medium were inoculated with a plastocyanin colony of the masterplate and incubated overnight at 37℃ and 180 rpm.
- 900 µL overnight culture and 900 µL 30% glycerol were combined in a cryo vial and mixed gently with a pipette.

- The cryo-culture was stored at –80℃.

3.1.2.4.5. Sequencing of plasmid DNA

As in chapter 3.1.1.11 the plasmid DNA was extracted and sent to sequencing.

3.2. Heterologous overexpression of recombinant proteins

3.2.1. Expression of SUIIc3 cox2, SUIIj3 cox2, SUIId2 cox1

- **Principles**

The soluble part of cytochrome *c* oxidase 2 Subunit II was expressed in the pET-3a plasmid in the *E. coli strain* BL21(DE3)pLysS. The protein was obtained in soluble form in the cytoplasm.

- **Protocol**

- 30 mL of LB_{AmpCm}-medium were inoculated with cells from a cryo-culture and incubated overnight at 37℃ and 180 rpm.
- 12 mL of this overnight culture were taken as inoculum for 1 L $M9ZB_{AmpCm}$-Medium, which was divided into two sterile 2 L erlenmeyer flasks.
- The inoculated $M9ZB_{AmpCm}$-medium was incubated at 37℃ and 180 rpm until OD_{600} reached 1.4.
- Subsequently the expression was induced by the addition of IPTG to a final concentration of 1 mM IPTG.
- The expression culture was incubated 20 h at 16℃ f or SUIIc3 and SUIIj3 cox2 and 4.5 h at 37℃ for SUIId2 cox 1 and 180 rpm and subsequently centrifuged for 10 min at 6000 rpm and 25℃.

- The pellet was resuspended in some supernatant and transferred to four falcon tubes.
- The cell suspension was centrifuged for 15 min at 4000 rpm and 25℃. The supernatant was discarded.
- The cell-pellet was stored at –80℃

- **Chemicals and equipment**

Autoclave: Fritz Gössner, Hamburg
Orbital Shaker: Infors HT, Ecotron, Infors AG
UV-VIS Spectrophotometer: U-1100 Spectrophotometer, Hitachi
RC-5C Centrifuge: Sorvall Instruments, Du Pont
SLA-Rotor 1500

3.3. Purification of recombinant proteins

To avoid blocking the columns with particles and introducing gas bubbles to the systems, all buffers and solutions for chromatographic application were filtrated through 0.45 µm filters using a Nalgene filter-funnel and degassed for 7 min in the ultrasonication bath. Protein solutions were filtrated in the same manner, but degassed under vacuum.

3.3.1. Purification of SUIIc3 cox2, SUIIj3 cox2, SUIId2 cox1

3.3.1.1. Cytoplasmatic release of SUIIj3 cox2

- **Principles**

SUIIj3 cox2 was released from the cytosol by lysis of the *E. coli* cells. Protease inhibitors were immediately added to protect the protein from proteolytic digestion. The high viscosity due to release of genomic DNA was reduced by sonication, and

insoluble proteins and cell components were separated from the soluble protein fractions by centrifugation. The separation of the recombinant protein from the large amount of soluble *E. coli* proteins in the supernatant was performed by several chromatographic steps. It is necessary that the reduced state of the two cysteine residues in the active site of the CuA-domain are preserved for reconstitution of the apoprotein with Cu^{2+}, therefore DTT, a reducing agent, was added to the lysis buffer and to the buffers used after the first chromatographic step.

Protocol

- The cell pellet of 1 L *E. coli* culture was suspended in 80 mL lysis buffer
- The lysis suspension was sonicated on ice (3 x 40 sec of short pulses) and centrifuged for 20 min at 18 000 rpm and 4℃. The p ellet was discarded.
- The supernatant containing the soluble proteins was filtrated and degassed for 15 min using a vacuum flask.

- **Chemicals and equipment**

Lysis buffer: See Chapter 3.1.1.9.2
200 mM PMSF and 0.5 M DTT: See Chapter 3.1.1.9.2

Ultrasonic probe: Sonics & Materials Inc., Vibra-Cell, type CV17
RC-5C Centrifuge: Sorvall Instruments, Du Pont
SS-34 Rotor
Membrane-vacuum pump: vacuubrand CVC 2
Nalgene filter funnel: 315-0047
Millipore filter: HVP04700 (pore size 0.45 µm)

3.3.1.2. Chromatographic purification of SUIIj3 cox2

SUIIj3 cox2 was purified with a two step purification protocol, that comprise exchange chromatography with Q-sepharose and a size exclusion chromatography with

Superdex^{TM} 75. Between the two chromatography steps, copper reconstitution was performed (See Chapter 3.3.1.3). Q-sepharose anion exchange chromatography was performed at 4℃ and Superdex^{TM} 75 size exclusion chromatography at room temperature.

3.3.1.2.1. Anion exchange chromatography with Q-Sepharose Fast Flow

- **Principles**

In ion exchange chromatography the separation is based on the reversible interactions between a charged protein and an oppositely charged chromatographic medium. The net surface charge of a protein varies according to the surrounding pH, hence a PH above its isoelectric point (pI) causes a negatively-charged protein that will bind to the positively charged functional groups on the matrix of an anion exchange column. At pH below its pI, a protein binds to a cation exchange column. Q-Sepharose Fast Flow is a strong anion exchange column. Quaternary amine groups $–N^{+}(CH_3)_3$, which are stable between pH 2 – 12, build the ion exchange groups. The bound protein can be eluted by changing the conditions like decreasing the pH or increasing the salt concentrations. The change of pH or the salt concentration can be performed stepwise or with a continuous gradient. Usually NaCl gradients are used for elution. During binding the target protein is concentrated and collected.

- **Protocol**

a) Preparation of the column:

- The gel was washed with distilled water in a suction filter and degassed under vacuum.
- Using a syringe, a small volume of degassed water was injected into the column from the bottom side in order to remove trapped air bubbles.
- The column was filled using a glass stick.

- After the gel had sedimented, the column was closed on the top, connected to a peristaltic pump and washed with degassed water until the gel was packed (flow rate: 2 mL/min).
- The prepared column had to be kept in 20 % ethanol.

b) Protein purification

- The column was washed with 2 CV (column volume) of degassed water at a flow rate of 2 mL/min to remove ethanol.
- The column was equilibrated with 2 CV of buffer A at a flow rate of 3 mL/min.
- The sample prepared as described in Chapter 3.3.1 was loaded onto the gel at a flow rate of 3 mL/min. The column was washed with 2 CV of buffer A at a flow rate of 3 mL/min.
- The bound protein was eluted by a stepwise increase of NaCl-concentration in buffer A, 90 mL of gradient 1, followed by 180 mL of gradient 2 and collected in 6 mL fractions.
- After elution the column was washed with 2 CV of buffer B, 2 CV of water at a flow rate of 3 mL/min, and for storage with 2 CV of 20% ethanol at a flow rate of 2 mL/min.
- Fractions were tested for recombinant protein by SDS-PAGE (see Chapter3.3.3).
- 200 mM PMSF and 0.5 M DTT were added to the pooled fractions to a final concentration of 200 µM PMSF and 0.5 mM DTT.

- **Chemicals and equipment**

20% Ethanol

200 mM PMSF and 0.5 M DTT:
See Chapter 3.1.1.9.2

Buffer A

50 mM phosphate buffer pH 7.0:
6.8 g KH_2PO_4
RO-water added to 1000 mL
pH was tested and adjusted
0.5 mM DTT was added before use

Buffer B:
Buffer A + 1 M NaCl
0.5 mM DTT was added before use

Gradient 1:
Buffer A + 100 mM NaCl
0.5 mM DTT was added before use

Gradient 2:
Buffer A + 200 mM NaCl
0.5 mM DTT was added before use

Column media: Q-Sepharose Fast Flow,
Amersham Pharmacia Biotech
stored in 20% ethanol
Column: Pharmacia, Ø = 2.6 cm; l = 28 cm; V = 150 mL
Peristaltic pump: Pharmacia, Peristaltic Pump P-1; Ø = 3.1 mm
Membrane-vacuum pump: vacuumbrand CVC2
Nalgene filter funnel: 315-0047
Millipore filter: HVP04700 (Pore size 0.45 µm)
Ultrasonication bath: Bandelin Sonorex Super RK 510 H

3.3.1.2.2. Size exclusion chromatography with Superdex^{TM} 75

- **Principles**

The underlying principle of size exclusion chromatography (SEC) is that particles of different sizes, or more precisely their hydrodynamic volume, will elute through a stationary phase with different flow rates. When gels are used as stationary phases, the technique is more specifically known as gel permeation chromatography (GPC). In a GPC column, gel particles formed as beads are packed onto a separation bed. The eluent, a buffer solution passes through. Molecules that are to be separated are

added to the solution on the top of the bed. The small molecules, which diffuse into the gel beads, are delayed in their passage down the column, compared to larger molecules that cannot diffuse into the beads and move continuously down the column with the flowing eluent. Larger molecules thus leave the column first, followed by the smaller in the order of their size. In GPC gels the pores have carefully controlled ranges of size, and the matrix is chosen for its inertness (lack of adsorptive properties) as well as its chemical and physical stability.

- **Protocol**

For this purification step a ÄktaPurifier FPLC system was used. All steps were carried out at room temperature. The buffers were filtrated and degassed before use and the reconstituted protein solution (See Chapter 3.3.1.3) concentrated to 1 mL with Amicon Centripreps (See Chapter 3.3.1.4). The maximum pressure was set to 1.8 MPa, the UV-detector to 280, 480 and 535 nm and the flow rate to 1 mL/min.

- The (pre-packed) column was washed with 2 CV of degassed water to remove ethanol.
- Subsequently the column was equilibrated with 2 CV of SEC-buffer.
- Before loading the protein solution, it was centrifuged for 5 min at 14000 rpm. 90 µL aliquots per run were loaded onto the column, using a 100 µL loop. 10 mL fractions were collected before and 0.3 mL fractions during protein elution, which was followed using an UV-VIS detector.
- Between each run the column was washed with 10 mL SEC-buffer. After the last run, the column was washed with 2 CV of SEC-buffer and 2 CV of water.
- Subsequently the column was washed with 2 CV of 20% ethanol at an altered flow rate of 0.5 mL/min.
- UV-VIS-absorption-spectra of the fractions were recorded.
- The collected fractions were checked for purity by SDS-PAGE (see Chapter 3.3.3). The protein fractions were pooled and 200 mM PMSF was added to a final concentration of 200 µM.
- The protein pool was concentrated using Amicon Centripreps (see Chapter 3.3.1.4).

- **Chemicals and equipment**

SEC-buffer:	**200 mM PMSF**
9.12 g $KH2PO_3$ [67 mM]	See Chapter 3.1.1.9.2
11.18 g KCl [150 mM]	**20% Ethanol**
6 M NaOH added to pH 7.0	
RO-water added to 1000 mL	

Column media: SuperdexTM75, 24 mL bed volume, exclusion limit: 100 000 Mr (globular proteins), 1.8 MPa maximal back pressure, Pharmacia Biotech
Stored in 20% ethanol
ÄKTApurifier: Pharmacia
Pump P-900
UV-detector UV-900
pH-meter/conductivity cell pH/C-900 Box 900
Fraction collector: Frac-900, Pharmacia
Membrane vacuum pump: vacuumbrand CVC2
Nalgene filter funnel: 315-0047
Millipore filter: HVP04700 (Pore size 0.45 µm)
Ultrasonication bath: Sonorex Super RK 510 H, Bandelin

3.3.1.3. Copper reconstitution

- **Principles**

Recombinant SUIIj3 cox2 does not have copper in the active center as *E. coli* cells show a lack of the required chaperones. There are two ways to attach copper into the active site.

3.3.1.3.1. Dialysis with urea

One way to reconstitute SUIIj3 cox2 is to unfold the protein with urea and remove the denaturating agent step by step by dialysis. Once the protein is refolded copper is added as $Cu(His)_2$.

- **Protocol**

- 3 M solid urea, 0.5 M DTT (1:2400) and 200 mM PMSF (1:1000) were added to the unconcentrated Q-Sepharose pool and incubated under stirring at 4℃ for 1 hour.
- The dialysis tube was boiled in distilled water for 1 hour.
- The protein solution was filled into the dialysis tube as completely as possible, to still allow sealing but prevent increasing the volume during dialysis as far as possible.
- The protein solution was dialysed for 1.5 hours under stirring at 4℃ in 50 mM phosphate buffer pH 6.5 and 1 M solid urea with a protein solution to buffer ratio of 1:20.
- The buffer was replaced by a 50 mM phosphate buffer pH 6.5 without urea (protein solution to buffer ratio of 1:50). The solution was dialysed for 1.5 hours under stirring at 4℃.
- The buffer was replaced by a 50 mM phosphate buffer pH 6.5 with 1.5 mM $Cu(His)_2$ (protein solution to buffer ratio of 1:20). After dialysis overnight under stirring at 4℃ the solution changed from greenish blue to purple.
- The protein was concentrated with Amicon Centripreps (See Chapter 3.3.1.4) and a UV-VIS-spectrum was recorded.

- **Chemicals and equipment**

50 mM phosphate buffer pH 6.5:
6.8 g KH_2PO_4
RO-water added to 1000 mL
pH was tested and adjusted

0.5 mM DTT was added before use

Urea: Sigma-Aldrich

200 mM PMSF and 0.5 M DTT

1.5 mM $Cu(His)_2$

Dialysis Tube: Sigma

Dialysis tubing cellulose membrane

diameter: 25 mm

exclusion limit: 12 000 Da

Magnetic Stirrer: IKAMAG RET (IKA, Germany)

3.3.1.3.2. Ultrafiltration with decreasing steps of urea

- **Principles**

Ultrafiltration is based on the use of regenerated cellulose or polyethersulfone membranes of different pore sizes. Small molecules can pass the membranes, whereas bigger molecules are retained, according to the membranes' molecular weight cut off level. Stirred cells are devices for ultrafiltration (see Figure 3.6). The pressure forces the fluid to pass the membrane, magnetic stirring prevents concentration polarisation and accumulation of macromolecules on the membrane surface.

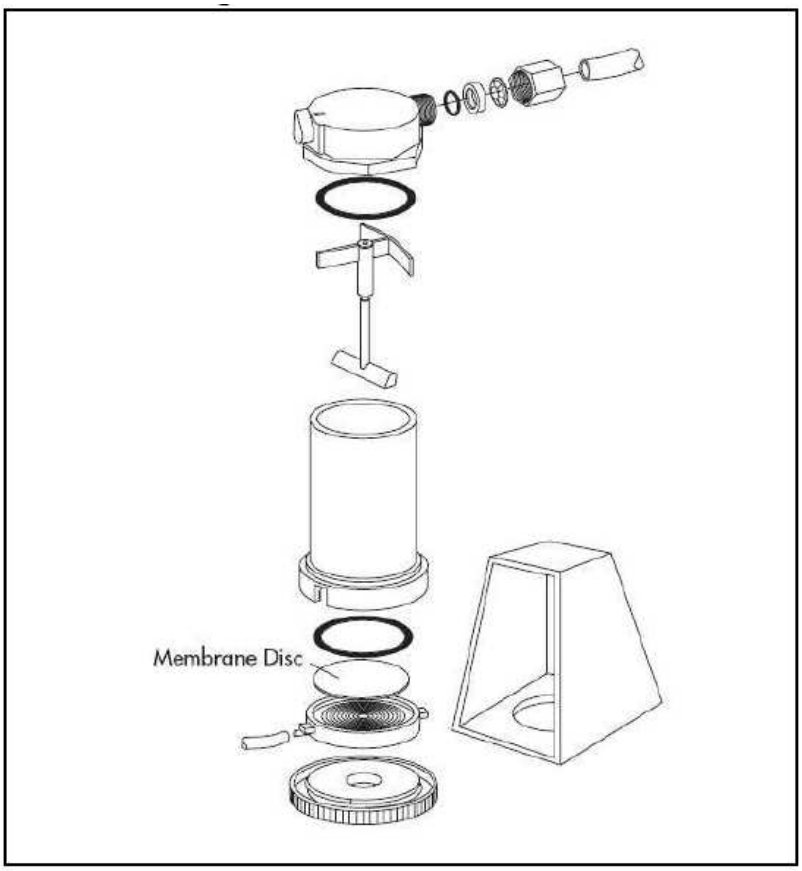

Figure 3.3: Ultrafiltration cell

- **Protocol**

- The membrane was placed with the glossy side up at the bottom of the ultrafiltration cell.
- All sealings were greased; the cell was assembled and filled with RO water.
- The lid was attached; the device was put into the rack and fixed on a magnetic stirrer.
- Nitrogen was applied to the device via the connection at the lid with a maximum pressure of 4.5 bar. The magnetic stirrer was switched on.
- The membrane was rinsed with water 3 times for 10 minutes.
- The remaining water was discarded (gas supply was closed, the pressure discharged).
- All purification steps were performed at 4℃.

- 0.5 M DTT (1:2400) and 200 mM PMSF (1:1000) and the concentrated protein pool after Q-Sepharose purification were added to 50 mL 50 mM phosphate buffer, pH 6.5 + 3 M urea and incubated under stirring at 4℃ for 1 hour.
- Pressure was applied until the starting volume was halfed.
- The filtrate was discarded and 25 mL 50 mM phosphate buffer, pH 6.5 with 1 M urea were added. Pressure was applied until the starting volume was halfed.
- The filtrate was discarded and 25 mL 50 mM phosphate buffer, pH 6.5 were added. Pressure was applied until the starting volume was halfed.
- The filtrate was discarded and 25 mL 50 mM phosphate buffer, pH 6.5 were added. Pressure was applied until the starting volume was halfed.
- The filtrate was discarded and 25 mL 50 mM phosphate buffer, pH 6.5 were added. Pressure was applied until the desired final volume of 25 mL was reached, the concentrated protein solution was kept in the cell and 150 mM $Cu(His)_2$ were added and incubated under stirring at 4℃ for 1.5 hours. The solution changed from greenish blue to purple.
- To get rid of the remaining $Cu(His)_{2,}$ which would disturb the spectroscopic investigations the protein solution was afterwards loaded onto an Anion exchange chromatography column with DEAE (See Chapter 3.3.3).
- The protein was concentrated with Amicon Centripreps (See Chapter 3.3.4) and a UV-VIS-spectrum recorded.
- Membranes were stored in 10% ethanol at 4℃.

- **Equipment**

Amicon Stirred Ultrafiltration Cells:
Modell 8010, 10 mL capacity, 1 mL minimal volume, 25 mm membrane diameter
Modell 8200, 200 mL capacity, 5 mL minimal volume, 62 mm membrane diameter
Membranes:
Millipore Ultrafiltration Membranes, Regenerated Cellulose, Filter Code PLBC,NMWC 3000
Magnetic Stirrer: IKAMAG RET (IKA, Germany)

3.3.2. Anion exchange chromatography with DEAE

- **Principles**

Diethylaminoethyl (DEAE) sepharose is a weak anion exchange column with –O-CH_2CH_3-N^+-$(C_2H_5)_2H$ groups bound to a highly cross-linked agarose matrix. It is stable between pH 2 - 9.5.

- **Protocol**

a)Preparation of the column

See Chapter 3.3.1.2.1

b)Protein purification

- The column was washed with 2 CV of degassed water at a flow rate of 2 mL/min to remove ethanol.
- Equilibration of the column was performed with 2 CV of buffer A at a flow rate of 3 mL/min.
- The sample was loaded onto the column at a flow rate of 3 mL/min.
- The protein was recovered by washing the column with 2 CV of buffer A at 3 mL/min, followed by gradient 1.
- 6 mL fractions were collected, analysed spectrophotometrically and pooled.
- After elution, the column was washed with 2 CV of buffer B, 2 CV of water, both at a flow rate of 3 mL/min, and 2 CV of 20% ethanol at a flow rate of 2 mL/min.

- **Chemicals and equipment**

Buffer A:	**Buffer B**:
10 mM Tris/HCl pH 8.0	Buffer A + 1 M NaCl
1.21 g Trizma base	**Step gradient 1**:
900 mL RO water	Buffer A + 0.05 M NaCl
6 M HCl added to pH 8.0	**20% Ethanol**
RO water added to 1000 mL	

Column media: Fractogel EMD 650s
DEAE (“tentacle”), Merck
Column: Pharmacia, V = 50 mL
Peristaltic pump: Pharmacia, Peristaltic Pump P-1; Ø = 3.1 mm
Membrane-vacuum pump: vacuumbrand CVC2
Nalgene filter funnel: 315-0047
Millipore filter: HVP04700 (Pore size 0.45 µm)
Ultrasonication bath: Bandelin Sonorex Super RK 510

3.3.3. Concentrating protein solution with Amicon Centripreps

- **Principles**

Centripreps are based on the principle of ultrafiltration. Their cellulose membrane is permeable for small molecules, whereas bigger components, like proteins, are retained in the sample container. The small molecules are collected in the filtrate collector by centrifugation.

- **Protocol**

- 15 mL of protein solution were filled into the sample container.
- The Centriprep was put together and centrifuged for 20 min at 4000 rpm and 4℃.

- The filtrate was discarded.
- Step 1-3 were repeated until the desired volume was reached.

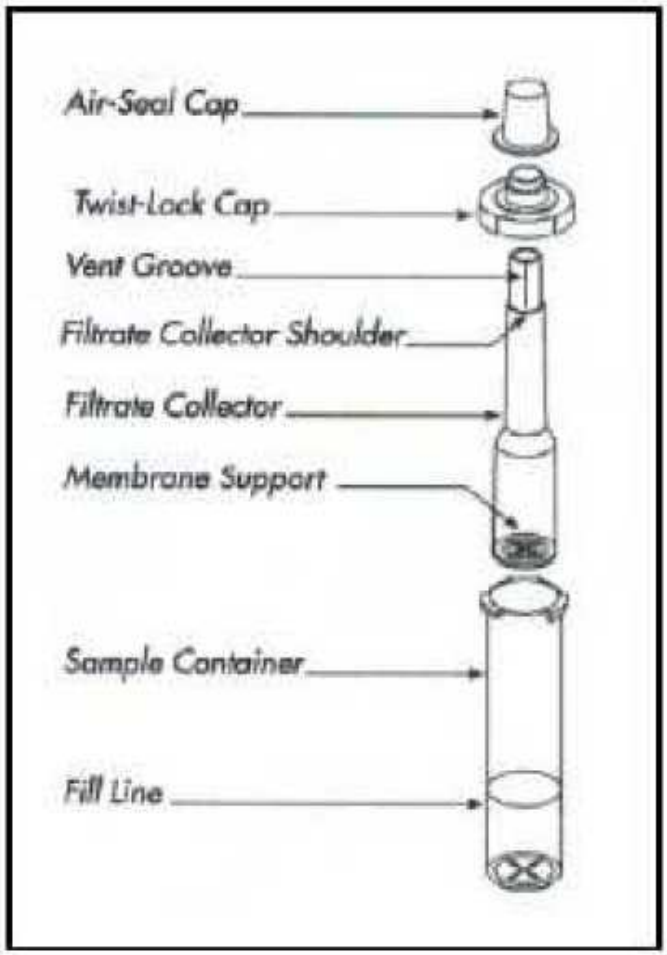

Figure 3.4: Amicon Centriprep

- **Equipment**

Amicon Centriprep YM-10:

Max. fill volume: 15 mL

End volume: 0.6 - 0.7 mL

Max. RCF: 3000 g

RC-5C Centrifuge: Sorvall Instruments, Du Pont

SLA-rotor 1500

3.3.4. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

- **Principles**

Many biomolecules carrying a charge can move in an electric field. The mobility of the ions depends on charge, size and shape of the molecules. These differences are the basis of electrophoresis.
The electrophoretic separation occurs in inert, homogenous gels like polyacrylamide, agar, starch. Due to the decreased diffusion within the gel, the separated components will move in discrete and separated bands. Polyacrylamide forms inert, homogenous gels, and exhibits excellent properties for protein separation. The pore size of the gel can be controlled by choosing various concentrations of acrylamide and methylenebisacrylamide (a cross-linking reagent) at the time of polymerisation.
Sodium dodecyl sulfate (SDS) is an anionic detergent, which binds dominantly to proteins causing denaturation. This kind of polyacrylamide gel electrophoresis is one of the most commonly used methods to separate complex protein mixtures and also to determine the relative molecule masses of the proteins. Under saturated conditions, about 1.4 g SDS are bound per gram protein, all proteins get a certain negative amount of charges per unit of mass. So in most cases the mobility of the protein-SDS-complex, caused by the kind of molecular sieve of the gel, is proportional to the logarithm (log10) of the relative mass (Mr).
To achieve further denaturation of proteins (reduction of disulfide linkages in tertiary and quaternary structure) they may also be boiled in the presence of dithiothreitol (DTT) or 2-mercaptoethanol before application to the gel.

- **Protocol**

<u>a)Preparation of the gel:</u>
Mixture for 2 gels: Polymerization ratio 1:37.5

Table 3.21: Components of Separating and Stacking Gels.

	Separating gel	**Stacking gel**
Monomer conc.	15%	4%
RO water	2.29 mL	2.97 mL
1.5 M Tris/HCl	2.50 mL	---
0.5 M Tris/HCl	---	1250 µL
Acrylamide/Bis	5.0 mL	670 µL
10% (w/v) SDS	100 µL	50 µL
10% APS	100 µL	50 µL
TEMED	10 µL	10 µL

b) Procedure

- The separating gel was cast and covered with 2-butanol to get a plain surface.
- Polymerisation of the gel for 30 min.
- The butanol was discarded, the gel was rinsed with water and the surface was dried using filter paper.
- The stacking gel was added and the comb (10 slots) was set in place.
- Polymerisation for 15 min.
- The comb was removed and the electrophoresis device assembled.
- The gels were put into the electrophoresis chamber that was then filled with 1 x running buffer.
- The samples were diluted 1:2 with 2 x sample buffer and boiled for 4 min.
- 15 µL of each sample and the marker (5 µL) were loaded on the gel using a Hamilton syringe.
- Electrophoresis was performed at constant 200 V and max. 70 mA

c) Coomassie staining

After the electrophoresis was finished the gel was rinsed with distilled water and incubated in coomassie staining solution at room temperature for 30 minutes on a shaker. Then the gel was destained in destaining solution until bands could be visualised on a clear background.

- **Chemicals and equipment**

Acrylamide/Bis:	**2x Sample buffer stock solution**:
(30% T; 2.67% C, 1:37.5):	2.0 mL Tris / HCl pH 6.8
146.0 g Acrylamide	1.6 mL Glycerol (20%)
4.0 g Bis	3.2 mL 15% SDS (6%)
RO-water added to 500 mL	0.4 mL 0.5% (w/v) Bromphenol Blue
filtrated and stored at 4℃	**TEMED**
1.5 M Tris/HCl pH 8.8:	**10% (w/v) SDS**:
54.45 g Tris base	1.0 g SDS in 10 mL RO-water
120 mL RO-water	**10% (w/v) APS**:
6 M HCl added to pH 8.8	50 mg Ammoniumperoxodisulphate
RO-water added to 300 mL	500 µl RO-water
0.5 M Tris/HCl pH 6.8:	**2x Sample buffer**:
6.0 g Tris base	900 µl 2x Sample buffer stock solution
60 mL water	100 µl β-Mercaptoethanol
6 M HCl added to pH 6.8	**Staining solution**:
RO-water added to 100 mL	0.1% (w/v) Coomassie Blue R-250
5x Running Buffer, pH 8.3:	40% (v/v) Methanol
15.15 g Tris base	10% (v/v) Glacial actic acid
72.0 g Glycine	50% (v/v) RO-water
5.0 g SDS	**Destaining solution**:
RO-water added to 1000 mL	40% (v/v) Methanol
stored at 4℃	10% (v/v) Glacial actic acid
1x Running buffer, pH 8.3:	50% (v/v) RO-water
160 mL 5x Running buffer, pH 8.3	
640 mL RO-water	

Marker: Page Ruler^{TM} Unstained Protein Ladder, Fermentas

Broad range: 10-200 kDa.

stored at –20℃

BioRad Mini-Protean III Dual Slab Cell

Spacer: 1.00 mm

Voltage source: BioRad Power Supply, Type 500/1000

3.4. Spectrophotometric investigations

After each purification and reconstitution step of the CuA-domain UV-VIS absorption spectra of the obtained protein solutions were recorded in order to determine the concentration and reconstitution efficiency.

3.4.1. Determination of protein concentration

- **Principles**

From the measured absorptions at specific wavelengths the protein concentration was calculated according to Lambert-Beer's Law using the molar extinction coefficient ε at different wavelengths.

Lambert-Beer's Law: $A = c \times \varepsilon \times d$

A…absorption

c…concentration [mol / l]

ε…molar extinction coefficient [$M^{-1}cm^{-1}$]

d…diameter of the cuvette: 1 cm

ε_{480nm}…2.8 mM^{-1} cm^{-1}

ε_{535nm}…3.1 mM^{-1} $cm^{-}1$

ε_{280nm}SUIIj3/c3 cox 2…21.0 mM^{-1} cm^{-1}

ε_{280nm}SUIId2 cox 1…21.0 mM^{-1} cm^{-1}

3.4.1.1. Determination of SUIIj3 cox2 protein concentration

- **Protocol**

- The sample was centrifuged at 14000 rpm for 4 min at RT, and if necessary it was diluted before the measurement.
- The holoprotein concentration was determined with the molar extinction coefficients of SUIIa3 of *Synechocystis PCC 6803*.
- The total protein concentration was determined with the molar extinction coefficient at 280 nm.

- **Equipment**

Spectrophotometer: Diode Array Specord UV-VIS S 10, Zeiss Instruments
Software: Aspect plus version 1.5
Cuvettes: Quartz-cuvettes (1 cm light-path)
Centrifuge: Sigma 1-15 (Sigma, Germany)

4. Results

4.1. Cloning of recombinant proteins

The soluble part of cytochrome *c* oxidase 2 subunit II (SUIIj3 cox2) and plastocyanin (PC) of *Nostoc sp.* PCC 7120 were cloned in the same *E. coli* strain to allow recombinant expression of the proteins.

4.1.1. Cloning of cox2 subunit II soluble domain (SUIIj3 cox2)

The truncated subunit II CuA-domain of the cyanobacterium *Nostoc sp. PCC 7120* was successfully cloned. DNA- and protein-sequences of subunit II cox2 were based on the sequences provided by Cyanobase, a genome database for cyanobacteria and previous successful cloning protocols. Primers were designed for expression of the periplasmic domain encoding for amino acid residues 137 to 327 of cytochrome *c* oxidase subunit II. The primer suIIj3_cox2_p1 introduced an *Nde*I restriction site and an ATG start codon, whereas primer suIIj3_cox2_p2 introduced a *BamH*I restriction site and a TGA stop codon.

4.1.1.1. Amplification by PCR

SUIIj3 cox2 was amplified by PCR. The obtained PCR-fragment was purified with Gene JET^{TM} Plasmid Mini Prep-Kit. To verify the length of the insert agarose gel electrophoresis was performed. Figure 4.1 shows the gel with the DNA visualised under UV-light. The intensity of the bands corresponds to the amount of DNA. The PCR-fragment was concentrated using the GFX PCR DNA and Gel Band Purification Kit. The band in lane 1 exhibits a size of about 560 bp, which corresponds well with the calculated length of SUIIj3 cox2 (573 bp).

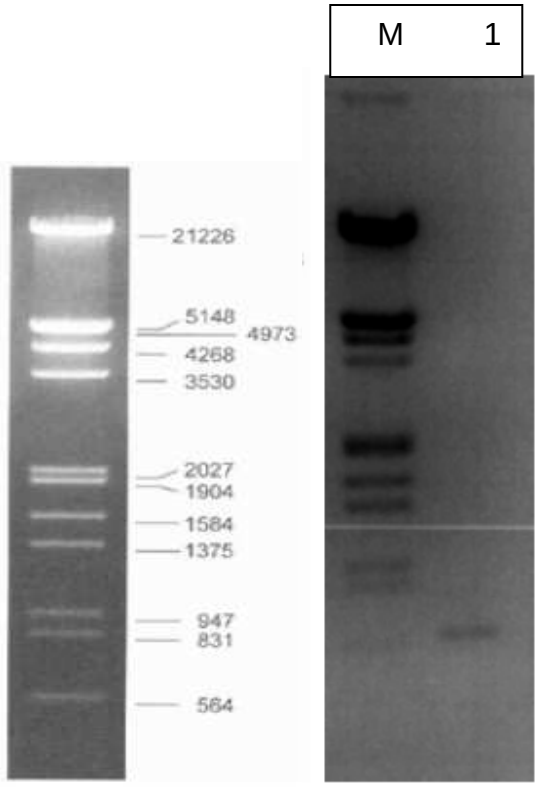

Figure 4.1. λ-III-marker and agarose gel electrophoresis of purified and concentrated DNA lane M: λ-III-marker; lane 1: concentrated PCR-fragment suIIj3 cox2,

4.1.1.2. Preparation of the pET-3a vector

The pET-3a vector was prepared using the QIAGEN plasmid purification kit. Agarose gel electrophoresis was performed to estimate the concentration of vector DNA. Figure 4.2. shows the agarose gel with the vector DNA visualised under UV-light. The intensity of the bands corresponds to the amount of vector-DNA.

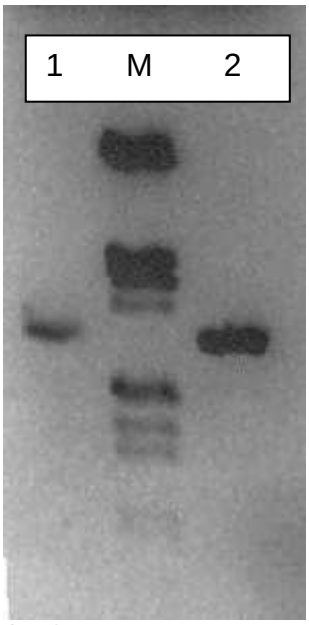

Figure 4.2. *Agarose gel electrophoresis of pET-3a vector DNA; lane 1: 1 µL pET-3a vector, lane M: λ-III-marker, lane 2: 5 µL pET-3a vector*

4.1.1.3. Digestion of insert and vector DNA and ligation

The purified insert coding for SUIIj3 cox2 and the pET-3a vector were digested with the restriction enzymes *BamH*I and *Nde*I. Alkaline phosphatase was added to the digestion approach of vector DNA to prevent ligation of vector DNA without an insert. Agarose gel electrophoresis (Figure 4.2) was performed in order to determine the concentration before ligation. After the separation step, the DNA positioned at the appropriate height was marked by the help of a long wave UV transilluminator and excised. The amount of DNA was roughly estimated by the intensity of its corresponding band on the agarose gel on figure 4.2. Longer fragments exhibit a more intensive colour because they incorporate more of the sybr safe bromide dye. Based on the concentration ratio between vector and insert DNA, which should be approximately 1:10, the components for the ligation and negative control without insert DNA were mixed. Vector and insert DNA were ligated by T4-DNA-ligase. The band at position 4640 bp shown in the figure represents the excised vectorial DNA, meanwhile the band at 573 bp represents the insert DNA of SUIIj3 cox 2.

Table 4.1 components of ligation assay

	assay	negative control
T4-DNA-Ligase	1 µL	1 µL
10x Ligase buffer	2 µL	2 µL
Vector DNA	2 µL	2 µL
Insert DNA	12 µL	---
Sterile water	3 µL	15 µL

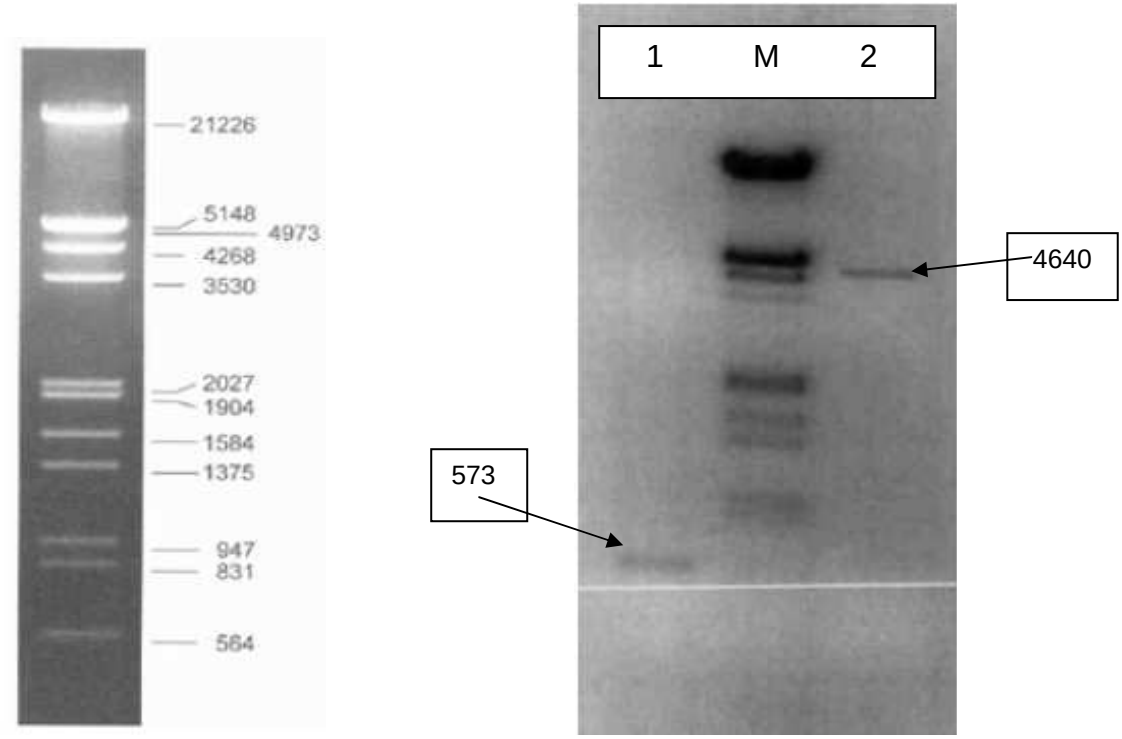

Figure 4.2: Agarose gel electrophoresis of digested insert and pET-3a vector DNA; lane 1: insert DNA, lane M: λ-III-marker, lane 2: pET-3a vector

4.1.1.4. Screening for positive clones

Screening for positive clones carrying the recombinant protein was performed by different methods. Colonies were picked from LB_{AmpCm}-plates and screened by PCR. If the clone carries the recombinant protein it can be amplified with PCR using the primers suIIj3_cox2_p1 and suIIj3_cox2_p2. Additionally a masterplate containing colonies of each picked clone was established. Agarose gel electrophoresis was performed and the amplified DNA was visualised under UV-light (shown in Figure 4.3.). Clone 3 and 7 were tested positive by PCR screening.

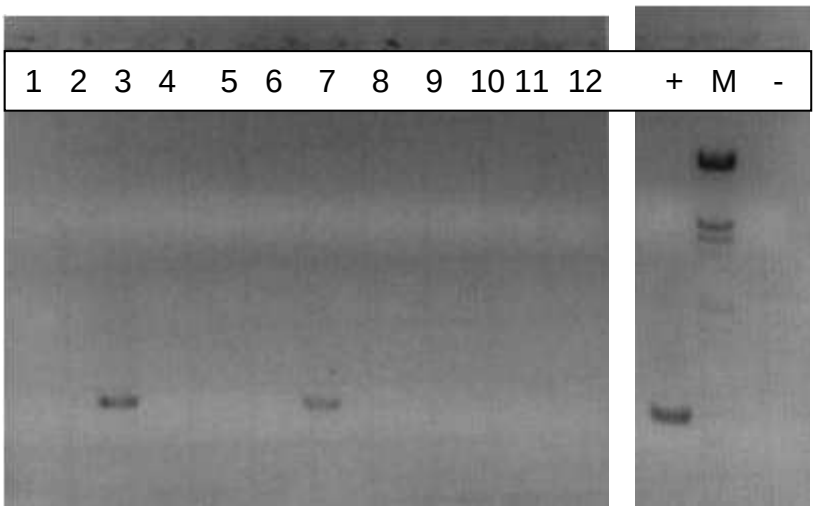

Figure 4.3. Agarose gel electrophoresis of clones 1-12; lane +: positive control, lane M: λ-III-marker, lane -: negative control

The expression performance of positive clones was checked by a small-scale expression experiment. Using SDS polyacrylamide gel electrophoresis, the expression of all proteins both in the supernatant and in the pellet could be inspected (shown in Figure 4.4.). Their molecular weight was compared to the calculated molecular weight of the suIIj3 cox2 fragment (20219 Da). Although the band of the expressed protein was closer to the 25000 Da band of the marker than to the 20000 Da band, it was referred to the SUIIj3 cox2.

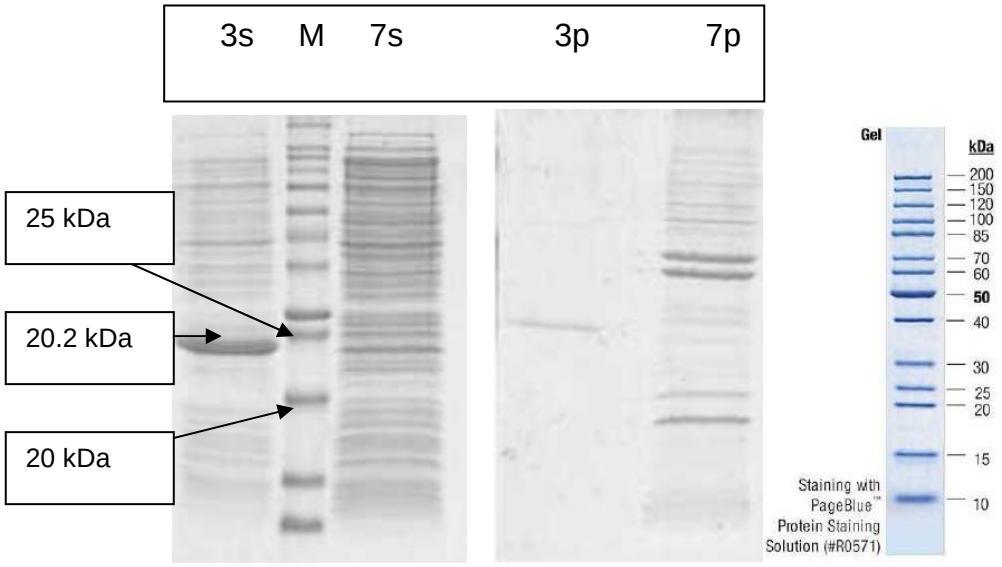

Figure 4.4. SDS-PAGE of proteins expressed by clone 3 and 7
s…proteins of the supernatant, p…proteins of the pellet, M…marker

Clone 3 expressed a protein in adequate concentration in the supernatant. Additional and former results support the fact that more SUIIj3 cox2 is found in the supernatant than in the pellet. To justify the assumption that SUIIj3 cox2 is produced, the plasmid DNA was sequenced.

4.1.1.5. Sequencing of plasmid DNA

The sequence of purified plasmid DNA of clone 3 was determined to assure that the plasmid DNA and as a consequence also the expressed protein does not contain any mutations.

DNA sequencing was conducted by I.B.L., Dennisgasse 23, 1200 Vienna. Plasmid DNA was purified with Gene Jet^{TM} Plasmid Mini-Prep Kit and T7 promoter and terminator primer were provided. Figure 4.5 shows the agarose gel with the visualised DNA.

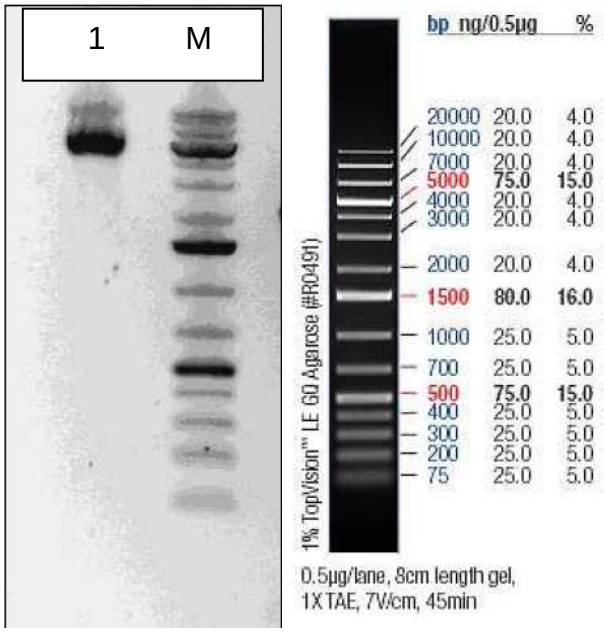

Figure 4.5. Agarose gel electrophoresis of purified DNA of clone 3 for sequencing lane 1: plasmid DNA of clone 3, lane M: Marker

The software tool DNAStar SeqMan 4.0 was used to compare the obtained sequence (shown in Figure 4.6.a. and 4.6.b.) with the genom from *Synecocystis* PCC 6803. No mutations were found that led to alterations of the proteins' amino acid sequence.

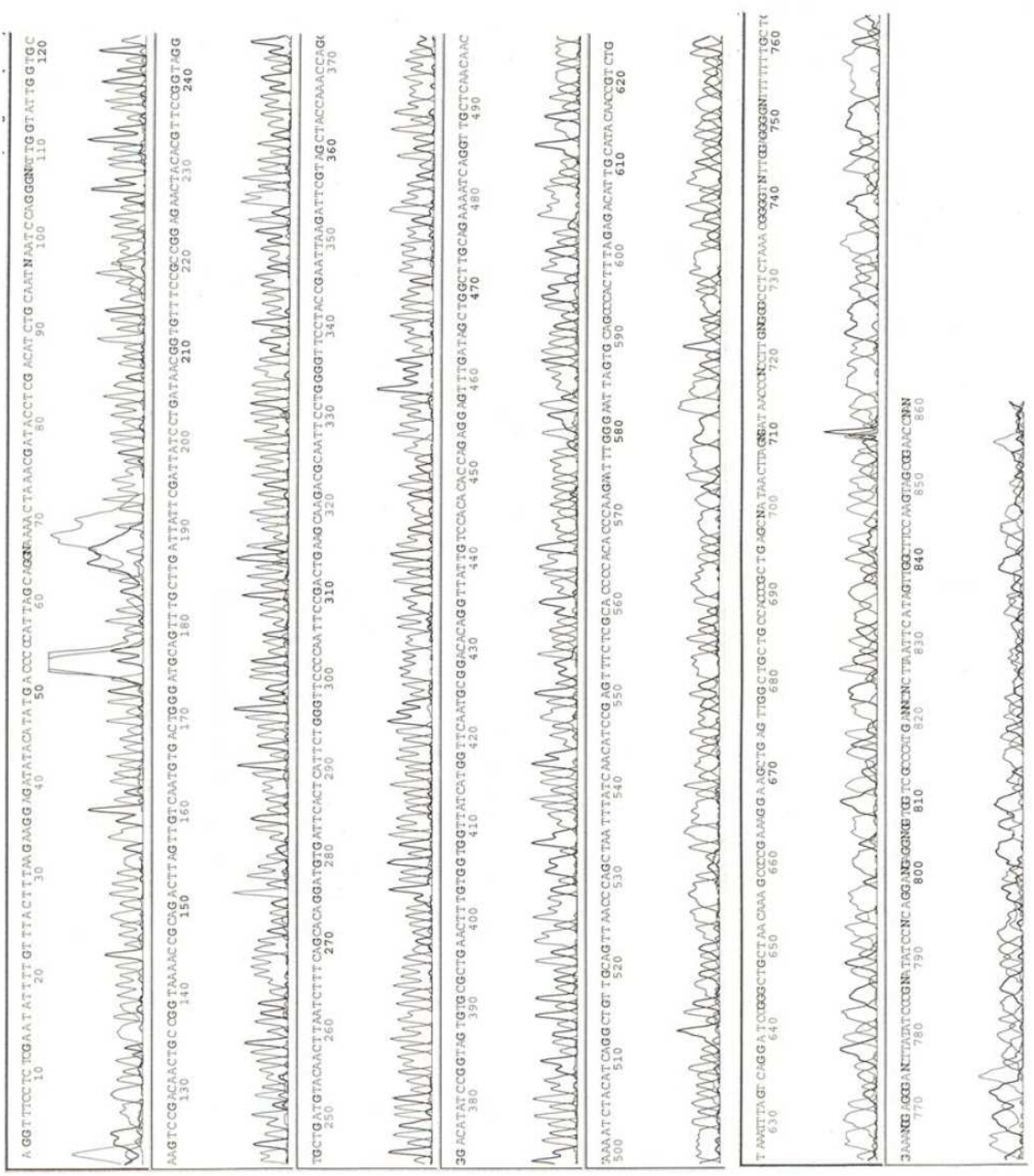

Figure 4.6.a. DNA sequence of clone 3, T7-promoter-primer was used for sequencing

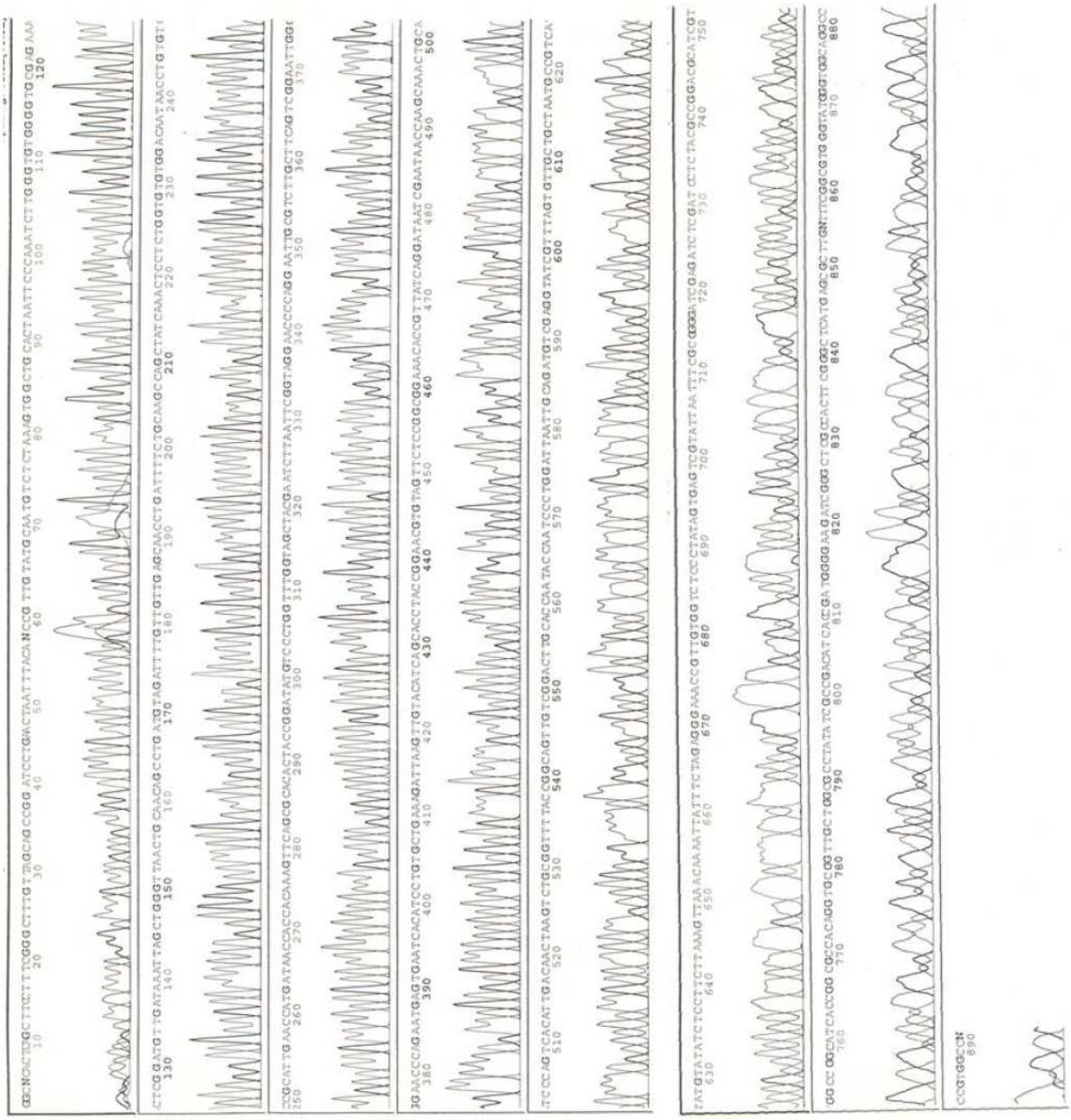

Figure 4.6.b. DNA sequence of clone 3, T7-terminator-primer was used for sequencing

4.1.2. Cloning of plastocyanin

Plastocyanin was also successfully cloned in *E. coli* BL21(DE3)Star carrying the pEC86 plasmid, using the pET-3a plasmid.

4.1.2.1. Amplification by PCR

Plastocyanin was amplified by PCR. The obtained PCR-fragment was purified with Gene JET^{TM} Plasmid Mini Prep-Kit. To verify the length of the insert agarose gel electrophoresis was performed. Figure 4.7 shows the gel with the DNA visualised under UV-light. The bands in lane 1 & 2 exhibit a size of about 300 bp, which corresponds well with the calculated length of plastocyanin (315 bp).

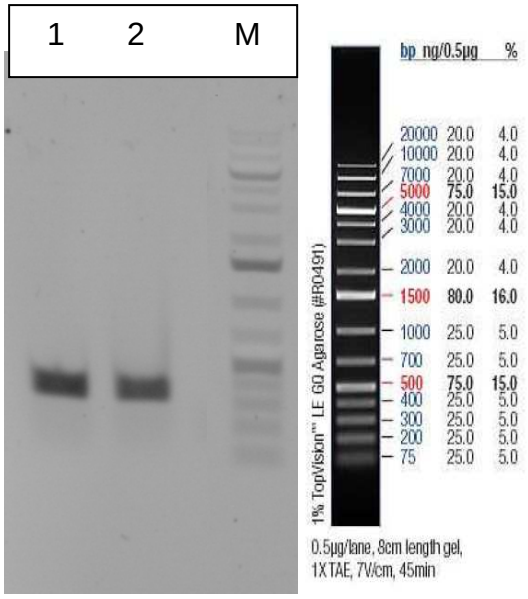

Figure 4.7. 1 kb ladder marker and agarose gel electrophoresis of purified and concentrated DNA lane 1 and lane 2: concentrated PCR-fragment plastocyanin, lane M: Marker

4.1.2.2. Digestion of insert and vector DNA and ligation

The purified DNA and the pET-3a vector were digested with the restriction enzymes *BamH*I and *Nde*I. Alkaline phosphatase was added to the digestion of vector DNA to prevent ligation of vector DNA without an insert. Gel electrophoresis (Figure 4.8) was performed in order to determine the concentration before ligation. After the separation step, the DNA positioned at the appropriate height was marked by the help of a long wave UV transilluminator and excised. The amount of DNA was roughly estimated by the intensity of its corresponding band on the agarose gel on figure 4.7. Longer fragments exhibit a more intensive colour because they

incorporate more of the sybr safe dye. The intensity ratio between insert and vector was calculated 1.5 →2:1. The length of the vector (4640 bp minus 40 bp, which is the range between the two cutting sites of *BamH*I and *Nde*I) is compared to the length of the insert (315 bp), giving a ratio of 14.6:1. Based on the concentration ratio between vector and insert DNA, which should be approximately 1:8, various amounts of vector and insert were mixed to perform the ligation assay. The negative control contained the same components apart from insert DNA. Vector and insert DNA were ligated by T4-DNA-ligase. Figure 4.8. shows the digested insert and vector DNA.

Table 4.2: Components for ligation

	assay	**negative control**
T4-DNA-Ligase	1 µL	1 µL
10x Ligase buffer	2 µL	2 µL
Vector DNA	7.5 µL	7.5 µL
Insert DNA	8 µL	---
Sterile water	1.5 µL	9.5 µL

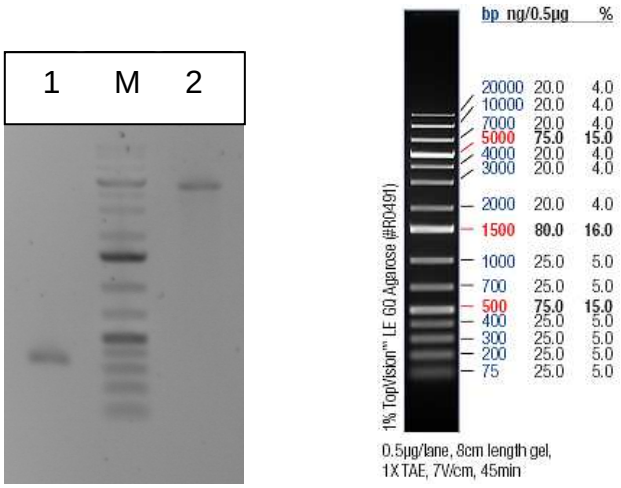

Figure 4.8. Agarose gel electrophoresis of digested insert and pET-3a vector DNA; lane 1: insert DNA, lane M: 1 kb-marker, lane 2: pET-3a vector

4.1.2.3. Screening for positive clones

Randomly picked single colonies on the LB_{AmpCm}-agar-plate were analysed by PCR screening. An expression screening was then performed to confirm that the selected clone is also expressing the target protein. Agarose gel electrophoresis (Figure 4.9) shows two positive clones in lane 7 and 19 found by PCR screening at the right length.

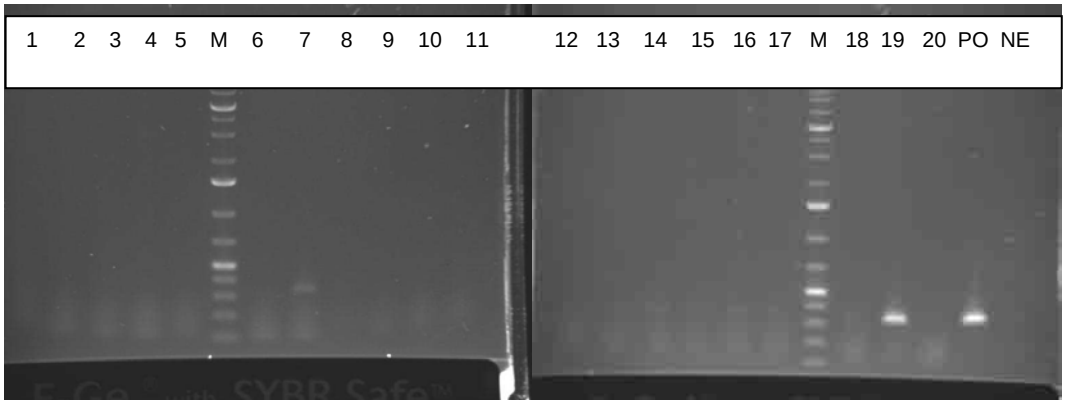

Figure 4.9: Agarose gel electrophoresis of PCR screening; lane M: 1 kb ladder, lane 7 and 19: positive clones; lane PO+: positive control; lane NE: negative control.

A small-scale expression of the positive clones was performed to investigate its expression abilities. The molecular weight of the expressed proteins was checked by SDS-PAGE and compared to the theoretical molecular weight of plastocyanin. UV-VIS spectra were also recorded.

SDS-PAGE (Figure 4.10) shows that almost all protein is found in the supernatant as soluble protein. Clone 7 expressed the desired protein in a sufficient amount. Comparison of the expression screening with the marker showed that the protein size is equal to the expected theoretical protein weight (10.3 kDa). A cryo-culture of clone 7 was set up.

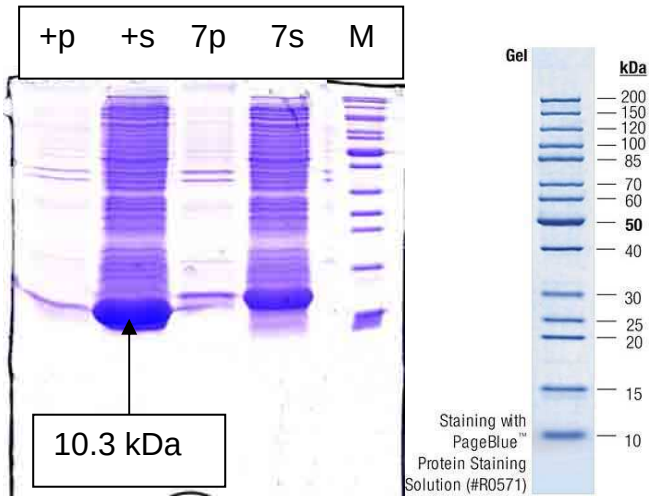

Figure 4.10: Comparision SDS-PAGE of the expression screening with marker lane +p : positive control = PC from Synechocystis *pellet; lane +s: positive control = PC from* Synechocystis *supernatant; lane 7p: clone 7 pellet; lane 7s: clone 7 supernatant*

4.1.2.4. Sequencing of plasmid DNA

The sequence of purified plasmid DNA of clone 7 was determined to assure that the plasmid DNA and as a consequence also the expressed protein does not contain any mutations.

DNA sequencing was conducted by I.B.L., Dennisgasse 23, 1200 Vienna. Plasmid DNA was purified with Gene Jet™ Plasmid Mini-Prep Kit and T7 promoter and terminator primer were provided. Agarose gel electrophoresis was performed to estimate plasmid DNA concentration (Figure 4.12).

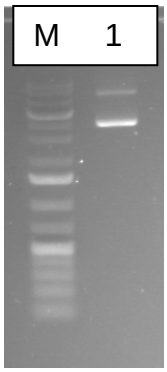

Figure 4.11: Agarose gel electrophoresis of the plasmid DNA of plastocyanin; lane M: 1 kb ladder; lane 1: 2 µL plastocyanin

No mutations were found that led to alterations of the proteins' amino acid sequence.

4.1.2.5. Screening for ideal expression conditions

A small-scale expression of the positiv clone 7 was performed to investigate the best expression conditions to produce as much soluble protein as possible. The expression time and temperature after addition of different concentrations of IPTG were varied as shown in Table 4.3 and the cells after harvest and lysation were analysed with SDS-Page (Figure 4.12).

Table 4.3: Expression screening conditions

Nr.	Temperature	Time	IPTG
1	37℃	4 h	1 mM
2	37℃	4 h	0.5 mM
3	37℃	4 h	0.2 mM
4	30℃	4 h	1 mM
5	RT	20 h	1 mM

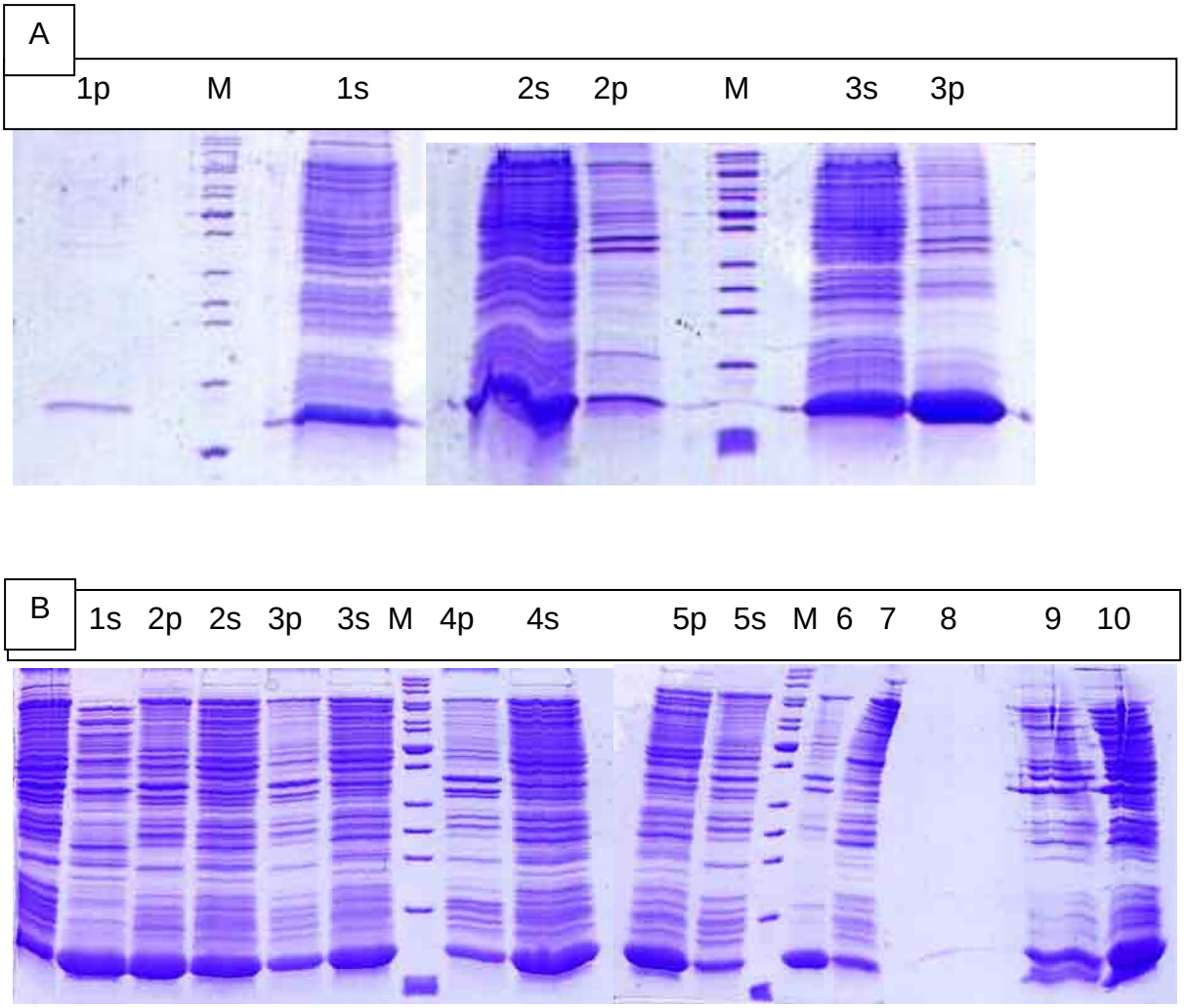

*Figure 4.12: **A**: SDS – Page expression screening plastocyanin lane M: marker; lane 1-3: pellet and supernatant at 20, 16 and 37°*

***B**: SDS – Page expression screening plastocyanin lane M: marker; lanes 1-5 are as mentioned in Table 4.1; 6: PC -Solution before NH_4SO_4-Addition; 7: PC - Solution after 60% NH_4SO_4; 8: PC - Solution after 100% NH_4SO_4; 9: Pellet after 60% NH_4SO_2; 10: Pellet after 100% NH_4SO_4*

Most of the protein at 37℃ is expressed as inclusi on bodies, however the yield of soluble protein is higher than with expressions at 16℃ or 20℃.

As a conclusion it can be said, that the amount of soluble protein at different conditions does not change significantly, meanwhile the fraction of inclusion bodies varies. According to this results, the highest yield can be achieved at 37℃ and 4 hours expression time.

4.2. Expression of recombinant proteins

The following recombinant proteins, shown in Table 3.20 were expressed in *E. coli*, using the pet-3a expression system.

Table 4.4: Recombinant proteins and the number of the various clones used

Protein	Number	Amino acid residues	Molecular Weight	*E. coli* strain	Expression vector
Cytochrome *c* Oxidase 2 Subunit II	c3-clone	113-237	22.5 kDa	BL21(DE3)pLysS	pET-3a
Cytochrome *c* Oxidase 2 Subunit II	j3-clone	137-327	20.2 kDa	BL21(DE3)pLysS	pET-3a
Cytochrome *c* Oxidase 1 Subunit II	d2-clone	113-355	26.4 kDa	BL21(DE3)pLysS	pET-3a

4.2.1. Expression of SUIIj3 cox2, SUIIc3 cox2 and SUIId2 cox1

SUIIj3 and SUIIc3 cox2 and SUIId2 cox1 were expressed in the *E. coli* strain BL21(DE3)pLysS. 2 L of $M9ZB_{AmpCm}$ were inoculated with 24 mL overnight culture prepared from a cryo-culture. The cells were incubated at 37℃ and 180 rpm until an OD_{600} of 1.4 was reached. Subsequently the expression was induced by the addition of IPTG to a final concentration of 1 mM IPTG, and incubated for 20 h at 16℃ for SUIIj3 and SUIIc3 cox2 and 4.5 h at 37℃ for SUIId2 cox1. A longer expression time at low temperature promotes the correct folding of the protein.

4.3. Purification of recombinant proteins

4.3.1. Purification of SUIIj3 cox2

4.3.1.1. Chromatographic purification

The overexpressed protein was released from the cytosol by lysis of the *E. coli* cells. Protease inhibitor was immediately added to protect the proteins from proteolytic digestion. It is necessary that the reduced state of the two cysteine residues in the active site of the CuA-domain is preserved for reconstitution of the apoprotein with Cu^{2+}, therefore DTT, a reducing agent, is added to the lysis buffer and to the buffers used in the first chromatographic step before copper reconstitution. SUIIj3 cox2 was purified by two chromatographic steps: anion exchange chromatography with Q-Sepharose Fast Flow and by size exclusion chromatography with Superdex^{TM} 75.
Figure 4.13 shows selected fractions after the Q-Sepharose Fast Flow chromatography. SUIIj3 cox2 was eluted from the column with gradient 1 (buffer A + 100 mM NaCl), whereas with gradient 2 (buffer A + 200 mM NaCl) only a small amount of SUIIj3 cox2 but high amount of other protein was eluted. Only fractions with high amount of SUIIj3 cox2 and a small amount of other proteins were pooled; in this case fractions 3-7. PMSF and DTT were added to the protein pool to a final concentration of 200 µM PMSF and 0.5 mM DTT. The protein was afterwards reconstituted.

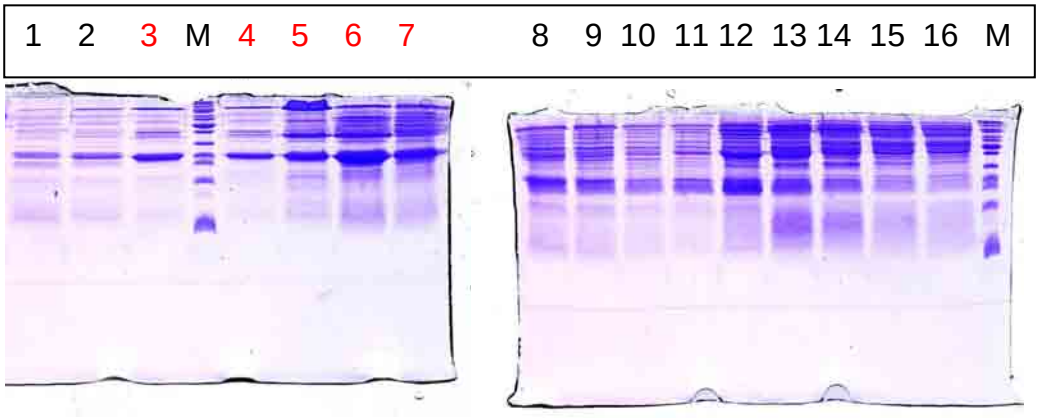

Figure 4.13: SDS-PAGE of SUIIj3 cox2 after Q-Sepharose Fast Flow.
Numbers 1-16 indicate the fractions collected during elution; JS are non SUIIj3 samples, fractions numbers pooled are coloured red; lane M: marker

4.3.1.1.1. Purification and Separation with Superdex^{TM} 75

To get rid of the remaining impurities and to separate the successfully reconstituted holoprotein from the non-reconstituted apoprotein size exclusion chromatography with Superdex^{TM} 75 was performed. The isoelectric point of SUIIj3 cox2 is known at pH 5.1, hence the pH of the buffer has to be 6.5 to keep the protein stable. It is known that apoprotein elutes from the Q-Sepharose gel at about 100-200 mM NaCl, therefore the NaCl concentration for the Superdex^{TM} 75 was set to a gradient starting from 80 mM to 300 mM NaCl in 50 mM PP pH 6.5. Figure 4.14 shows a typical chromatogram. The purification results in a prominent protein peak with a pronounced shoulder. The main peak refers to SUIIj3 cox2, whereas the shoulder might contain non-heme proteins.

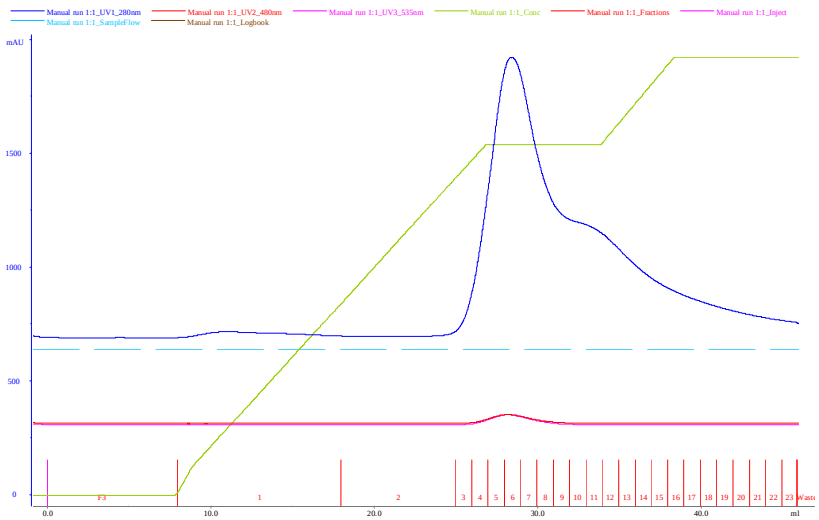

Figure 4.14: Chromatogram of SUIIc3 cox2 of the SuperdexTM 75 chromatographical step. blue line: 280 nm; red line: 480 nm; pink line: 535 nm

The fractions with significant absorbance at 480/535 nm were analysed by UV-VIS (Figure 4.15) to sort out the active protein containing fractions and to calculate the amount of holoprotein.

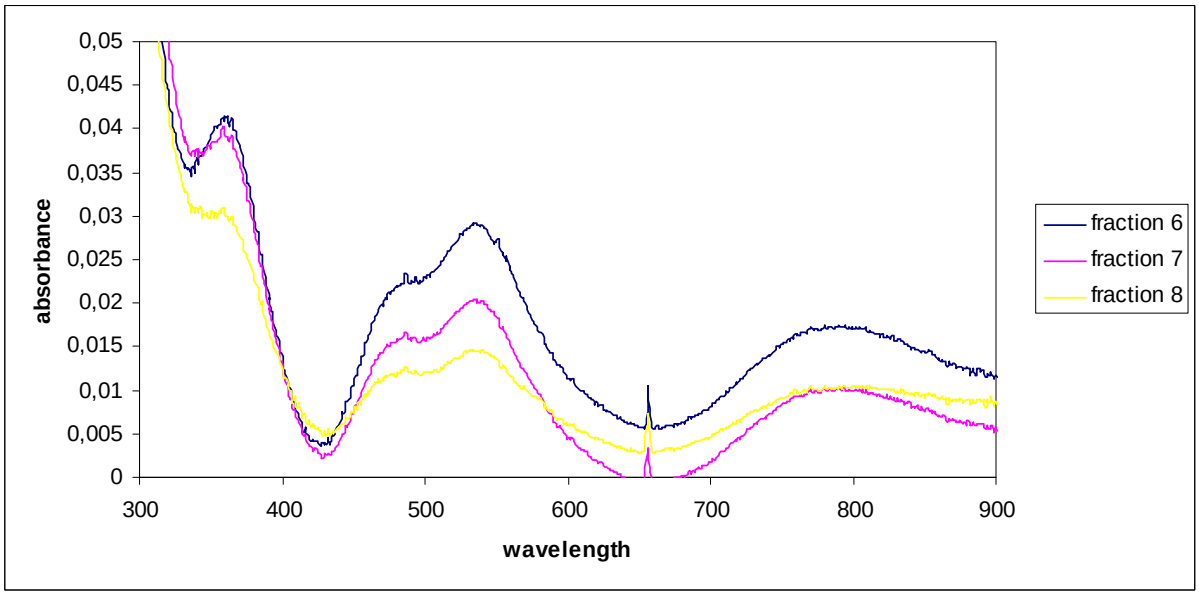

Figure 4.15 Comparision of the eluated Superdex fractions

Fractions 6-8 were pooled and concentrated and the amount of holoprotein was calculated:

Fraction 6: Holoprotein 18%
Fraction 7: Holoprotein 13.5%
Fraction 8: Holoprotein 12.8%

Although the protein solution is now free of any other protein impurities shown by SDS-PAGE in figure 4.16, the purification effect of this chromatographic step is too low to compensate the loss of holoprotein during this purification. The peak at 480/535 nm on the Superdex is based mainly on SUIIj3 cox2 but it is not possible with this method to differentiate between apo- and holoprotein.

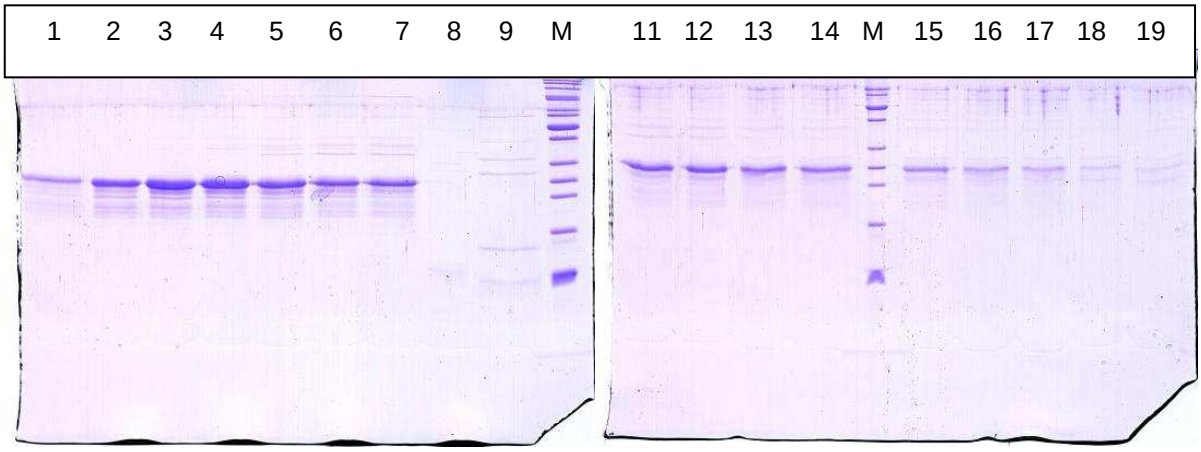

Figure 4.16. SDS-PAGE of suIIj3 cox2 after size exclusion chromatography lanes 1-19: fractions collected during elution; lanes 8+9: non-SUIIj3 probes; lane M: marker

4.3.1.2. Copper reconstitution

Recombinant SUIIj3 cox2 does not have copper in the active center as *E. coli* cells show a lack of the required chaperones. The reconstituted, oxidized CuA-domain is characterised by its purple colour and exhibits two strong absorbance maxima at about 480 nm and 535 nm, arising from the interaction of the two copper ions with each other and with the ligands. Two approaches were tried to reconstitute the protein: Dialysis after unfolding with urea and ultrafiltration after unfolding with urea.

4.3.1.2.1. Dialysis with urea

One way to reconstitute SUIIj3 cox2 is to unfold the protein with urea and refold it again by removing the denaturating agent step by step by dialysis. This was achieved by incubating the unconcentrated Q-Sepharose protein pool with 3 M urea at 4℃ for 1 hour. The protein solution was then fi lled into a dialysis tube and dialysed for 1.5 hours at 4℃ in 50 mM phosphate buffer pH 6 .5 and 1 M solid urea with a protein solution to buffer ratio of 1:20. The buffer was then replaced by a 50 mM phosphate buffer, pH 6.5, without urea and a protein solution to buffer ratio of 1:50. The solution was dialysed for 1.5 hours under stirring at 4℃. The buffer was once again substituted by a 50 mM phosphate buffer, pH 6.5, with 1.5 mM $Cu(His)_2$ and a protein solution to buffer ratio of 1:20. After overnight dialysis at 4℃ the solution showed a slightly purple color.

The reconstituted SUIIj3 cox2 solution was concentrated with Amicon Centripreps. During concentration the remaining $Cu(His)_2$ solution was washed out and the solution became more and more purple. The recorded UV-VIS spectrum (Figure 4.18) showed the typical absorbance maxima at about 480 nm and 535 nm but only 20%, as calculated in Chapter 4.4.2.1, of total SUIIj3 cox2 was reconstituted.

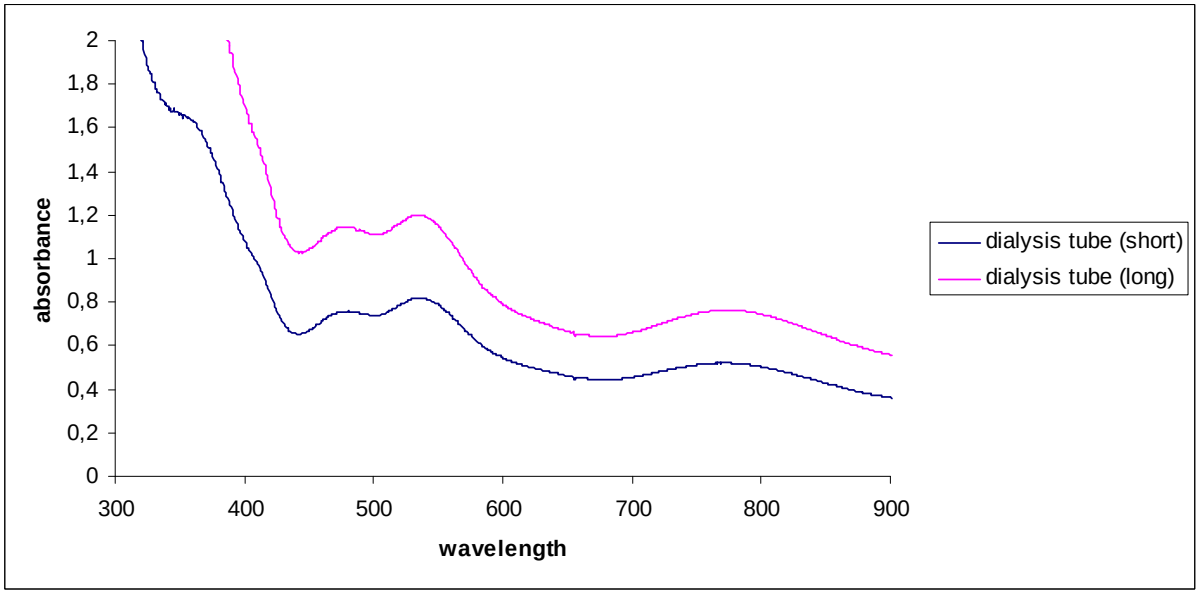

Figure 4.18: UV-VIS spectrum of the reconstituted CuA-domain of 2 dialysis experiments

4.3.1.2.2. Ultrafiltration with urea

An alternative way to reconstitute SUIIj3 cox2 is to unfold the protein with urea and refold it by removing the denaturating agent step by step using ultrafiltration. This was achieved by incubating the unconcentrated Q-Sepharose protein pool with 3 M urea at 4℃ for 1 hour. The protein solution was th en filled into an ultrafiltration cell and concentrated by reducing the volume. The cell was refilled with 50 mM phosphate buffer, pH 6.5, and 1 M solid urea and again concentrated. This was repeated three times with the exception that no urea was added anymore. Finally 1.5 mM $Cu(His)_2$ was added to the solution and incubated 1.5 hours under stirring at 4℃.
The colour of the protein solution changed quite fast from yellow-green to dark blue/violet. The reconstituted protein solution was filtrated and degassed and was loaded on a DEAE column to get rid of the remaining $Cu(His)_2$. The recorded UV-VIS spectrum (Figure 4.19) showed the typical absorbance maxima at about 480 nm and 535 nm but only 47% of total SUIIj3 cox2 was reconstituted. Nevertheless this was an increase of 50% in the yield of reconstituted holoprotein compared to the method using dialysis.
However, the reconstituted protein was not stable. Figure 4.19 shows the spectrum of the reconstituted protein after storage overnight at 4℃ (green), compared with the spectrum recorded immediately after ultrafiltration (red). The amount of reconstituted protein decreased dramatically.

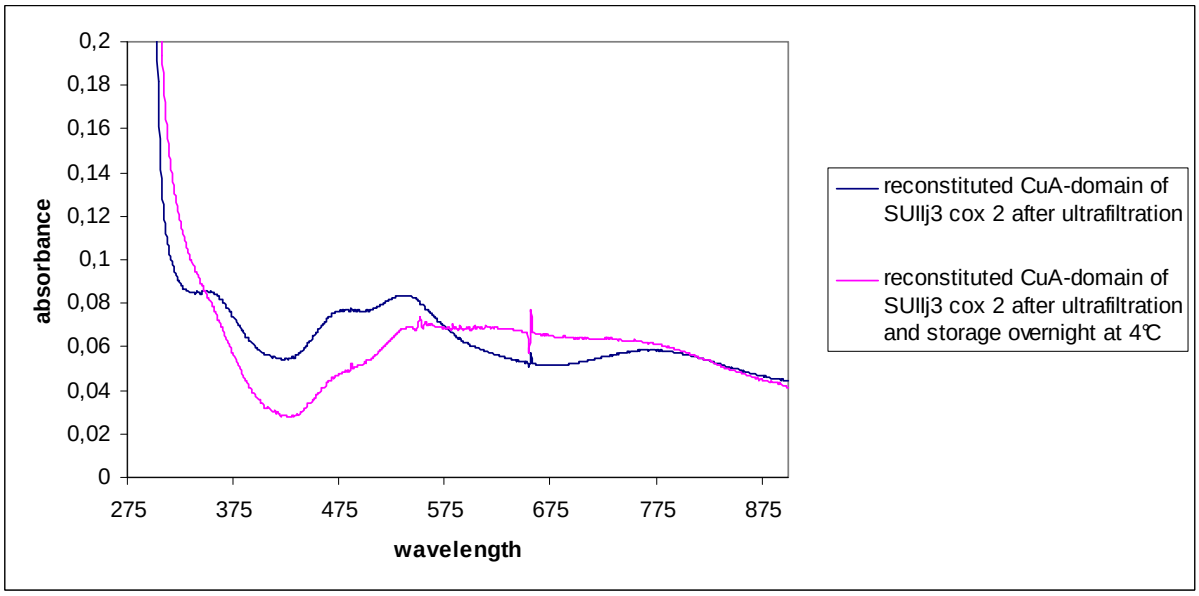

Figure 4.19: Overlay of UV-VIS spectra of the reconstituted CuA-domain of SUIIj3 cox 2 after ultrafiltration and after overnight storage at 4°

The loss of reconstituted protein was about 50% per day when stored at 4℃ and around 30% per day when stored at -80℃. According to calculations most of the precipitated protein is non-functional apoprotein. Generally, during storage active holoprotein was converted to non-functional apoprotein.

4.3.2. Purification of SUIIc3 cox2

4.3.2.1. Chromatographic purification

Chromatographic purification of SUIIc3 cox2 was performed with the same protocol as shown in Chapter 4.3.1.1. Figure 4.20 shows selected fractions after the Q-Sepharose Fast Flow chromatography. Fractions 2-9 were pooled.

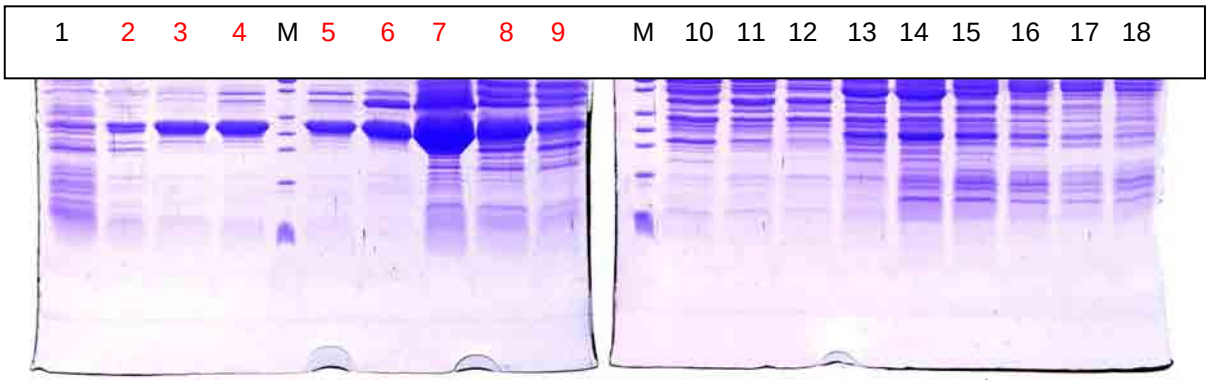

Figure 4.20: SDS-PAGE of SUIIc3 cox2 after Q-Sepharose Fast Flow.
Numbers 1-18 indicate the fractions collected during elution; fractions numbers pooled are coloured red; lane M: marker

4.3.2.2. Copper reconstitution

Recombinant SUIIj3 cox2 does not have copper in the active center as *E. coli* cells show a lack of the required chaperones. The reconstituted, oxidized CuA-domain is characterised by its purple colour and exhibits two strong absorbance maxima at about 480 nm and 535 nm, arising from the interaction of the two copper ions with each other and with the ligands.
To reconstitute the protein the above mentioned protocol, that combines unfolding by urea with ultrafiltration, was used.

4.3.2.2.1. Ultrafiltration with urea

The recorded UV-VIS spectrum (Figure 4.21) after ultrafiltration and concentration shows the typical absorbance maxima at about 480 nm and 535 nm but only 25%, as calculated in Chapter 4.4.2.2, of total SUIIc3 cox2 was reconstituted. The same stability problem as with SUIIj3 cox 2 was observed.

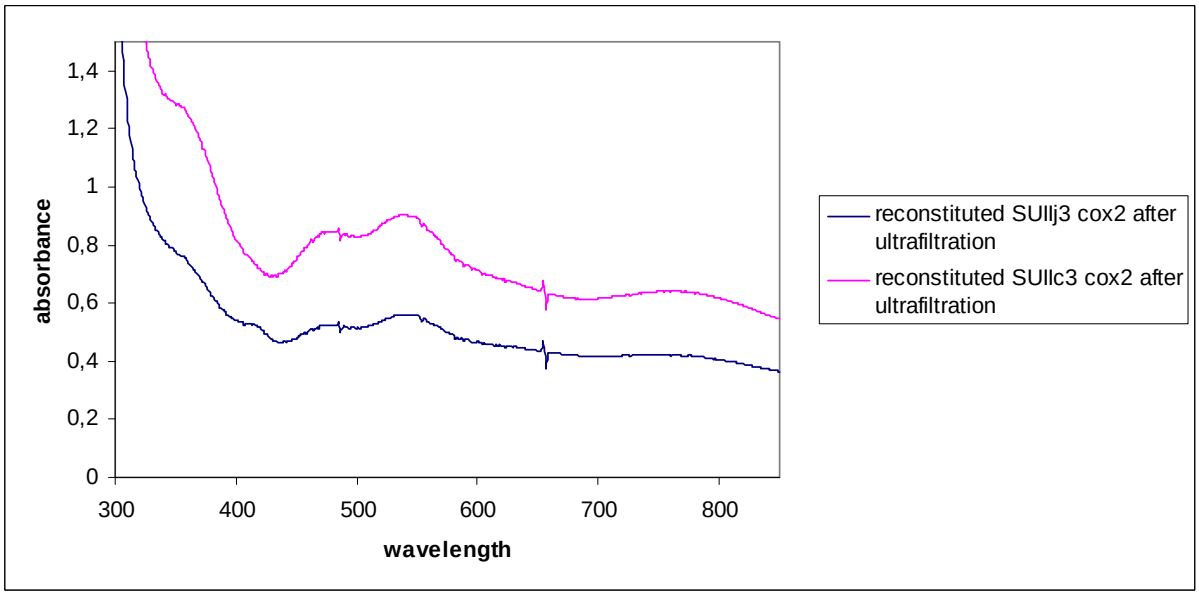

Figure 4.21: UV-VIS spectrum of the reconstituted CuA-domain of SUIIc3 cox 2 after ultrafiltration and after overnight storage at -80℃.

4.3.3. Purification of SUIId2 cox1

4.3.3.1. Chromatographic purification

Chromatographic purification of SUIId2 cox1 was performed with the same protocol as shown in Chapter 4.3.1.1. Figure 4.22 shows selected fractions after the Q-Sepharose Fast Flow chromatography. Fractions 6-11 were pooled.

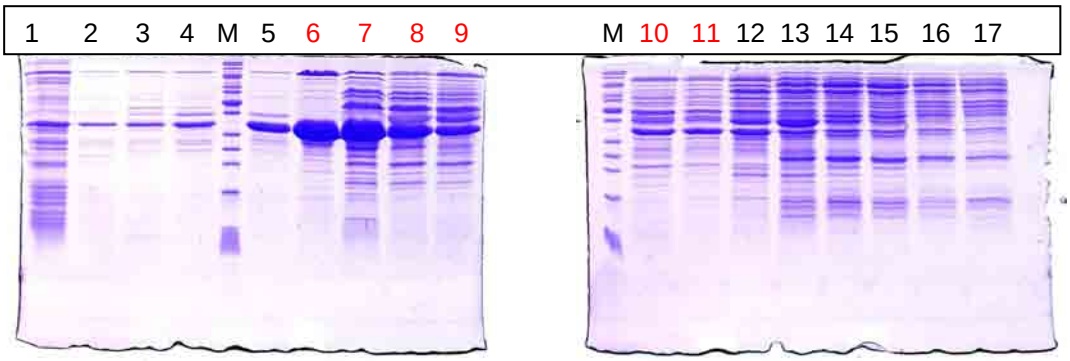

Figure 4.22: SDS-PAGE of SUIId2 cox1 after Q-Sepharose Fast Flow
Numbers 1-17 indicate the fractions collected during elution; fractions numbers pooled are coloured red; lane M: marker

4.3.3.2. Copper reconstitution

Recombinant SUIIj3 cox2 does not have copper in the active center as *E. coli* cells show a lack of the required chaperones. The reconstituted, oxidized CuA-domain is characterised by its purple colour and exhibits two strong absorbance maxima at about 480 nm and 535 nm, arising from the interaction of the two copper ions with each other and with the ligands.
To reconstitute the protein the above mentioned protocol, that combines unfolding by urea with ultrafiltration, was used.

4.3.3.2.1. Ultrafiltration with urea

The recorded UV-VIS spectrum (Figure 4.23) after concentration shows the typical absorbance maxima at about 480 nm and 535 nm but only 11%, as calculated in Chapter 4.4.2.3, of total SUIId2 cox1 was reconstituted.

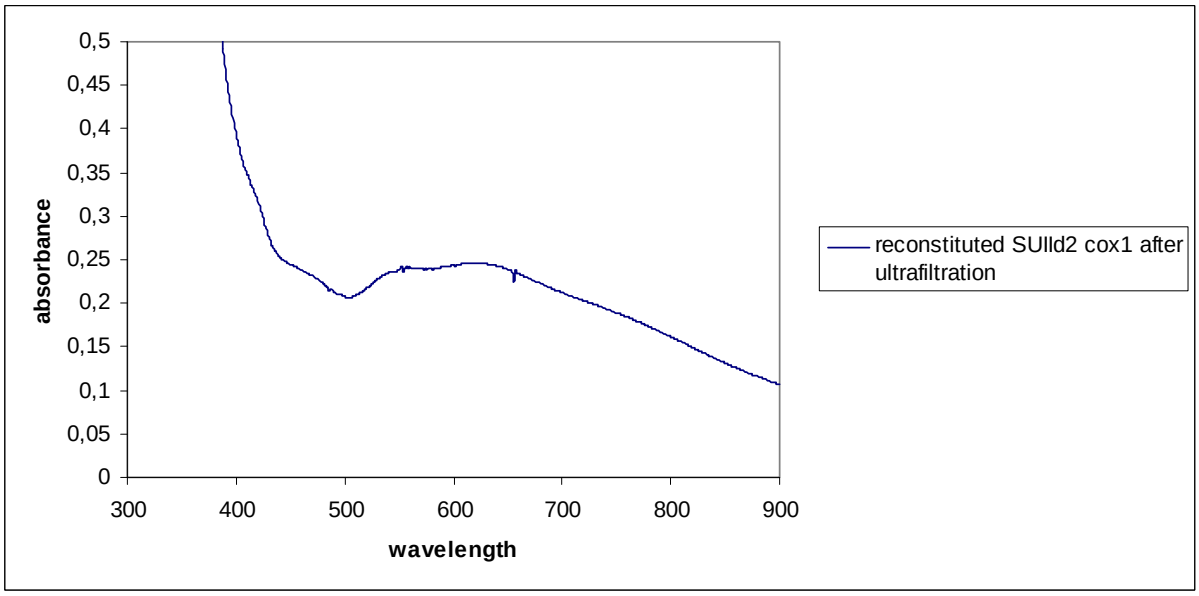

Figure 4.23: UV-VIS spectrum of the reconstituted CuA-domain of SUIId2 cox1 after ultrafiltration

The same stability problem as with SUIIj3 cox 2 was observed.

4.4. Spectrophotometric investigations

4.4.1. Spectra of the recombinant proteins

The reconstituted, oxidized CuA-domain exhibits two strong absorbance maxima at about 480 nm and 535 nm, arising from the interactions between the two copper ions with each other and with the ligands. A characteristic spectrum of the reconstituted SUIIj3 cox2 is illustrated Figure 4.24.

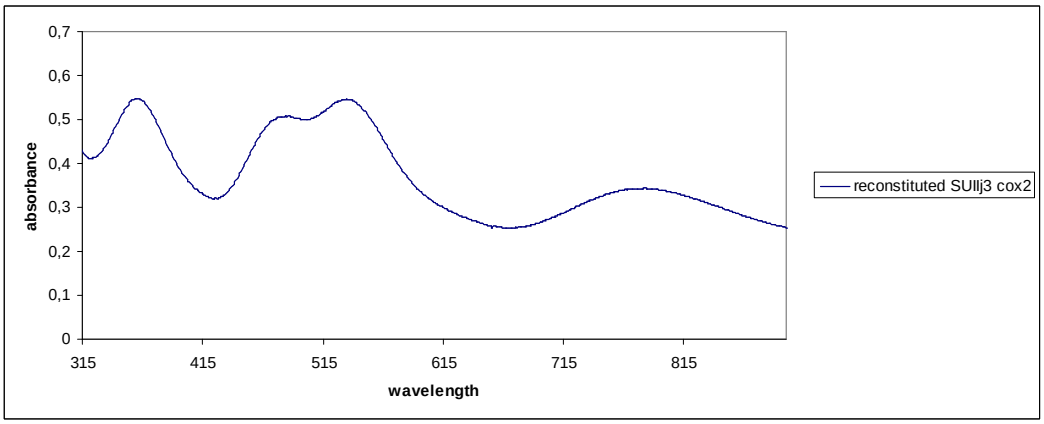

Figure 4.24: characteristic spectrum of reconstituted SUIIj3 cox2

4.4.2. Determination of protein concentration

4.4.2.1. Determination of SUIIj3 cox2 protein concentration

The protein concentration was calculated according to Lambert Beer's law as:

$$c = A / \varepsilon \times d$$

using ε_{280} = 21.0 mM^{-1} cm^{-1} for total protein concentration and ε_{480} = 2.8 mM^{-1} cm^{-1} or ε_{535} = 3.1 mM^{-1} cm^{-1} for the holoprotein concentration. Copper reconstitution efficiency was calculated as (mg total protein/mg holoprotein) x 100. The results of the copper reconstitution with ultrafiltration are given in Table 4.5 up to 4.7 with one calculation example above.

280 nm:

A = 0.4332

c = 0.4332 x 10 / 21000 $M^{-1}cm^{-}1$ x 1 cm = 2.06 x 10^{-4} mol/L

n = 0.2063 mM x 0.003 L x 20200 mg/mmol

m = 12.5 mg total protein

480 nm:

A = 0.2783

c = 0.2783 / 2820 $M^{-1}cm^{-1}$ x 1cm = 9.8 x 10^{-5} mol/L

n = 0.09869 mM x 0.003 L x 20200 mg/mmol

m = 5.98 mg holoprotein

535 nm:

A = 0.2978

c = 0.2978 / 3080 $M^{-1}cm^{-1}$ x 1 cm = 9.7 x 10^{-5} mol/L

n = 0.09669 mM x 0.003 L x 20200 mg/mmol

m = 5.85 mg holoprotein

copper reconstitution efficiency: 5.98/12.5 = 47.8%

wavelength [nm]	ε [mM^{-1} cm^{-1}]	concentration [mM]	total protein / L *E. coli* culture [mg]	copper reconstitution efficiency [%]
280	21.0	2.0	12.5	47.8
480	2.8	0.99	6.0	
535	3.1	0.97	5.8	

Table 4.5: Concentration and yield of the copper reconstitution of SUIIj3 cox2after purification and ultrafiltration

4.4.2.2. Determination of SUIIc3 cox2 protein concentration

wavelength [nm]	ε [mM^{-1} cm^{-1}]	concentration [mM]	total protein / L *E. coli* culture [mg]	copper reconstitution efficiency [%]
280	21.0	2.33	66	25.3
480	2.8	0.6	17	
535	3.1	0.58	17	

Table 4.6: Concentration and yield of the copper reconstitution of SUIIc3 cox2 after purification and ultrafiltration

4.4.2.3. Determination of SUIId2 cox1 protein concentration

wavelength [nm]	ε [mM^{-1} cm^{-1}]	concentration [mM]	total protein / L *E. coli* culture [mg]	copper reconstitution efficiency [%]
280	21.0	0.77	33.7	11.1
480	2.8	0.08	3.7	
535	3.1	0.07	3.6	

Table 4.7: Concentration and yield of the copper reconstitution of SUIId2 cox1 after purification and ultrafiltration

5. Discussion

5.1. Cloning of SUIIj3 cox2 and plastocyanin

The pET-3a plasmid carrying the cytochrome *c* oxidase 2 subunit II insert (amino acid residues 137-327) was successfully transformed into *E. coli* BL21(DE3)pLysS. The protein has the same cleavage site as SUIId2 cox1 (amino acid residues 113-355), comprising both the electron binding site and the electron entry site but without the two transmembrane helices at the N-terminus.
The 573 bp long PCR-product was digested with *BamH*I and *Nde*I, ligated with the pET-3a vector and transferred in *E. coli*. This expression system was already used by Judith Schachinger **[111]**. PCR-screening showed that clone j3 was carrying the insert at the right length. SDS-PAGE after expression screening confirmed that the clone was also expressing a good amount of soluble protein with 22.5 kDa molecular weight, which corresponds to the calculated molecular weight of SUIIj3 cox2. Sequencing of DNA purified from the transformed cells proofed that it contained no mutations that lead to differences in the proteins' amino acid sequence.

The electron carrier protein plastocyanin was also successfully cloned in *E. coli* BL21(DE3)pLysS using the pET-3a plasmid. The insert coding for plastocyanin was 315 bp long. After amplification by PCR, it was digested with *BamH*I and *Nde*I, ligated with the pET-3a vector and transferred in *E. coli* BL21(DE3)pLysS.
Two positive clones were found after PCR-screening, and both were expressing a protein with 11.1 kDa molecular weight. Spectra of the supernatant after cell lysis showed that clone c7 was expressing the highest amount of soluble protein. Sequencing of DNA confirmed finally that it contained no mutations that lead to differences in the proteins' amino acid sequence.

5.2. Expression and purification of SUIIj3 cox 2, SUIIc3 cox 2, SUIId2 cox 1

SUIIj3 cox2 and SUIIc3 cox2 cloned previously by Chantal Lucini were expressed in the *E. coli* strain BL21(DE3)pLysS using a protocol similar to that of a former expression of SUIIj3 cox2, but with a longer expression time (20 h) and at low temperature (16℃) to promote the correct folding of the protein. SUIId2 cox1 was successfully expressed with the same protocol at shorter expression time (4.5 h) and higher temperature (37℃)

Purification and copper reconstitution were established with SUIIj3. Purfification included two chromatographic steps, namely Q-Sepharose and Superdex^{TM} 75.

Between these two steps the reconstitution of SUIIj3 cox2 with copper was performed following two strategies. Reconstitution by dialysis with urea was successful but the amount of reconstituted SUIIj3 cox2 was only 20% of the total protein amount (an average of 10 mg per liter culture volume). However, SDS-PAGE showed that the protein solution is not pure.

Higher yields of purified, functional holoprotein were obtained using ultrafiltration. Up to 40 mg total protein could be obtained per liter culture volume with about 45% holoprotein of high purity. Compared to the method using dialysis the ultrafiltration provides a much more stable and reproducible protocol.

However, observed low protein stability did not allow kinetic measurements. It is not known yet, why the already incorporated copper was lost upon storage. One reason could be that the protein was still not properly folded and by this the copper was not properly coordinated to the active site, hence got lost within time.

As a conclusion it can be said that even if these results are better than those obtained with the standard protocol, they are far away to be statisfying because of the reconstitution efficiency and the stability of the achieved protein. Due to the low stability it was not possible to obtain a protein pool with desired concentration and pureness to move on to kinetic measurements.

6. References

[1] Bryant, D. A. (edt.) (1994) in The Molecular Biology of Cyanobacteria, Kluwer Academic Publishers, Dordrecht.

[2] Whitton, B. A.(edt.), Potts, M. (edt.) (2000) in The Ecology of Cyanobacteria, Kluwer Academic Publishers, Dordrecht.

[3] Peschek, G. A., Obinger, C., Paumann, M. (2004) Physiol. Plant. 120, 358-369.

[4] Paumann, M., Lubura, B., Regelsberger, G., Feichtinger, M., Köllensberger, G., Jakopitsch, C., Furtmüller, P.G., Peschek, G. A., Obinger, C. (2004) J. Biol. Chem.279, 10293-10303.

[5] Broda, E., Peschek, G. A. (1979) J. Theor. Biol. 81, 201-212.

[6] Raven, J. A., Allen, J. F., (2003) Genome Biol. 4: 209 – 217.

[7] Waterbury, J. B., Watson, S. W., Guillard, R., Brand, L. E. (1979) Nature 277, 293-294.

[8] Berg, J. M., Tymoczko, J. L., Streyer, L. (edt.), (2007) in Biochemistry, W.H. Freeman, New York, pp. 561-576 and 603-620.

[9] Paumann, M., Regelsberger, G., Obinger, C., Peschek, G. A. (2005) Biochim. Biophys. Acta 1707, 231-253.

[10] Mitchell, P.(1961) Nature 191, 144-148.

[11] Hill, R., Bendall, D. S. (1960) Nature 186, 136-137.

[12] Mitchell, P. (1961) Nature 191, 144-148.

[13] Vidal R, López-Maury L, Guerrero MG, Florencio FJ. J Bacteriol. 2009 Jul;191(13):4383-91. Epub 2009 May 1.

[14] Peschek, G. A. (1984) Subcell. Biochem. 10, 85-191.

[15] Hervas, M., Navarro, J. A., Diaz, A., Bottin, H., De la Rosa, M. A. (1995) Biochemistry 34, 11321-11326.

[16] Sandmann, G., Böger, P. (1980) Plant. Sci. Lett. 17, 417-424.

[17] Zhang, L., McSpadden, B., Pakrasi, H. B., Whitmarsh, J. (1992) J. Biol. Chem. 267, 19054-19059.

[18] Malakhov, M. P., Malakhova, O. A., Murata, N. (1999) FEBS Lett. 444, 281-284.

[19] Duran, R. V., Hervas, M., De la Rosa, M. A., Navarro, J. A. (2004) J. Biol.Chem. 279, 7229-7233.

[20] Pils, D., Schmetterer, G. (2001) FEMS Microbiol Lett. 203, 217-222.

[21] Navarro, J. A., Duràn, R. V., De la Rosa, M. A., Hervàs, M. (2005) FEBS Lett. 579, 3565-3568.
[22] Malakhov, M. P., Malakhova, O. A., Murata, N. (1999) FEBS Lett. 444, 281-284.
[23] Balme, A., Hervàs, M. Campos, L. A., Sancho, J., De la Rosa, M., Navarro, J. A. (2002) Photosyn. Res. 70, 281-289.
[24] Chitnis, P. R. (1996) Plant. Physiol. 111, 661-669.
[25] Paumann, M., Feichtinger, M., Bernroitner, M., Goldfuhs, J., Jakopitsch, C., Furtmüller, P.G., Regelsberger, G., Peschek, G. A., Obinger, C. (2004) FEBS Lett. 576, 101-106.
[26] Peschek, G. A., Zoderer, R. (2001) in: Algal Adaption to Environmental Stresses (Rai, L. C. and Gaur, J. P., Eds.), pp. 203-258, Springer,Berlin/Heidelberg/New York.
[27] Jones, L. W., Myers, J. (1963) Nature 199, 670-672.
[28] Jones, K. M., Buikema, W. J., Haselkorn, R. (2003) J. Bacteriol. 185, 2306-2314.
[29] Pils, D., Wilken, C., Valladares, A., Flores, E., Schmetterer, G. (2004) Biochim. Biophys. Acta 1659, 32-45.
[30] Ernst, A., Black, T., Cai, Y., Panoff, J. M., Tiwari, D. N., Wolk, C.P. (1992) J. Bacteriol. 174, 6025-6032.
[31] Mulligan, M. E., Haselkorn, R. (1989) J. Biol. Chem. 264, 19200-19207.
[32] Haselkorn, R. (1978) Annu. Rev. Plant Physiol. 29, 319-344.
[33] Kaneko, T., Nakamura, Y., Wolk, C. P., Kuritz, T., Sasamoto, S., Watanabe, A., Iriguchi, M., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kohara, M., Matsumoto, M., Matsuno, A., Muraki, A., Nakazaki, N., Shimpo, S., Sugimoto, M., Takazawa, M., Yamada, M., Yasuda, M., Tabata, S. (2001) DNA Research 8, 205-213.
[34] Klint, J., Rasmussen, U., Bergman, B., (2006) J. Plant. Physiol. 164, 11-18.
[35] Jones, K. M., Haselkorn, R. (2002) J. Bacteriol. 184, 2491-2499.
[36] Fay, P., Cox, R. M., (1967) Biochim. Biophys. Acta 143, 562-569.
[37] Fleming, H., Haselkorn R., (1973) Proc. Natl. Acad. Scid. 70, 2727-2731.
[38] Haystead, A., Robinson, R., Stewart, W. D. (1970) Arch. Mikrobiol. 74, 235-243.
[39] Adams, D. G. (2000) Curr. Opin. Microbiol. 3, 618-624.
[40] Murry, M. A., Wolk, C. P. (1989) Arch. Microbiol. 151, 469-474.
[41] Valladares, A., Herrero, A., Pils, D., Schmetterer, G., Flores, E. (2003) Mol. Microbiol. 47, 1239-1249.

[42] Bagchi, S. N., Ernst, E., Böger, P. (1991) Z. Naturforsch. 46c, 407-415.

[43] Ernst, A., Kirschenlohr, H., Diez, J., Böger P. (1984) Arch. Microbiol. 140, 120-125.

[44] Houchins, J. P., Hind, G. (1984) Plant Physiol. 76, 456-460.

[45] Reich, S., Böger, P. (1989) FEMS Microbiol. Lett. 58, 81-86.

[46] Walsby, A. E. (1985) Proc. R. Soc. Lond. B 226, 345-366.

[47] Hart, S. E., Schlarb-Ridley, B. G., Bendall, D. S., Howe, C. J. (2005) Biochem. Soc. Trans. 33, 832-835.

[48] Fay, P. (1992) Microbiol. Rev. 56, 340-373.

[49] Bryant, D. A. (edt.) (1994) in The Molecular Biology of Cyanobacteria, Kluwer Academic Publishers, Dordrecht.

[50] Ludwig, B., Bender, E., Arnold, S., Hüttemann, M., Icksoo, L., Kadenbach,B. (2001) Chembiochem 2, 392-403.

[51] Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621-628.

[52] Junge, W., Lill, H., Engelbrecht S. (1997) Trends Biochem. Sci. 22, 420-423.

[53] Stock, D., Leslie, A. G. W., Walker, J. E. (1999) Science 286, 1700-1705

[54] Garcia-Horsman, J. A., Barqueras, B., Rumbley, J., Ma, J., Gennis, R. B. (1994) J. Bacteriol. 176, 5587-5600.

[55] Gennis, R: B., Ferguson-Miller, S. (1996) Curr Biol. 6, 36-8.

[56] Haddock, B. A., Jones, C. W. (1977) Bacteriol. Rev. 41, 47-99.

[57] Kelly, M. J., Poole, R. K., Yates, M. G., Kennedy, C. (1990) J. Bacteriol. 172, 6010-6019.

[58] Wikström, M. (1977) Nature 266, 271-273.

[59] Paumann, M., Obinger, C., Peschek, G. A. (2005) Biochem. Biophys. Acta; 1707(2-3, 231-53

[60] Capaldi, R. A. (1990) Annu. Rev. Biochem. 59, 569-596.

[61] Kadenbach, B. (1983) Angew. Chem. 95, 273-281.

[62] Kadenbach, B. (1983) Angew. Chem. Int. Ed. Engl. 22, 275-282.

[63] Kadenbach, B., Stroh, A., Ungibauer, M., Kuhn-Nentwig, L., Büge, U., Jarausch, J. (1986) Methods Enzymol. 126, 32-45.

[64] Kadenbach, B., Kuhn-Nentwig, L., Büge, U. (1987) Curr. Top. Bioenerg. 15, 113-161.

[65] Geier, B. M., Schägger, H., Ortwein, C., Link, T. A., Hagen, W. R., Brandt, U., von Jagow, G. (1994) Eur. J. Biochem. 227, 296-302.
[66] Iwata, S., Ostermeier, C., Ludwig, B., Michel, H. (1995) Nature 376, 660-669.
[67] Kadenbach, B. (1986) Biochemistry 18, 39-54.
[68] Ostermeier, C., Iwata, S., Michel, H. (1996) Curr. Opin. Struct. Biol. 6, 460-[69] Malatesta, F., Nicoletti, F., Zickermann, V., Ludwig, B., Brunori, M. (1998) FEBS Lett. 434, 322-324.
[70] Brzezinski, P., Sundahl, M., Ädelroth, P., Wilson, M. T., El-Agez, B., Wittung, P., Malmström, B. G. (1995) Biophys. Chem. 54, 191-197.
[71] Pan, L. P., Hibdon, S., Liu, R.-Q., Durham, B., Millett, F. (1993) Biochemistry 32, 8492-8496.
[72] Ädelroth, P., Brzezinski, P., Malmström, B. G. (1995) Biochemistry 34, 2844-2849.
[73] Brzezinski, P., Malmström, B. G. (1987) Biochim. Biophys. Acta 894, 29-38.
[74] Boelens, R., Wever, R., van Gelder, B. F. (1982) Biochim. Biophys. Acta 682, 264-272.
75] Brzezinski, P. (1996) Biochemistry 35, 5611-5615.
[76] Verkhovsky, M. I., Morgan, J. E., Wikström, M. (1995) Biochemistry 34, 7483-7491.
[77] Saraste, M. (1990) Rev. Biophys. 23, 331-366.
78] Wilmanns, M., Lappalainen, P., Kelly, M., Sauer-Eriksson, E., Saraste, M. (1995) Biochemistry 92, 11955-11959.
[79] Malmström, B. G., Aasa, R. (1993) FEBS Lett. 325, 49-52.
[80] Hill, B. C. (1994) J. Biol. Chem. 269, 2419-2425.
[81] Lappalainen, P., Aasa, R., Malmström, B. G., Saraste, M. (1993) J. Biol. Chem. 268, 26416-26421.
[82] Abramson, J., Svensson-Ek, M., Byrne, B., Iwata, S. (2001) Biochim. Biophys. Acta 1544, 1-9.
[83] Kannt, A., Michel, H. (2001) in Handbook of Metalloproteins (Messerschidt, A. Huber, R., Poulos, T., Wieghardt, K., eds.), pp 331-347, John Wiley and sons, LTD.
[84] Pereira, M. M., Santana, M., Teixeira, M. (2001) Biochim. Biophys.Acta 1505, 185–208.
[85] Pereira, M. M., Gomes, C.M., Teixeira, M. (2002) FEBS Lett. 522, 14–18.

[86] Andrews, D., Mattatall, N. R., Arnold, D., Hill, B. C. (2005) Protein Expr. Purif. 42, 227-235.

[87] Adman, E. T. (1991) Adv. Protein Chem. 42, 145-197.

[88] Alge, D., Peschek, G.A. (1993) Biochem. Mol. Biol. Int. 29, 511 – 525.

[89] Slutter, C. E., Sanders, D., Wittung, P., Malmström, B. G., Aasa, R., Richards, J. H., Gray, H. B., Fee, J. A. (1996) Biochemistry 35, 3387-3395.

[90] Salgado, J., Warmerdam, G., Bubacco, L., Canters, G. W. (1998) Biochemistry 37, 7278-7289.

[91] Beinert, H. (1997) Eur. J. Biochem. 245, 521-532.

[92] Robin, M. B., Day, P. (1967) Adv. Inorg. Chem. Radiochem. 209, 247-422.

[93] Fee, J. A., Sanders, D., Slutter, C. E., Doan, P. E., Aasa, R., Karpefors, M., Vänngård, T. (1995) Biochem. Biophys. Res. Commun. 212, 77-83.

[94] Molina-Heredia FP, Hervás M, Navarro JA, De la Rosa MA. Biochem.. Biophys Res Commun. 1998 Feb 4;243(1):302-6.

[95] Schmetterer, G., Valladares, A., Pils, D., Steinbach, S., Pacher, M., Muro-Pastor, A. M., Flores, E., Herrero, A. (2001) J. Bacteriol. 183, 6429-6434.

[96] Hervas, M., Navarro, J. A., De la Rosa, M. A. (2003) Accounts Chem. Res. 36,798-805.

[97] Moser, D., Nicholls, P., Wastyn, M., Peschek, G. A. (1991) Biochemistry International 24, 757-768.

[98] Lockau, W. (1981) Arch. Microbiol. 128, 336-340.

[99] Manna, P., Vermaas, W. (1997) Plant Molecular Biology 35, 407-416.

[100] Zhang, L., McSpadden, B., Pakrasi, H. B., Whitmarsh, J. (1992) J. Biol. Chem. 267, 19054-19059.

[101] Malakhov, M.P., Malakhova, O.A. Murata, N. (1999) FEBS Lett. 444, 281- 284.

[102] Wastl, J., Bendall, D.S. Howe, C.J. (2002) Trends in Plant Science 7, 244-245.

[103] Molina-Heredia, F. P., Wastl, J., Navarro, J. A., Bendall, D. S., Hervas, M., Howe, C. J. De la Rosa, M. A. (2003) Nature 424, 33-34.

[104] Schmetterer, G, Pils, D. in Respiration in Archea and Bacteria, pp. 262-276, Springer Verlag Berlin, Heidelberg, New York.

[105] Reuter, W., Wiegand, G., (2001) Cytochrome c_6 . In Handbook of Metalloproteins (Messerschidt, A., Huber, R., Poulos, T., Wieghardt. K., eds), pp 87-99, John Wiley and sons, LTD.

[106] Freeman, H., Guss, J. M. (2001) Plastocyanin. In Handbook of Metalloproteins (Messerschidt, A., Huber, R., Poulos, T., Wieghardt. K., eds), pp 1153-1169, John Wiley and sons, LTD.

[107] Díaz, A., Navarro, F., Hervás, M., Navarro, J. A. De la Rosa, M. A. (1998) Biochem. Biophys. Res. Comm. 243, 302-306.

[108] Paumann M., Bernroitner M., Lubura B., Peer M., Jakopitsch C., Furtmuller P. G., Peschek G. A., Obinger C. (2004) FEMS Microbiol Lett. 239, 301-307.

[109] Daniela Tangl, Cyanobacterial Cytochrome c_M (Diploma thesis)

[110] Pils, D., Wilken, C., Valladares, A., Flores, E., Schmetterer, G. (2004) Biochim. Biophys. Acta 1659, 32-45.

[111] Judith Schachinger, Copper A Domain in Cytochrome C Oxidases of Cyanobacterium Nostoc Sp. PCC 7120 (Diploma thesis)

[112] Doris Gusenbauer, Diploma thesis

[113] Chantal Lucini, Diploma Thesis