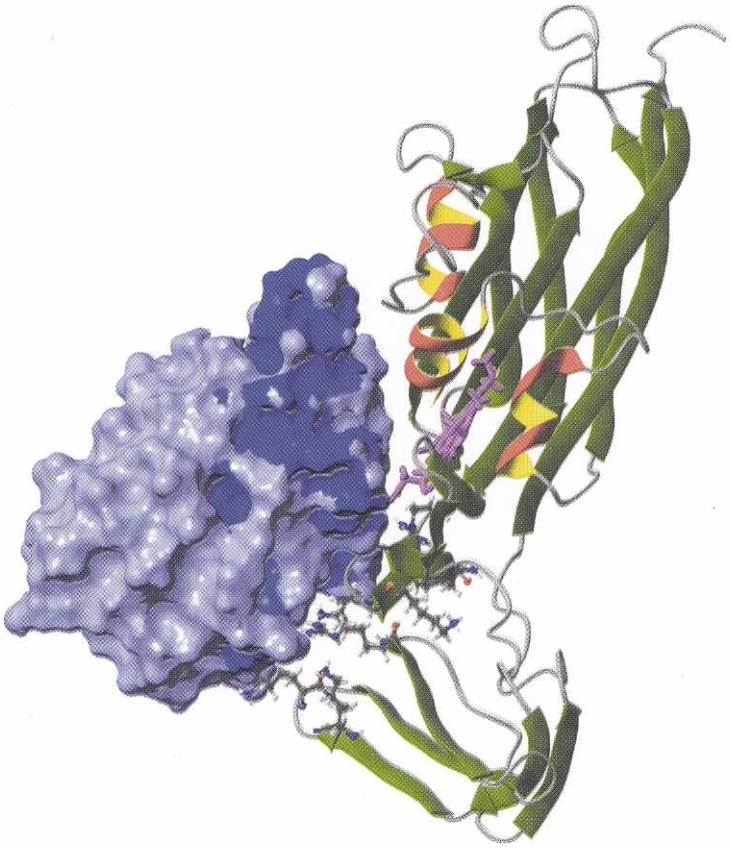
Studies on spinach plastocyanin and mutants

Expression in Escherichia coli, folding and function



Mikael Ejdebäck

Department of Chemistry
Biochemistry and Biophysics
Göteborg University
Göteborg 1999

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Expression in Escherichia coli, folding and function

Mikael Ejdebäck

Institutionen för Kemi
Biokemi och Biofysik
Göteborgs Universitet
405 30 Göteborg

AKADEMISK AVHANDLING

för filosofie doktorsexamen i kemi som med medgivande av Institutionen för kemi vid Göteborgs Universitet kommer att försvaras offentligt
fredagen den 21:e maj 1999 klockan 10.15
i föreläsningssal K2320, Medicinaregatan 9B, Göteborg.

Fakultetsopponent: Professor Miguel A. De la Rosa, Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla y CSIC, Sevilla, Spanien.

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Göteborg 1999

Dissertation abstract

Photosynthesis is a process in which photons from sunlight excite chlorophylls in the thylakoid membranes of plants, algae and cyanobacteria. The photo-oxidised reaction centre chlorophyll P700 is re-reduced by an electron transferred from the soluble, blue copper protein plastocyanin. The oxidised plastocyanin dissociates and binds to the cytochrome b_6f complex, where it accepts an electron and a new redox cycle can begin. Plastocyanin has three regions of importance for the interaction with its redox partners, a hydrophobic patch and two acidic (negatively charged) patches. Electrostatic interactions between opposite charges are important for the association and the specificity and stability of the formed complexes.

In this work the interactions with photosystem 1 and cytochrome *c* have been studied using mutants of plastocyanin. The mutations introduced in the small acidic patch and position 88 of plastocyanin had small effects on the binding to photosystem 1 as compared to the weak binding reported for mutants in the large acidic patch. The affinity was increased by the Glu60Gln, Glu60Lys and Asp61Lys mutations and a more efficient electron transfer was observed for the Gln88Lys mutation. The association between Pc mutated in the small acidic patch and cytochrome *c* was weakened and the rearrangement hindered by lysines in positions 59 and 60.

The development of an efficient expression system for spinach plastocyanin in the bacterium *Escherichia coli* made it possible to produce sufficient amounts of isotopically labelled plastocyanin for NMR experiments. This technique was used for solving the structure of the complex between plastocyanin and cytochrome *f*. The hydrophobic patch on plastocyanin binds to an area close to the heme on cytochrome *f*. Electrostatic interactions between opposite charges on the two proteins are also important. The short distance from the heme to the copper ligand His87 suggests an electron transfer from the heme via Tyr1 or Phe4 on cytochrome *f*.

The involvement of specific amino-acid residues in copper binding or folding of plastocyanin has also been examined by site-directed mutagenesis. The copper-binding histidines have been replaced by other amino acids, but no blue protein could be produced. The stability of the different redox forms of copper plastocyanin as well as the zinc protein has also been determined by guanidinium-induced unfolding.

Keywords: plastocyanin, electron transfer, copper, protein-protein interactions, protein expression, folding, site-directed mutagenesis, NMR

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Cover: The structure of the complex between spinach plastocyanin (blue) and turnip cytochrome f (green) as determined by NMR (papers II and IV). The backbone of residues on Pc that show chemical-shift changes upon complex formation is indicated in a darker blue. Positively charged residues on cytochrome f that are proposed to take part in the interaction with plastocyanin are shown as ball-and-stick, while the heme group is coloured lilac. The illustration was made using the MolMol software [1].

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Göteborg 1999

To my mother and father

and to María

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List of original publications

This thesis is based on the following publications, which will be referred to by their Roman numerals:

I Mikael Ejdebäck, Simon Young, Anita Samuelsson and B. Göran Karlsson: Effects of codon usage and vector-host combinations on the expression of spinach plastocyanin in *Escherichia coli.*
(1997) *Protein expression and purification* **11**, 17–25.

II Marcellus Ubbink, Mikael Ejdebäck, B. Göran Karlsson and Derek S. Bendall: The structure of the complex of plastocyanin and cytochrome *f* determined by paramagnetic NMR and restrained rigid-body molecular dynamics.
(1998) *Structure* **6**, 323–335.

III Maja M. Ivkovic–Jensen, G. Matthias Ullmann, Simon Young, Örjan Hansson, Milan M. Crnogorac, Mikael Ejdebäck and Nenad M Kostic: Effects of single and double mutations in plastocyanin on the rate constant and activation parameters for the rearrangement gating the electron-transfer reaction between the triplet state of zinc cytochrome c and cupriplastocyanin.
(1998) *Biochemistry* **37**, 9557–9569.

IV Mikael Ejdebäck, Anders Bergkvist, B. Göran Karlsson and Marcellus Ubbink: Side-chain interactions in the plastocyanin-cytochrome *f* complex as determined by 1H–NMR chemical shift analysis.
Manuscript.

V Kenneth Olesen, Mikael Ejdebäck, Milan M. Crnogorac, Nenad M. Kostic and Örjan Hansson: Electron transfer to photosystem 1 from spinach plastocyanin mutated in the small acidic patch: Dependence on magnesium ion concentration and comparison of mechanistic models.
(1999) *Biochemistry, submitted.*

Abbreviations and nomenclature

CD	Circular dichroism
Cyt	Cytochrome
E. coli	*Escherichia coli*
EPR	Electron paramagnetic resonance
GuHCl	Guanidine hydrochloride
IPTG	Isopropyl–β–D–thiogalactopyranoside
Pc	Plastocyanin
PCR	Polymerase chain reaction
PPI	Peptidyl prolyl isomerase
SEHC	Small exterior hydrophobic cluster

Mutant names:

Example: His87Gln denotes a substitution of the histidine in position 87 for a glutamine. Plastocyanin residues are numbered according to the processed spinach plastocyanin sequence. Amino acids are generally abbreviated according to their three-letter codes:

Amino acid	Three-letter code	One-letter code	Amino acid	Three-letter code	One-letter code
Alanine	Ala	A	Lysine	Lys	K
Aspartate	Asp	D	Leucine	Leu	L
Asparagine	Asn	N	Methionine	Met	M
Arginine	Arg	R	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamate	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

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Chapter 1. Introduction

A process crucial for all organisms is the acquisition, conversion and conservation of energy that occurs by photosynthesis or respiration. Plants, algae and some bacteria take energy from the sunlight, convert it and store it in chemical compounds by photosynthesis. This energy can be metabolised by the organism itself or by others, to power anabolic processes, transport of substances over membranes or organism movement. Energy is converted in the electron-transfer chain to a proton gradient and reducing power. This conversion is done in a series of oxidation-reduction reactions where an electron is transferred through a chain of redox factors in membrane-bound protein complexes connected by soluble redox proteins. Plastocyanin (Pc) is one of the soluble redox proteins in the photosynthetic electron-transfer chain and its function has been studied in detail in this work. Knowledge about protein-protein interactions and the structure of protein complexes is important for the understanding of how proteins function. A combination of molecular biological, biochemical and biophysical techniques has been used to investigate these interactions. The studies can be divided into three different areas.

- Improved procedures for production and purification of the recombinant spinach protein and mutants expressed in *Escherichia coli (E. coli)*. This has resulted in the possibility to produce larger amounts of unlabelled mutant proteins for functional studies and isotopically labelled protein for nuclear magnetic resonance (NMR) studies.
- Folding and stability studies of the two redox forms of the copper protein, the inert zinc-derivative and some mutants. The large variation in expression levels observed for mutants of spinach Pc can be explained in terms of changes in protein stability upon mutation.
- Interaction of wild type Pc and mutants with the physiological redox partners cytochrome (cyt) *f* and photosystem 1 as well as the non-physiological partner cyt *c*. The results from these studies support the hypothesis that both hydrophobic and electrostatic interactions are important in these interactions. A special role for residue Glu60 in the interaction with photosystem 1 is also proposed.

Chapter 2. Photosynthesis

Photosynthesis is a process in which electrons from chlorophyll molecules, excited by photons from light, are passed through a series of proteins and small organic carriers. This process takes place in the thylakoid membrane of cyanobacteria, algae and higher plants. The photosynthetic apparatus consists of three major components: light harvesting antennas, reaction centres and an electron-transfer chain. In the latter, photosystems 1 and 2 and the cyt b_6f complex are connected in series by the soluble electron carriers plastoquinone and Pc as shown in figure 1. Water is used as an electron donor to photosystem 2 whereby molecular oxygen is formed.

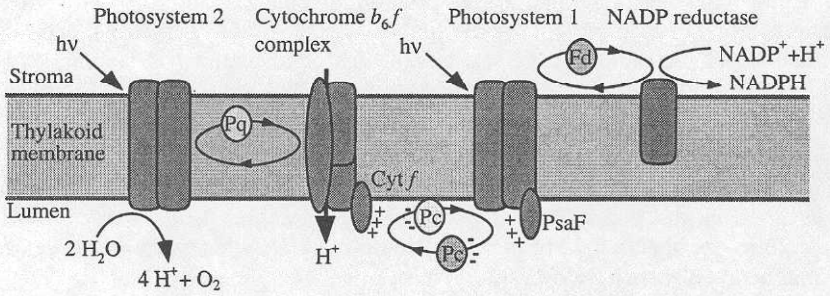

Figure 1. A schematic picture of the components of the photosynthetic electron-transfer chain. Ferredoxin and plastoquinone are abbreviated as Fd and Pq respectively. Not all subunits of photosystems 1 and 2 are shown.

The antennas consist of membrane-bound proteins with a large number of associated chlorophyll molecules, which absorb photons and transfer the excitation energy to a reaction centre. In the reaction centre, a chlorophyll is excited and an electron is passed on to the electron-transfer chain. As the electron is passed through the electron-transfer chain, protons are pumped across the membrane creating a proton gradient that can be used to generate ATP.

Some components of the electron-transfer chain are of special interest for this thesis because of their interaction with Pc. These are the cyt b_6f complex and photosystem 1.

2.1 Photosystem 1

The three–dimensional structure of photosystem 1 from the cyanobacterium *Synechococcus elongatus* has been determined by X–ray crystallography at a resolution of 4 Å [2]. Photosystem 1 from this organism is a complex of 11 subunits denoted PsaA to PsaN and a total of 34 transmembrane helices and nine surface α–helices have been identified [2, 3]. The PsaA and PsaB subunits form

the heterodimeric core of photosystem 1 and they contain most of the antenna chlorophylls, transmembrane helices and redox co–factors. The reaction-centre P700 is a chlorophyll dimer located between PsaA and PsaB at the lumenal side of the membrane. The electron-transfer reactions within photosystem 1 have recently been reviewed [3]. P700 is excited by light to $P700^*$ followed by a charge separation to $P700^+$ and the reduced form of the electron acceptor A_0 (a chlorophyll *a* molecule). The electron is then transferred through a series of electron carriers: A_1 (phylloquinone), the iron-sulphur centres F_X, F_A and F_B and leaves the photosystem when it is donated to a soluble ferredoxin (or flavodoxin). The electron is transferred to ferredoxin-NADP reductase, which reduces $NADP^+$. An electron transferred from Pc finally re-reduces the $P700^+$. The PsaA and PsaB subunits contain a surface α–helix each and it has been suggested that Pc docks with its hydrophobic patch to a shallow hydrophobic pocket created by the helices, the ends of transmembrane helices and neighbouring loops [4].

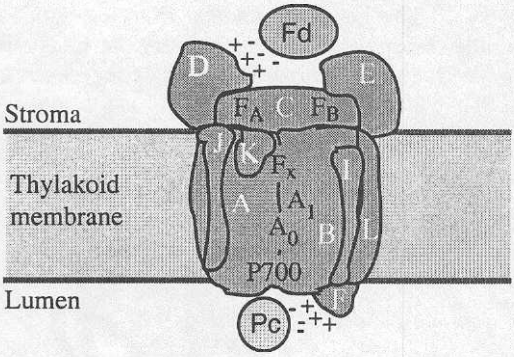

Figure 2. Schematic drawing of photosystem 1 based on [5]. Only subunits present in both eukaryotes and cyanobacteria are illustrated. The names of the subunits are written in white text. Ferredoxin is abbreviated as Fd.

One additional subunit of 15 kDa, PsaF, has been suggested to be important for the interaction between photosystem 1 and Pc in algae and plants [6, 7]. A prolonged lysine-containing N–terminal helix on PsaF makes the interaction with Pc possible. In this amphipathic helix, six conserved lysines are aligned on one side. Site-directed mutagenesis of lysines on PsaF or negatively charged residues on Pc followed by cross-linking and/or kinetic studies have shown a plausible interaction between these positive residues and negative residues on Pc [7-10]. Removal of the PsaF subunit abolishes the electron transfer from Pc to photosystem 1 in plants, severely reduces it in green algae but has no effect on the electron transfer in cyanobacteria [11-13].

The interaction between Pc and photosystem 1 may have developed from a simple collisional mechanism in most cyanobacteria, to a formation of a transient complex in algae and a process that involves both a formation of a transient complex as well as a rearrangement before electron transfer in plants [14]. It has been proposed that the interaction between charged residues on PsaF and Pc has evolved to allow a fast electron transfer to photosystem 1 of higher plants and algae by orienting the Pc into a tight docking complex [15].

2.2 The cytochrome b_6f complex

The cyt b_6f complex is the thylakoid counterpart to the mitochondrial and bacterial cyt bc_1 complexes. It is a dimeric complex of 210 kDa with 22–24 transmembrane helices [16]. The subunits are cyt *f*, cyt b_6, the Rieske iron-sulphur protein and subunit IV. Electrons from photosystem 2 are delivered by a mobile plastoquinol to the Rieske protein and cyt b_6, further on to the heme in cyt *f* and finally to Pc. The difference in reduction potential between the acceptor and donor in the complex is used for pumping two protons per electron across the membrane in the Q–cycle [17].

Cyt *f*, being the largest subunit (285 residues in turnip cyt *f*) in the cyt b_6f complex, is anchored to the membrane by a C–terminal helix. The crystal structure of the soluble part (252 residues) of turnip cyt *f* has been solved at 1.96 Å resolution and it is shown in figure 3 [18]. Cyt *f* differs from other *c*–type cytochromes in that it consists of two domains of predominantly β–strand secondary structure and in that the heme is axially ligated to the N–terminal α–amino group of Tyr1. The heme is shielded from the aqueous phase by Tyr1 and two other aromatic residues, which can explain the unusually high reduction potential of +365 mV. This tyrosine may be a part of the electron-transfer pathway to Pc [paper II].

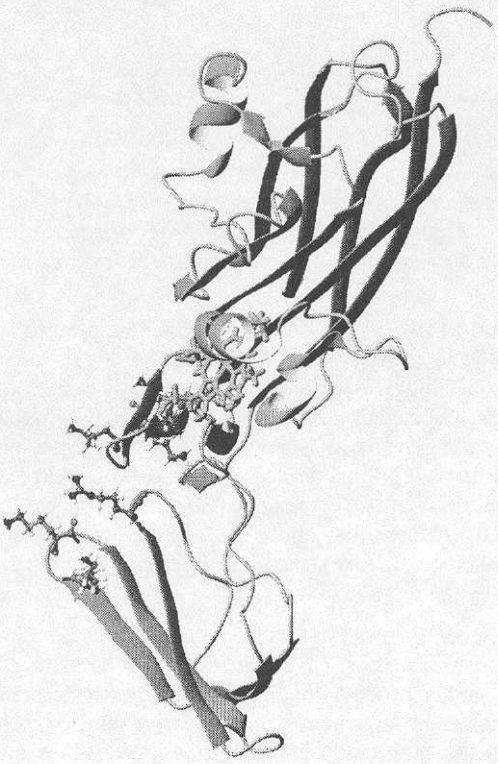

Figure 3. Picture of the soluble part of cyt f showing the positively charged residues and the heme group.

The total charge of cyt *f* from algae and higher plant is negative, but clustering of basic residues create regions of local positive charge. Lysine residues are conserved in positions 58, 65, 66 and 187 and so are the positive charges in positions 122, 185 and 209. Other factors that influence the local charge are the heme carboxylates, which are masked by positive charges in the neighbourhood at low ionic strength but becomes visible at higher. The redox state of the heme has only a small effect on the total charge. *[19]*

The interaction between cyt *f* and Pc has been studied by cross-linking, chemical modification, site-directed mutagenesis and NMR [8, 20-22][papers II and IV]. The ionic-strength dependence suggests that the complex formation is governed mainly by electrostatic interactions between positive residues on cyt *f* and negative residues on Pc. NMR data on the diprotein complex also show strong interactions between the heme area on cyt *f* and the hydrophobic patch on Pc [papers II and IV].

The positive patch on cyt *f* is missing or reversed in most cyanobacterial cyt *f* and so is the corresponding negative patch in Pc. Some of the positive charges on cyt *f* from algae are also missing. Mutations of the remaining positive charges on cyt *f* from the alga *Chlamydomonas reinhardtii* had only a small effect on the electron transfer to Pc and cyt c_6 *in vivo*, but a larger effect *in vitro* [23, 24].

A linear internal water chain has been found in cyt *f* and proposed to function as a proton wire [18]. In a study where residues that provided hydrogen bonds to this water chain were removed, wild type-like oxidation rates of cyt *f* were observed. The concerted reduction of cyt b_6 and cyt *f* was however lost and the reduction rate of cyt *f* retarded [25].

Chapter 3. Plastocyanin

Copper proteins can be divided into three classes with regard to their spectral characteristics. The different spectral properties are caused by variation in ligation and geometry of the copper site as is shown in figure 4. The blue type 1 copper proteins, all have an intense blue colour and they are represented by proteins such as Pc, azurin, pseudoazurin, amicyanin and stellacyanin. The type 2 copper proteins, such as superoxide dismutase and galactose oxidase, are non-blue but paramagnetic. The type 3 proteins have a pair of copper atoms and a strong absorbance at 330 nm but are usually neither blue nor paramagnetic. Examples from this family are tyrosinase and hemocyanin. Other copper proteins, binding more than one type of copper, are ascorbate oxidase, ceruloplasmin, nitrite reductase, laccase and cyt oxidase.

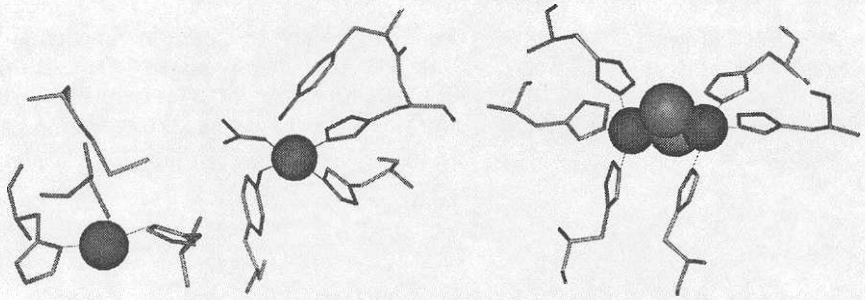

Figure 4. Shown from left to right is the type 1 copper site of Pc [26], the type 2 site of galactose oxidase [27] and the oxygenated type 3 site of hemocyanin [28].

3.1 Evolution

Evolution acts on the DNA level, which leads to a gradual change in the amino-acid sequence of proteins. While structurally and/or functionally important amino acids are conserved, others are more readily changed, sometimes resulting in improved properties or acquisition of new functions. By comparing the conservation pattern of amino-acid sequences or the three-dimensional structures of related proteins, important residues can be identified and the evolutionary history deduced.

3.1.1 The evolution of copper proteins

The first photosynthetic organisms that developed about three billion years ago were anoxygenic bacteria that used inorganic or organic compounds as electron donors to the electron-transfer chain. Later, oxygenic photosynthetic bacteria (cyanobacteria) that could use water as the electron donor developed. Molecular oxygen was formed and the oxygen content in the atmosphere rose. When the oxygen content was high enough to allow Cu(II) to be solubilised, copper proteins

developed and among them Pc [29, 30]. A chloroplast was formed by the engulfment of a photosynthetic bacterium, probably a cyanobacterium, and the first photosynthetic eukaryotic cells developed [31]. In some organisms, Pc replaced cyt c_6 as the electron donor to photosystem 1 whereas both are still used in others (see 3.1.2). Nowadays, copper is bound in a large number of proteins with diverse functions and copper proteins can be found in most organism groups: prokaryotes, fungi, algae, plants and animals.

It has been suggested that all copper proteins have a common origin and that Pc diverged from azurin upon the appearance of the oxygenic cyanobacteria. By reduction of the loop between β–strands 4 and 5, the pseudoazurin, amicyanin and Pc diverged. A subsequent addition of a disulphide bridge separated the phytocyanin sub-family containing stellacyanins, plantacyanins and uclacyanins from the other copper proteins [29]. The three domains of oxidases have been created by duplication of a small blue protein ancestor, and they have sometimes lost copper binding sites or formed new sites at the domain interfaces [29]. This has resulted in new properties of these multidomain proteins such as the capacity to reduce oxygen to water. Examples of proteins that have subunits with the cupredoxin fold are cyt oxidase and ceruloplasmin. Shown below in figure 5 is a similarity tree of the copper-binding loop sequences of some representative groups of type 1 copper proteins.

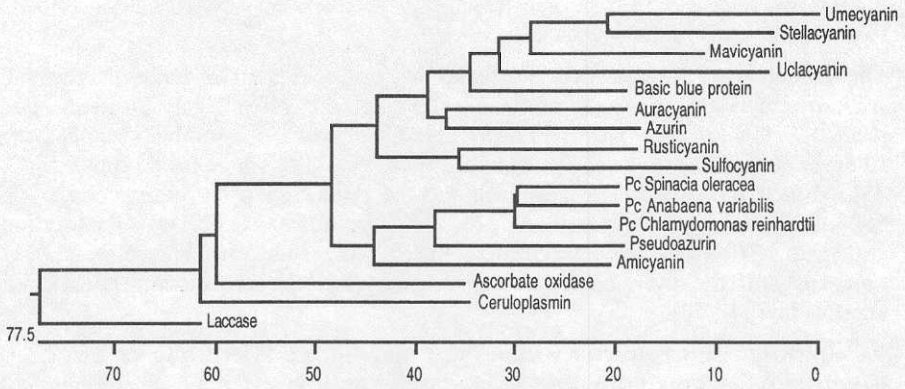

Figure 5. Similarity tree of the copper-binding loop. The sequence range was from Tyr80 to Gly94 (spinach Pc numbering). The scale shows the number of amino acid replacements that have occurred.

3.1.2 Cytochrome c_6 - an example of a convergent co-evolution

Pc and cyt c_6 have many properties in common; both are soluble, redox active metalloproteins that act as mobile electron carriers between the cyt b_6f complex and photosystem 1. Pc is synthesised in higher plants, whereas cyt c_6 is the only donor to photosystem 1 in some cyanobacteria. Both are synthesised by many algae and some cyanobacteria in a process regulated by the availability of copper [32-36]. Evidence for a competition for copper between Pc and a copper-binding cyt c_6–transcription factor has been published [37]. In the green alga *Chlamydomonas reinhardtii* the level of Pc is probably regulated post-translationally by the stability of the apoprotein [37].

Since the two proteins have the same physiological function a similar structure might be expected. However Pc is a β–sheet copper protein and cyt c_6 is a four α–helix heme protein. Despite this, the size and the reduction potential is similar. The isoelectric point and charge distribution on cyt c_6 and Pc isolated from the same organism also correspond well [38, 39]. The heme group of cyt c_6 and the copper ligand His87 of Pc are both surrounded by hydrophobic residues in the proposed area for electron transfer to photosystem 1. The negative charges on cyt c_6 are conserved in algae but not in cyanobacteria. Studies on the interaction between Pc and cyt c_6 with photosystem 1 show that both interact with the same kinetic mechanism in *Synechocystis* and algae [14]. Analysis of kinetic data from these studies suggests that the charge interaction first developed for cyt c_6 and that Pc later replaced cyt c_6 in plants and some algae [14, 38].

3.2 Structure & function of plastocyanin

An illustration of the conservation pattern in 24 plant, six algal and seven cyanobacterial Pc sequences are shown in figure 6. In plant Pc 45 % of the amino acids are identical and 63 % are homologous. If one also includes sequences from algae these values are 36 and 49 % respectively. The absence of acidic patches in cyanobacteria makes the homology to eukaryotic Pc low, only 19 % of the residues are identical and 33 % homologous. The most conserved residues are the copper ligands and some glycines and prolines. Glycines and prolines have the highest and lowest conformational freedom respectively and their function is often to define the ends of the β–strands.

The conservation pattern of the Pc sequences proposes similar three-dimensional structures. Crystal structures of Pc from poplar, the green algae *Enteromorpha prolifera, Chlamydomonas reinhardti* and *Ulva pertusa* and the cyanobacteria *Phormidium laminosum* and *Synechococcus* sp. PCC 7942 have been reported [40-45]. Mutant forms of Pc from spinach and the cyanobacterium *Synechocystis* spp 6803 have also been determined [26, 46]. In addition, the NMR solution structures of Pc from spinach, parsley, french bean, the green alga *Scenedesmus obliquus* and the cyanobacterium *Anabaena variabilis* are known [Bergkvist, unpublished][47-50].

A comparison of the structures shows that they all are folded into an elongated β–sandwich that consists of eight strands, which are connected by seven loops. All strands but one are contiguous β–strands whereas the remaining one also includes a short α–helix. The charge distribution on the Pc surface is asymmetric. Seven of the thirteen negative residues are located in the acidic patches on the "east" side of the molecule while positive residues dominate the opposite side. Nine surface-exposed hydrophobic residues at the "north" end create a hydrophobic patch. The hydrophobic interior between the two β–sheets contains seven phenylalanines and a copper ion bound in a distorted tetrahedral conformation by the copper ligands His37, His87, Cys84 and more weakly by Met92.

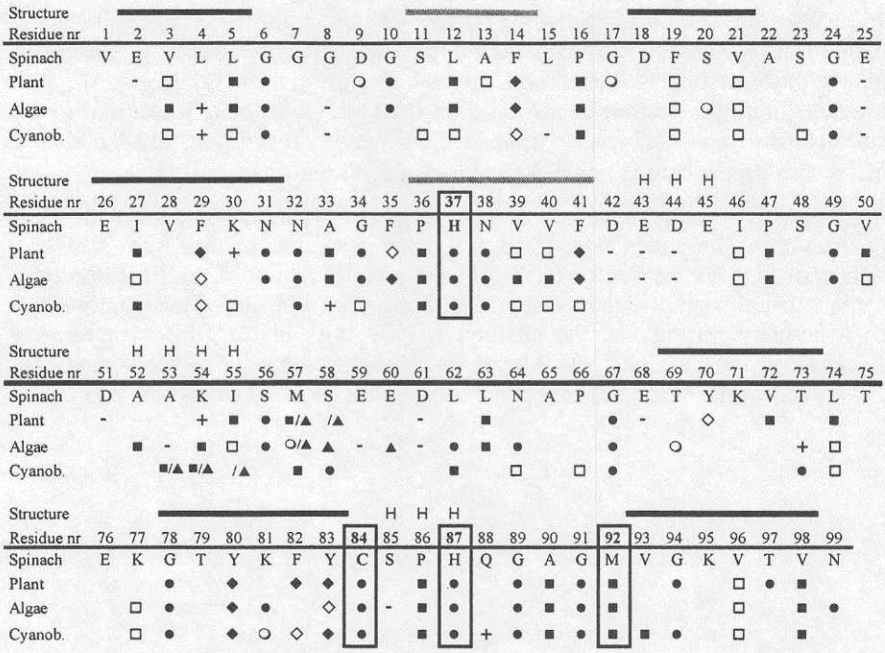

Figure 6. Illustration of Pc sequences aligned to the spinach Pc sequence. The β–strands from the crystal structure of spinach Pc are shown as black lines above the sequence and the α–helices with an H. Shown as grey lines are the two additional β–strands in the poplar and parsley structures. The conservation within each organism group is indicated below the sequence with the copper ligands boxed. Conserved residues are indicated with filled symbols and homologous conservation with open symbols:

●/○ *uncharged, polar* ■/□ *non-polar* ◆/◇ *aromatic residue*
-/+ *negative / positive charge* ▲ *deletion*

3.2.1 The copper site

All cupredoxins have a copper ion situated in, or close to, a plane created by the side chains of a cysteine and two histidines. The distance from the copper to the plane, the identity of the axial ligands as well as the bond lengths vary and so do also the properties of the copper site. Depending on the oxidation state of the copper, different co-ordination geometries are preferred. The cuprous ion, Cu(I), normally prefers a tetrahedral co-ordination whereas cupric copper, Cu(II), prefers a tetragonal geometry. The distorted tetrahedral geometry seen in the proteins is a compromise that makes interconversion easier [51]. This reduces the reorganisation energy and a higher electron-transfer rate can be achieved [52]. Removal or substitution of copper with other metals has only small effects on the overall structure nor does the oxidation state influence the structure to any large extent [53]. The copper site adapts to the changes by adjusting the ligand geometry to the size and charge of the metal ion [54].

The co-ordination of copper in cupredoxins gives them three characteristic features; an intense blue colour, a narrow hyperfine splitting in the EPR spectrum and a high reduction potential as compared to free copper. The blue colour is due to the intense absorption, ε=4700 $M^{-1}cm^{-1}$ at 597 nm for spinach Pc [paper I]. This originates from an electron excitation from the cysteine sulphur pπ orbital to the partially filled copper $3d_{x2-y2}$ orbital [55]. A weaker absorption, attributed to a histidine to copper charge transfer, can be seen at 460 nm.

The properties of the unpaired copper 3*d*–electron can be studied with EPR spectroscopy. The interaction of this electron with the nuclear spin causes a characteristic hyperfine splitting ($A_{||}$) in the parallel region of the EPR spectrum that is unusually small due to electron delocalisation [55, 56]. The magnitude of the hyperfine splitting and the position ($g_{||}$) of this in the EPR spectrum are characteristic for the copper site. Mutations can induce changes in the copper site, which can be seen in the EPR spectrum (see figure 7, table 2 in section 5.5).

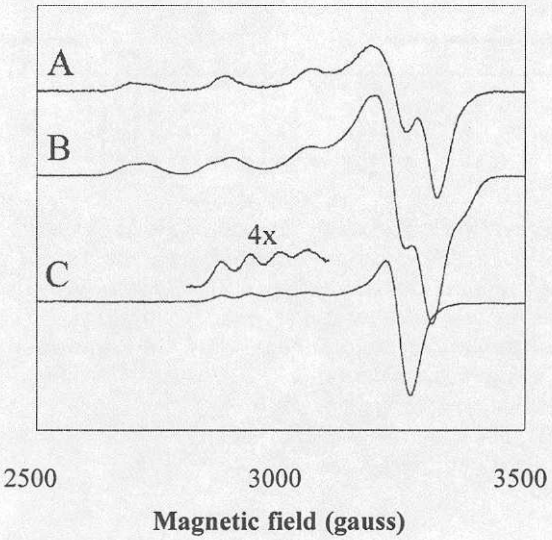

Figure 7. EPR spectra of the wild type Pc type 1 copper site (C) and the type 2 copper sites observed in the His87Gln (B) and His87Asp (A) mutants.

The reduction potential, $E°$, gives a measure of the electron affinity of a compound. The more positive it is, the higher is the affinity for an electron and the more stable is the reduced form of the compound. In electron-transfer chains, electrons are transferred from molecules with low $E°$ values to molecules with higher values. The larger the difference in $E°$ between electron donor and acceptor is, the larger is the driving force for electron transfer. In the cyt *f*–Pc–P700 chain, the reduction potentials for the unassociated proteins are +360, +384 and +490 mV, respectively [57, 58]. The reduction potentials can however change upon complex formation [59][paper V]. Differences in ligation, solvation and the hydrophobic environment around the copper site cause the reduction potentials to vary among the cupredoxins, from +187 mV in stellacyanin to +670 mV in rusticyanin [60]. A stronger axial ligation lowers the reduction potential while a

greater extent of hydrophobic encapsulation raises it [61]. The solvent exposed His87 becomes protonated at low pH (pK_a is 4.9 in spinach Pc) and it moves away from the copper, which becomes three-co-ordinated and the reduced form becomes stabilised [62]. This increases the reduction potential at low pH [60].

3.2.2 The acidic patch

The sequence homology between Pc from higher plants and algae is high while the homology to cyanobacterial Pc is considerably lower. The acidic (negative) residues in the large acidic patch (residues 42–45) are highly conserved among plants, however the residues in the small acidic patch (residues 59–61) are frequently substituted for the other negatively charged amino acid. In Pc from algae, residues 58 and 60 are deleted but negative residues in positions 53 and/or 85 could compensate for this. This deletion and compensation can also be seen in the parsley and barley sequences. No prominent acidic patch can be seen in the cyanobacterial sequences and the glutamate in position 60 is replaced by a positive lysine in *Synechocystis* and *Anabaena* Pc. The high variation of the charged residues in Pc from different organism groups makes the isoelectric point vary from acidic (about 4) in higher plants and algae to basic (about 9) in the cyanobacterium *Anabaena*.

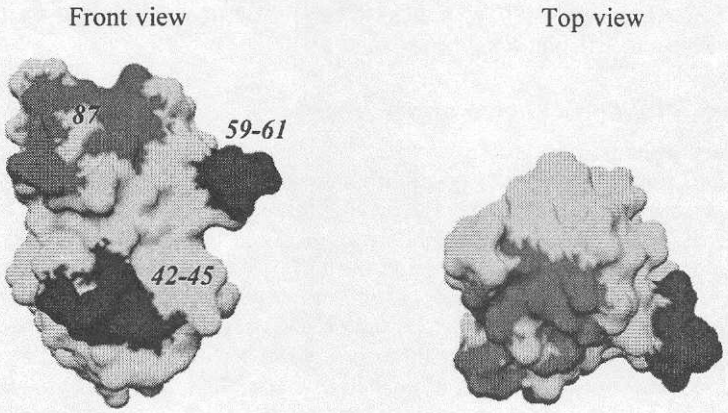

Figure 8. Surface pictures of Pc. The residues of the acidic patches are shown in dark grey and the residues belonging to the hydrophobic patch in a lighter grey.

Cross-linking, competitive inhibition by positively charged inorganic compounds as well as chemical modification and site-directed mutagenesis of the negative residues on Pc support an interaction between negative residues on Pc and positive residues on cyt *f* [21, 22, 63-65]. NMR studies of the complex between Pc and cyt *f* gives further support for such an interaction [papers II and IV]. Tyr83 is located between the acidic patches, and has also been suggested to be important for the interaction with cyt *f* [65, 66]. Studies on the reaction between Pc and photosystem 1 suggest a similar electrostatic interaction for this complex [6, 10, 67, 68][paper V].

3.2.3 The hydrophobic patch

While Pc and amicyanin are the only known cupredoxins that have an acidic patch that surrounds a potential electron-transfer site, all known structures of cupredoxins have a hydrophobic patch which surrounds the solvent-exposed histidine. A flat surface of about 550 $Å^2$ is created in Pc by highly conserved hydrophobic residues located in β–strand loops at the northern end of the molecule. These are residues Gly10, Leu12, Ala33, Gly34, Phe35, Pro36, Pro86, Gly89 and Ala90 of higher plants and algae. His87 is exposed at the centre of the hydrophobic patch and the copper is positioned about 6 Å beneath the hydrophobic surface.

The importance of the hydrophobic patch has been studied by site-directed mutagenesis. In our laboratory residues 12, 35, 36 and 90 have been mutated. The results show that a charged or bulky amino acid at position 12 severely affects the electron transfer to photosystem 1 in a pH–dependent manner while the binding but not the electron transfer to cyt *f* is affected [58, 65, 68]. Phe35 can be changed to a tyrosine without altering the kinetics to photosystem 1 and cyt *f* [65, 68]. Pro36 is one of the two *cis*–prolines in spinach Pc and replacing this residue with the highly flexible glycine distorts the copper site and lowers the reactivity with photosystem 1 [69]. Haehnel et al. have replaced Gly10 and Ala90 with the more bulky leucine and observed an impaired electron transfer to photosystem 1 [70]. However, we found an Ala90Trp mutant to be similar to wild type in its reaction with photosystem 1 [Ejdebäck, unpublished].

3.3 Protein folding - in vivo *and* in vitro

The tertiary structure of a protein is determined by the amino-acid sequence since spontaneous folding *in vitro* is possible for small, single domain proteins as has been shown by Anfinsen [71, 72]. The driving force for protein folding is believed to be the hydrophobic effect and maximisation of the number of hydrogen bonds [73]. The protein structure is stabilised by burial of hydrophobic residues in the core, thereby shielding them from contact with water, whereas polar residues are predominantly exposed at the surface. Levinthal proposed folding to be a non-random process since it would take billions of years for a protein of the size of Pc to fold if all conformations had to be probed, the so-called Levinthal-paradox [74]. Most proteins fold in seconds to minutes and to explain this several models have been proposed. Originally, folding was believed to occur through a particular route involving a number of subsequent steps and well-defined intermediates (the pathway models). The "new view" of protein folding is the funnel model [75]. Here, folding molecules follow a number of routes which all lead to the energy minimum. A popular explanation is that the protein molecules are like skiers on a downhill skiing slope. They start from different starting points (random coil conformations) but sooner or later they all end up at the bottom of the landscape (the energy minimum and native state). Some of them get trapped in a valley (a local minimum) and have to climb (partially unfold) to reach the native state. Others may follow a canyon (pathway) and can therefore easily reach their native conformation.

All known cupredoxins have the same fold, the immunoglobulin fold [76]. Two sheets of β–strands are folded into a β–sandwich with a Greek–key topology. This folding pattern is shared by a large number of proteins with diverse functions and no obvious amino-acid sequence homology. Despite this, the structure of the hydrophobic core is conserved, consisting of two pairs of sequential β–strands

connected by β–arches. The cupredoxin fold differs from the folding pattern of other Greek–key proteins in that β–strands one and three are not anti-parallel but form a parallel interconnection between the two β–sheets. One of the proposed mechanisms for folding of β–sheet proteins can be seen in figure 9 [77]. The central β–strands first form a long hairpin that folds over and additional inter-strand interactions are made creating the β–sandwich. Finally, peripheral strands insert into the structure. This sequential pathway model is not supported by the modern funnel models for protein folding, but is anyway illustrative.

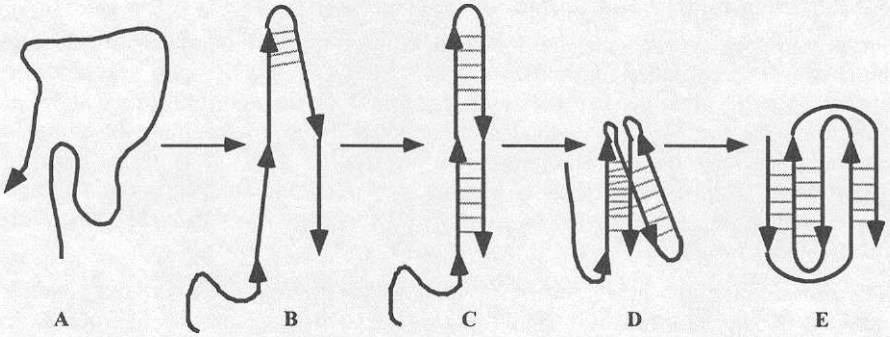

Figure 9. The folding of a β–sheet protein from its random coil conformation (A). First, the strand nucleates to form a β–hairpin (B) that propagates to an extensive secondary structure (C). A hydrophobic collapse follow and new interactions are formed (D). A flattened representation of the native Greek–key is also shown (E).

3.3.1 Protein folding in the cell

In vivo, small newly synthesised proteins spontaneously adopt their native conformation in a folding process that is often co-translational or co-translocational. Folding and assembly of larger and more complex proteins is often aided by chaperones or chaperonines as well as by folding catalysts. The periplasm of *E. coli* is an oxidising environment where disulphides can form in a process catalysed by the protein disulphide isomerases. Enzymes active in the isomerisation of prolines, the peptidyl-prolyl isomerases (PPI), have also been found here [78]. Proteins can be misfolded because of heat or other kinds of environmental stress. To handle this, the cells have developed a wide range of heat-shock proteins. These are chaperones or chaperonines that can stabilise unfolded protein, unfold misfolded proteins or prevent protein aggregation. Misfolded proteins are dangerous to the cell since they can form large aggregates by the interaction of their hydrophobic surfaces. Prion diseases like the mad-cow disease are caused by the aggregation of a misfolded form of a normal cellular protein into an extensive β–sheet structure [79, 80] and protein aggregation is also the cause of Alzheimer's disease [81, 82].

In eukaryotes, Pc is encoded in the nucleus and the mRNA is translated into the preapoprotein in the cytoplasm. To direct the protein into the correct compartment, the thylakoid lumen, the preapoprotein contains two signal sequences for translocation into the chloroplast and thylakoid lumen, respectively. The protein is folded in the thylakoid lumen and copper is incorporated.

Characteristic features of Pc that have implications for its folding are the presence of a redox active copper ion and two *cis*–prolines. The rate of proline isomerisation can be catalysed by a PPI recently discovered in the thylakoid lumen [83]. Copper incorporation is stimulated by the gene product Pcy2. The exact mechanism is not known but a copper transport or chaperone activity is suggested [84]. The incorporation of copper is important for maintaining high steady-state levels of Pc in the thylakoid lumen since a rapid degradation ($t_{1/2}$=16–18 min) of the apoprotein has been observed [85].

3.3.2 Protein folding and unfolding experiments

Protein folding *in vitro* can be followed with a number of spectroscopic and biochemical techniques. Circular dichroism (CD), NMR and fluorescence spectroscopy is often used in the folding studies. These methods probe different features of the protein. The secondary structure and the environment of aromatic amino acids (the tertiary structure) can be studied by CD and fluorescence spectroscopy respectively. NMR is a rather slow technique and it is mainly used in combination with deuterium-hydrogen exchange to provide structural information.

The most common way to unfold proteins is to destroy the hydrogen-bonding network by the addition of a chaotropic agent such as guanidine hydrochloride (GuHCl) or urea. Thermal denaturation can also be used, however this process is almost always irreversible. Folding and unfolding of small and globular proteins generally exhibit two-state kinetics, where the folded and unfolded states interconvert readily without observable intermediates. This co-operative process can be seen as a sigmoidal curve in a plot of degree of unfolding versus concentration of denaturing agent. The slope of this curve gives a measure of the degree of the exposure of hydrocarbons to the aqueous solvent and it is often proportional to the size of the protein. From the slope and the midpoint of the transition the stability of a protein can be calculated. A typical stability for a protein of the same size as Pc is –20 to –40 kJ/mol.

In this work, the unfolding of Pc has been studied by far–UV CD and NMR and the results have been interpreted according to a two-state model. The oxidised and the reduced copper proteins as well as the zinc protein were studied. The CD unfolding curves are shown in figure 10 and they show that the oxidised copper protein (–48 kJ/mol) is about twice as stable as the reduced protein (–20 kJ/mol) and the zinc protein (–19 kJ/mol) [Ejdebäck, unpublished]. The stability of the reduced protein increased to –39 kJ/mol, a value similar to the one reported for reduced azurin, upon correction for a linear phase (see below). NMR data on the zinc protein support the results from the CD measurements (not shown). The different stabilities can be explained by differences in the folded and unfolded states of the proteins. This is further complicated by the different co-ordination of the copper in the two redox states, which makes the entropic contribution to the free energy vary.

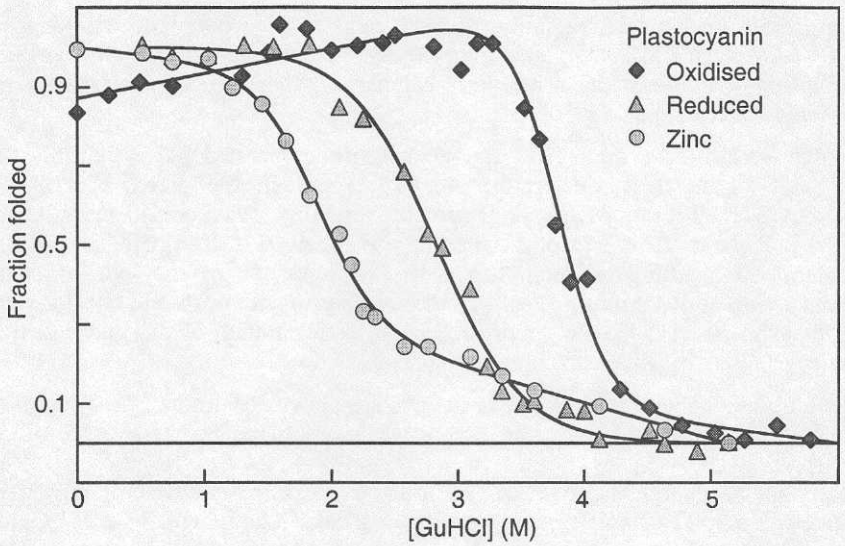

Figure 10. Normalised equilibrium unfolding curves of Cu(II), Cu(I) and Zn(II) Pc as measured by CD at 226 nm.

The unfolding of oxidised Pc is irreversible since the Cu(II) ion oxidises the thiol-group of the cysteine [86]. Refolding of the reduced protein can be studied, however the process is not fully reversible at longer denaturation times [Ejdebäck, unpublished]. One explanation may be that the copper in reduced Pc is more loosely bound and is easily lost in a time-dependent process upon denaturation. The resulting apoprotein has a lower stability than the reduced protein and continues to unfold during refolding, adding an additional phase to the refolding curve. Therefore, the redox inactive zinc-protein was included in the studies.

Unfolding of Pc is fast and completed in seconds at 5 M GuHCl. Refolding of Pc is considerably slower, and takes place in about 15 minutes [87]. One reason for this is the isomerisation of the X–Pro peptide bonds. These bonds can be in two different configurations, *cis* and *trans*. In Pc, prolines 16 and 36 exist in *cis*–configuration in the folded protein [88], while they prefer the *trans* configuration in the unfolded protein as have been shown with peptide fragments of Pc [89]. Conversion to the *cis*–form is sterically hindered in the folded protein and isomerisation is therefore slow. This isomerisation is catalysed *in vivo* by PPI, and a faster refolding of Pc *in vitro* was observed upon addition of PPI [87]. Double-jump experiments where the protein is first denatured for a time too short for proline isomerisation and then immediately refolded also result in a faster refolding [Ejdebäck, unpublished][87]. To simplify the refolding of Pc, either or both *cis*–prolines were changed to glycines using site-directed mutagenesis (see section 5.3.2). The Pro36Gly could be produced but not the Pro16Gly mutant. Preliminary refolding data show faster refolding species for Pro36Gly as compared to the wild type protein [Ejdebäck, unpublished].

The thermal stability of copper-containing Pc from *Chlamydomonas reinhardtii* and spinach has been studied by others [90, 91]. The reduced protein was more stable than the oxidised, with melting points of 71°C and 61°C respectively [90]. A sample of the spinach protein in 100 mM NaCl, containing a mixture of the two

redox forms, had a melting point of 68°C and a free energy of unfolding of 15–20 kJ/mol [91]. This deviates from the results presented in this work, where equilibrium unfolding was studied as compared to the irreversible process of thermal denaturation.

Results from folding studies on apo–Pc have been reported [87, 92]. The free energy of folding of was determined to –23 kJ/mol in the presence of 0.5 M Na_2SO_4 [87]. Without stabilising salt the stability was considerably lower (–9 kJ/mol), and about 2 % of the protein was unfolded at 0 M GuHCl [92]. A problem with handling the apoprotein is its low stability, the easy but unwanted incorporation of metal ions present in trace amounts in chemicals and on glassware and that the cysteine is easily oxidised. Upon concentration of the apoprotein it readily precipitates [Ejdebäck, unpublished].

The folding of another blue copper protein, azurin, has been studied by Leckner et al. [93, 94]. The oxidised copper protein was more stable than the reduced and zinc proteins, having ΔG values of –52, –44, –40 kJ/mol respectively. The tertiary and secondary structures of azurin and Pc are similar but some important features can explain the differences in the folding data. Azurin contains a disulphide bridge, a more prominent α–helix and a copper-binding bend that is one residue longer. The copper site also contains one extra axial ligand, Gly45, with a weak interaction with copper. No *cis*–prolines are present in azurin and refolding is considerably faster. Disulphide bridges are known to stabilise the native conformation by reducing the entropy of the unfolded state. An azurin mutant with the disulphide bridge removed by site-directed mutagenesis was destabilised by about 20 kJ/mol as compared to the wild type protein [B.G. Karlsson, personal communication].

Chapter 4. Expression of recombinant proteins

Recombinant DNA technology was developed in the 1970´s and it is now widely used in the medical and agricultural industry. Hormones such as insulin and growth hormones can be produced by bacteria and today, 25–45 % of the crops produced in the USA are modified for resistance against damage by frost, herbicides or insects. Recombinant DNA technology involves gene cloning, that is the insertion of a DNA fragment (in this case spinach DNA) into a DNA carrier molecule (plasmid) and introduction of this plasmid into a host cell (in this case an *E. coli* bacterium). The bacterium multiplies to identical cells, a clone, all carrying the plasmid. In this fairly simple way large amounts of DNA or protein can be produced without the need of large amounts of animal or plant material. Some important features of the plasmid and the host cell for the production of recombinant proteins will be discussed in the following sections of this chapter.

4.1 The plasmid construct

Plasmids are small circular DNA molecules that are located extrachromosomally in the cell. The polylinker hosts a number of restriction enzyme recognition sites needed for cloning. Important sequences needed for protein production are the promoter, ribosome binding site, signal peptide, transcription termination region and gene(s) for antibiotic resistance. Carrying a plasmid is a burden for the cell and to select for cells that containing plasmids, an antibiotic resistance gene (often the β–lactamase gene, which gives resistance to ampicillin) is harboured on the plasmid.

The Pc gene has been cloned into a plasmid together with the signal peptide, ribosome binding site and the termination region of azurin from *Pseudomonas aeruginosa* [95]. These flanking sequences are known to result in high yields (~100 mg protein/litre culture) of azurin in *E. coli* [96].

4.1.1 The promoter and termination sequence

The promoter is the sequence upstream of the coding sequence that regulates the synthesis of mRNA and thereby also the protein production. Gene expression is regulated by binding and dissociation of RNA polymerase and transcription factors to the promoter sequence. Promoters like the *lac* and T7 promoters are often used to regulate protein expression and both have been used in this work. The termination sequence secures an efficient termination of the transcription by forming a hairpin. Hairpins and other secondary structures protect mRNA from degradation by exoribonucleases [97]. Endonucleolytic removal of these sequences by endoribonucleases is required before exonucleolytic degradation can proceed [98].

The *lac* promoter is a weak promoter that normally controls the transcription of the *lac* operon, which contains genes for enzymes involved in lactose metabolism. It is turned on (induced) by allo-lactose, although a chemical analogue, isopropyl–β–D–thiogalactopyranoside (IPTG) is normally used. When glucose is

available, lactose metabolism is inhibited (repressed) and the genes controlled by the *lac* promoter are turned off by the presence of the *lac*–repressor and the absence of the cAMP–CAP complex. This repression is often unwanted in cultivations on minimal media cultivations since glucose is commonly used as a carbon source for the bacteria. A promoter, *lac*UV5 that is insensitive to catabolite repression by glucose has therefore been created [99].

The stronger T7ϕ*10* promoter originates from the T7 bacteriophage and normally regulates the high level production of the major T7 capsid protein. An expression system, consisting of a plasmid with the T7 promoter and a bacterium with a chromosomal copy of the gene for T7 RNA polymerase, has been developed [100]. Upon induction, often by addition of IPTG, T7 RNA polymerase is produced and starts to transcribe the gene regulated by the T7 promoter. The advantage with this system is the higher processivity and specificity of T7 RNA polymerase compared to *E. colis* own. It is also unaffected by the presence of glucose since the T7 RNA polymerase synthesis is regulated by the *lac*UV5 promoter. The high expression levels can cause inclusion bodies to form in the bacterial cytoplasm, which can either simplify or complicate the protein purification.

In this work the Pc gene is cloned into a plasmid (pCA), which contains the T7ϕ*10* promoter, Tϕ terminator as well as the T7 gene 10 translation start site. A comparison between the *lac* and the T7 expression systems show that more Pc is produced by the T7 system (see section 4.4). The *lac* system failed to produce Pc in combination with growth on minimal medium. Glycerol had to be used as the carbon source since the construct contained the *lac* promoter. This reduced the cell growth rate and an explanation for the low yield of Pc can be that it and/or its mRNA was broken down.

4.1.2 Codon usage

The translation of mRNA into a protein sequence is done by the ribosomes where individual amino acids, carried by tRNA–molecules, are connected by peptide bonds. Amino acids are encoded by triplets of nucleotide bases, and since there are 61 amino-acid encoding triplets and only 20 amino acids the genetic code is degenerate. Different organisms prefer different codons and their pool of different tRNA is varied in the same way [101]. This can cause serious problems in the translation process if the codon usage in the cloned gene is too different from the tRNA–pool in the expression host. It has been shown that the translation rate of several sequential codons, whose corresponding tRNA are of low abundance, is severely reduced [102]. This could cause stable secondary structures to form on the mRNA, frame shifting and a premature termination of the translation.

In two constructs pUG223t_r and pME223 a cluster of seven codons in the beginning of the Pc gene was changed to the codons most frequently used by *E. coli* to enhance the translation [paper I]. Not every rare codon in the Pc gene was replaced since the remaining ones are distributed over the gene without any large clustering. In the azurin gene the number of rare codons are few, evenly distributed and high yields of the protein are usually obtained. This is probably an effect of the closer genetic distance between the bacterium from which the azurin gene was isolated, *Pseudomonas aeruginosa*, and the expression host (*E. coli*). The results from the experiments are presented in section 4.4.

4.2 Expression

4.2.1. Expression host and strain

The optimal expression host modifies the protein in the same way as the original organism, gives high protein yields and grows easily and fast. Common expression hosts are bacteria, baculovirus, yeast, insect cells and transgenic plants. The bacterium *E. coli* is the most commonly used when no post-translational modification is needed and several strains have been developed for protein expression.

In this work, Pc was expressed in the periplasm of *E. coli*. Expression in the periplasm has a number of advantages [103]. First, toxic proteins that can be lethal to the cell are transported from the cytoplasm. Second, the more oxidising environment of the periplasm allows disulphide bridges to form. Third, purification is simplified by the lower amount of proteins in the periplasm. Finally, the number of proteases in this compartment is reduced. The strains used in this work were *E. coli* TG1, RV308 and BL21(DE3) [104-106]. *E. coli* TG1/RV308 and BL21(DE3) were used in combination with plasmids that contain the *lac*- and T7–promoters, respectively. High protein yields of azurin expressed in the periplasm of *E. coli* RV308 have been obtained [96]. This is probably due to a more efficient secretion and the same effect is also expected for Pc.

4.2.2. Proteolytic degradation

More than 25 proteases have been detected in *E. coli* but only some of these are associated with the cell envelope or present in the periplasm. Some proteases are induced by the heat-shock response and triggered by elevated levels of aggregated, abnormal or unprocessed proteins [107]. The Lon protease performs initial endoproteolytical cleavage of cytoplasmic proteins in an ATP–dependent process whereas the DegP protease degrades unfolded proteins by cleaving the peptide bond after valines and isoleucines [108, 109]. A set of protease-deficient strains has therefore been developed for efficient expression of foreign proteins in *E. coli* [110]. The inactivation of protease genes results in higher expression yields, however, cellular processes and cell growth can be affected as the number of inactivated genes increases [111]]. Other ways to reduce the proteolytic activity is to use a mildly acidic environment (pH 5.5–6.0), lower temperature or to add zinc or copper ions that inhibit the proteases [110].

In this work, Pc is synthesised as a preapoprotein in the cytoplasm of *E. coli* and directed to the periplasm by the signal peptide. This transport is important since the apoprotein is very unstable and the number of proteases in the periplasm is reduced compared to the number in the cytoplasm. In the periplasm Pc folds to the native protein in a process that competes with the formation of misfolded protein. As Pc contains two *cis*–prolines and folding involves a slow isomerisation step some Pc can exist as misfolded protein. Misfolded protein can form aggregates, unfold and/or be recognised and degraded by proteases [112, 113].

E. coli BL21(DE3) belongs to the B strains, all having an inactive gene for the Lon protease. In addition it lacks one of the outer membrane proteases, OmpT. This protease is specific for paired basic residues and it is inhibited by Cu^{2+} and Zn^{2+} ions [114]. Since Pc have no paired basic residues and copper or zinc ions are included in the cultivation media, inactivation of this gene should have no effects

on the expression of Pc. *E. coli* RV308 and TG1 have no inactivated protease genes.

4.3 Cultivation and purification

Four different kinds of growth medium have been used in this work. Three are so called rich media, TB and LB media and a medium made on algae that could be grown on ^{15}N- and/or ^{13}C–labelled media. The fourth is a poor medium, the M9 minimal medium that consists of a number of salts. It was mainly used in the production of ^{15}N- or ^{13}C–labelled Pc samples. Copper sulphate was included in all cultivations at a concentration of 0.1 mM to saturate the copper sites since the apoprotein is unstable. For the same reason copper was also included in the buffers of all purification steps.

Pc was purified by subjecting the cells to an osmotic shock that released the periplasmic proteins. The pH of the supernatant was then lowered to 5.0 to precipitate some contaminating proteins. Two chromatography steps followed, an ion-exchange step and a gel filtration. Highly homogeneous samples of Pc could be obtained by separating the oxidised and reduced forms of Pc on a Resource Q anion exchange column [paper I].

4.4 Expression and purification results

The amounts of Pc obtained increased by 21–33 % upon removal of the codon cluster. The yield could be further increased by 21–33 % by changing the expression strain from *E. coli* TG1 to RV308 and by another 30 % when using the T7 promoter and the BL21(DE3) strain. Altogether, changing the codons, the expression system and the cultivation procedure ended up in a yield of 38 mg/l [paper I]. This should be compared with the yield of 2 mg/l previously reported from our lab [95]. By using the T7 expression system, 10 mg/l of Pc could be produced on minimal medium [paper I]. The reasonably high expression levels on this media allowed production of ^{15}N- or ^{13}C–labelled Pc for structural and protein-protein interaction studies by NMR [paper II].

The oxidised protein could be purified with an A_{278}/A_{597} ratio of 1.0 that was stable for hours without any auto-reduction [paper I]. This should be compared with the ratio of 1.2 often used in the literature and considered as pure Pc [70, 95, 115]. These samples are probably heterogeneous, containing zinc-protein and/or apoprotein. The higher absorbance of reduced Pc at 278 nm could also account for this [116]. Based on amino-acid analysis and determination of total and oxidised copper, the absorption coefficient at 278 nm could be calculated and related to the absorbance at 597 nm. The absorption coefficients at both wavelengths were determined to 4700 $M^{-1}cm^{-1}$ [paper I]. This can be compared to values of 4500-5900 $M^{-1}cm^{-1}$ and 4500-4900 $M^{-1}cm^{-1}$ used for the absorbances at 278 and 597 nm considering an A_{278}/A_{597} ratio of 1.1 to 1.2 [51, 65, 70, 115, 117].

Chapter 5. Site-directed mutagenesis

Nature has tried amino-acid substitutions on Pc for billions of years through the action of evolution. However, in the plant Pc sequences about 50 % of the residues are still unchanged. Of the changes that have occurred in plant Pc, about half are homologous. These can be substitutions between residues of similar charge (Asp vs. Glu), hydrophobicity (Ile vs. Leu and Val), hydrophilicity (Ser vs. Thr) or size (Ala vs. Ser). Most common in Pc are substitutions between hydrophobic aliphatic residues. The evolutionary mutagenesis process can be speeded up *in vitro* by site-directed mutagenesis. In this work two methods have been used, a PCR–based method and a commercial kit from Stratagene.

5.1 A two-step PCR-based method for site directed mutagenesis

The polymerase chain reaction, PCR, is a method for "copying" DNA developed by Kary B. Mullis and for which he was awarded the Nobel price in 1993. A heat-stable polymerase, *Taq* DNA polymerase, from *Thermus aquaticus* is used together with two primers that are complementary to the flanking regions of the DNA that is to be copied. The reaction mixture is subjected to cycling temperatures. First, at about 94°C, double stranded DNA is separated into single stranded DNA. By lowering the temperature to about 55°C the primers bind to their complementary sequences. At 72°C the polymerase elongates the primers with the plasmid DNA as a template. In this way, the sequence between the primers is copied and it can be used as a template in the next cycle. By raising the temperature to 94°C and going through the cycle another n times, 2^n copies of the original sequence can be made. This method is now commonly used in screening for genetic disorders, amplification of genetic material in forensic analysis and also in mutagenesis work.

By using the PCR procedure in two steps, Landt et al. developed a method where a mutagenic primer is used [118]. This primer is complementary to the part of the DNA sequence that is to be mutated except for the nucleotides that constitute the mutation. In the first PCR reaction the region between the mutagenic primer and one of the flanking primers is amplified, incorporating the mutation. This fragment is purified and used as a megaprimer in the second PCR reaction together with the other flanking primer, producing a new fragment that covers the sequence between the two flanking primers. This product is purified, cloned into a plasmid and transformed into *E. coli*. The advantages with this method are that it is relatively cheap and that the mutagenesis efficiency is high. Some experience is needed in primer construction and optimisation of the PCR parameters. The main disadvantage is that *Taq* DNA polymerase has no proof-reading activity, which could cause additional mutations within the amplified region, and it is therefore necessary to sequence the gene to verify that it is correct.

5.2 The QuikChange kit

This mutagenesis kit from Stratagene is highly efficient in introducing specific base changes into virtually any double-stranded plasmid and it requires no specialised vectors, unique restriction sites or multiple transformations. The method works by simultaneously annealing two primers containing the mutation to the plasmid. The two primers, each complementary to the opposite strands of the plasmid, are extended by *Pfu* DNA polymerase during temperature cycling. This heat-stable polymerase is purified from the archaebacterium *Pyrococcus furiosus* and it has the lowest error rate (about 10x lower than *Taq* polymerase) of any known thermostable DNA polymerase. It is also with its half-life of 13 h at 95°C more thermostable than *Taq* polymerase (half-life 40 min). Upon incorporation of the primers, a mutated plasmid that contains staggered nicks is generated. After temperature cycling, the product is treated with *Dpn* I restriction enzyme. This enzyme is specific for the methylated and hemimethylated DNA (target sequence 5'–G^{m6}ATC–3') produced by most *E. coli* strains and is used to digest the parental DNA template and thereby select for mutation-containing DNA. The nicked plasmid DNA containing the desired mutations is then transformed into Epicurian Coli XL1–Blue cells.

The advantages with this method are its speed and high mutagenesis efficiency. Transformants are produced after one day and few unwanted mutations are detected due to the high fidelity of the *Pfu* DNA polymerase, low cycle number and low amount of starting DNA. The main disadvantages are that unwanted mutations can happen anywhere in the plasmid and that two mutagenic primers are needed.

5.3 Site-directed mutagenesis projects

Many questions about the structure and function of proteins can be solved by site-directed mutagenesis. However, it is not always possible to obtain the mutant protein because of a low protein stability. Although this in itself is an important observation, no characterisation of the mutant protein can be done. One possibility is then to express the protein *in vitro*. Expression *in vitro* is expensive and only small amounts of protein can be produced.

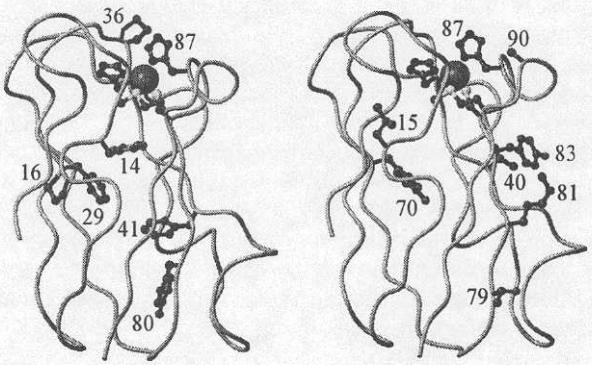

Figure 11. Backbone representations of Pc with copper ligands and mutated residues shown as ball-and-stick and indicated by their number in spinach Pc. Positions 59-61 and 88 are indicated in figure 14.

About forty mutants of spinach Pc have been created on the DNA level for this work but only twenty were obtained in the amounts needed for a full characterisation. I have used site-directed mutagenesis as a tool in the following projects:

5.3.1 Removal of a codon cluster

Clusters of rare codons can have effects on the protein synthesis as described in section 4.1.2. A codon cluster in the spinach Pc gene was removed by replacement with codons optimal for high-level expression in *E. coli*. This resulted in 21–33 % higher expression levels [paper I].

5.3.2 Removal of the *cis*–prolines

Two of the five prolines in Pc, Pro16 and Pro36, have their X–Pro bonds in *cis*–configuration in the folded protein. In the denatured state, these bonds adopt their preferred *trans*–configurations [89]. Refolding of this "*trans*–Pc" is slow *in vitro* and complicated by the isomerisation of the two prolines as have been described in section 3.3.2.

To determine the contribution of the *cis*–prolines to the refolding kinetics of Pc they were replaced by glycines. Glycine is the smallest amino acid and it has the highest conformational freedom. The Pro36Gly mutant has been expressed successfully but since this residue is next to a copper ligand the spectroscopic properties and the reaction with photosystem 1 were altered [69]. The expression of Pro16Gly as well as the Pro16Gly, Pro36Gly double mutant failed. Pro16 is situated in a tight turn that connects the two β–sheets [88] and it may be important for proper packing. The slow isomerisation of this residue has been identified as the rate-limiting step in the refolding of Pc, which causes accumulation of folding intermediates [87].

The folding mechanism of ribonuclease T1 has been shown to be dramatically simplified upon replacement of one of the two *cis*–prolines. A compensatory mutation of the residue that precedes the proline left the thermal stability of the double mutant almost unchanged [112]. A replacement of the other *cis*–proline destabilised the protein by about 20 kJ/mol and refolding was not simplified [119]. It was concluded that this residue was of major importance for the folding of the wild type protein. The replacement of two *cis*–prolines in ribonuclease A resulted in a thermal destabilisation by about 10 kJ/mol each [120, 121].

5.3.3 Insertion of a tryptophan into plastocyanin

Protein folding can be monitored by following the shift or intensity changes in the tryptophan or tyrosine fluorescence. The folding and unfolding of Pc, which contains three tyrosines but no tryptophan, should be possible to follow by measuring the change in fluorescence intensity upon denaturation. The change in the tyrosine fluorescence intensity was however found to be very sensitive to differences in Pc concentration and large errors were observed. Therefore it would be preferable to measure the tryptophan shift.

To make this possible, some aromatic residues in Pc were chosen for mutation into tryptophans [122][Ejdebäck, unpublished]. An optimal mutation would have a buried tryptophan with little influence on the protein structure and stability. To explore this, the mutations were modelled into the Pc structure using the program

Discover and then subjected to energy minimisation. Energy-minimised structures that did not deviate much from the wild type structure were found for the Phe14Trp, Phe29Trp, Phe41Trp and Tyr70Trp mutants and these were therefore chosen for site-directed mutagenesis.

The tyrosine in position 70 is not conserved and a phenylalanine, valine or threonine is found here in Pc from other organisms. Substitution of this residue by a tryptophan was successful and a protein with wild-type characteristics was obtained. The tryptophan showed no shift in the emission spectrum upon denaturation and it is probably surface exposed [122]. It was not possible to obtain any of the mutants where a phenylalanine was mutated. Possible explanations could be that Phe14 forms a corner of β–sheet 1, is conserved in eukaryotes and might be important for packing. The Phe29 is in fact a tryptophan in some algae and cyanobacteria and a successful expression of the mutant protein was expected, however other packing contacts might have influenced the expression. Phe41 is totally conserved in Pc and substitution might be deleterious. Phenylalanines 29 and 41 are located in two successive β–strands separated by a loop and they are parts of the β–zipper suggested to be the nucleation site for folding [123]. A mutation in these positions could therefore prevent a proper folding.

5.3.4 The tyrosine corner and SEHC

The tyrosine in the position corresponding to Tyr80 in spinach Pc is highly conserved among cupredoxins and non-related but structurally similar Greek-key β–barrel proteins. This residue is packed between the two β–sheets and it defines the loop between two of the β–strands. The hydroxyl group is typically hydrogen bonded to the backbone of a hydrophilic residue in position *i*–4, where *i* corresponds to the tyrosine, and also to the neighbouring β–strand [124]. The residue in position *i*–2 is almost always a glycine that helps the backbone to make a bend around the tightly packed phenyl ring of Tyr80 and it also forms hydrogen bonds to the neighbouring β–strand. These interactions have been suggested to be important in the folding of Greek-key β–barrel proteins and/or for protecting a cleft in the structure from entrance of water. To elucidate the function of this residue, it was replaced by a phenylalanine, leucine or a tryptophan. None of the mutants could be expressed, confirming the important role of this residue [Ejdebäck, unpublished]. A minimised theoretical structure with some structural perturbations could be obtained for the Tyr80Trp mutant. These perturbations could possibly be avoided by providing more space for the tryptophan with the additional mutations Ile46Ala and Val50Ala [122].

Pc contains 30 large hydrophobic residues. Most of these residues are buried in the hydrophobic core, however some are located in β–strands pointing outwards [125]. Two of the latter, valines 28 and 40 in Pc are highly packed and shielded from the solvent by side chains from hydrophilic residues. Val40 is highly conserved and, due to the shielding by residues at the acidic patch, its solvent exposure is only 7 %. A similar buried hydrophobic residue is found in the corresponding positions in azurins from *Pseudomonas aeruginosa* and *Alcaligenes denitrificans* and pseudoazurin from *Alcaligenes faecalis* [126-128]. In these proteins the residue is even more buried by the presence of an α–helix, suggesting a structural rather than functional importance [125]. A hypothesis is that the SEHC assists in protein folding by aligning the β–strands.

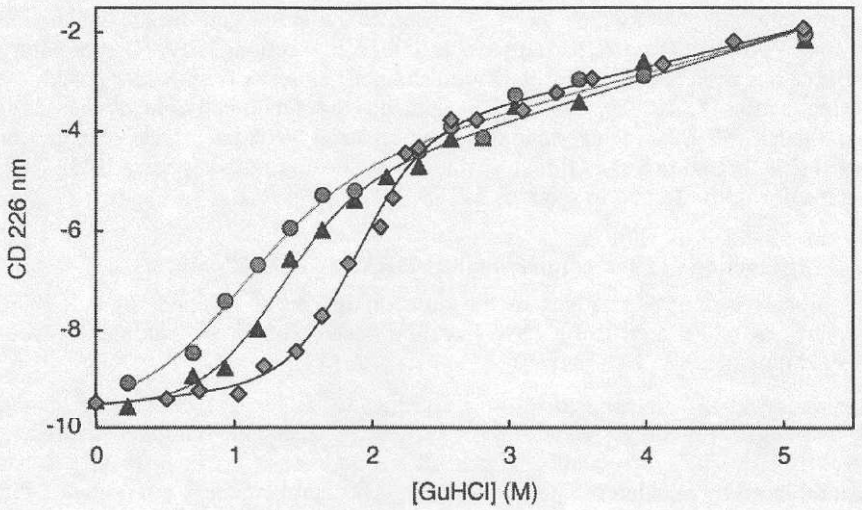

Figure 12. Normalised equilibrium unfolding curves of wild type Pc (diamonds) and two mutants, Val40Leu (circles) and Val40Met (triangles), as measured by CD at 226 nm.

To study the function of this residue, it was replaced by a methionine, leucine, alanine or glycine. Mutants with wild-type characteristics (see table 2 in section 5.5) were obtained except for Val40Gly, which did not express. The equilibrium unfolding curves of the zinc-substituted wild type protein and the mutants Val40Met and Val40Leu are shown in figure 12. The protein stability was reduced by the mutations and the largest effect was observed for the Val40Leu mutant, which starts to unfold already at low denaturant concentrations. Another interesting observation is that the expression level decreased as the size of the substituting amino acid was changed (see table 1 in section 5.4). This effect has also been observed for mutants of Tyr83 [Ejdebäck, unpublished]. A contact map shows many contacts between these two residues [92].

5.3.5 Introduction of residues for probe attachment

Intraprotein electron transfer can be studied by the attachment of a photo-active ruthenium complex to a histidine in the protein and then measure the flash-induced electron transfer between ruthenium and copper. Histidines have previously been introduced at position 83 and 12 in Pc and now also in positions 15, 79 and 81 [58, 129]. The intraprotein electron-transfer rates in the ruthenium-labelled proteins were measured and from these data the decay factor for the internal electron transfer and the reorganisation energy for the copper to ruthenium electron transfer could be calculated [130, 131].

In recent experiments Phe35 and Tyr83 have been replaced by a cysteine in order to attach an EPR spin probe to the cysteine and observe the behaviour of this probe upon docking with the redox partners of Pc. The Phe35Cys mutant could be expressed and modified while the Tyr83Cys mutant did not accumulate, possibly because of its close distance to the copper ligand Cys84 and effects on copper binding [Olesen and Ejdebäck, unpublished].

5.3.6 Reduction of the negative charge in the small acidic patch

Electrostatic interactions have been proposed to be important in the interaction between Pc and its redox partners as described in section 3.2.2. Contradictory evidence has been published for the involvement of either or both acidic patches in the interaction [8, 10, 22]. To further elucidate the functional role of the small acidic patch (59–61), each residue has been replaced by its neutral counterpart or by a lysine. In addition the Glu88Lys mutant has been studied [papers III and V]. The results are presented in sections 6.1 and 6.3.

5.3.7 Mutagenesis of the copper ligands His37 and His87

The highest conserved residues in the cupredoxins are the copper ligands. The copper sites of Pc and azurin have therefore been extensively studied by site-directed mutagenesis [132-137].

The buried His37 in spinach Pc was changed to an asparagine, aspartate and glycine but the mutant proteins did not express in detectable amounts [Ejdebäck, unpublished]. The corresponding ligand in *Pseudomonas aeruginosa* azurin has been replaced by all other amino acids [135]. All azurin mutants accumulated but only the aspartate mutant was blue and its properties were perturbed. A mixture of type 1 and type 2 copper sites was observed in the glycine mutant upon addition of external ligands [138]. The crystal structure of apo–His46Gly shows a wild-type like structure and that the hole created by the mutation is occupied by a water molecule [139]. This residue is obviously not essential for a type 1 copper site in azurin, but it may tune the redox properties, stabilise the protein and protect the copper site from solvent and ligand access [135].

To study the importance of His87, this residue was replaced by a number of amino acids. The uncharged glutamine and asparagine were chosen because of the similar size of their side-chains. Charged residues were introduced with the His87Asp, His87Glu and His87Arg mutations to evaluate the influence of charges on the copper co-ordination. The His87Gly, His87Gln, His87Asn and His87Asp proteins were produced as non-blue proteins whereas the His87Arg, His87Ile and His87Glu mutants could not be obtained [137]. The purified mutant proteins were EPR active but non-blue even in the presence of the oxidising agents ferricyanide ($E°$ +430 mV) and fungal laccase ($E°$ +780 mV) (see figure 7 in section 3.2.1). The number of copper ions per protein molecule was approximately one and this indicates a specific copper binding and that copper is not unspecifically bound to the protein surface. The mutants probably exist as their holoproteins since they are stable at room temperature for days, a condition that is known to degrade apo–Pc [85]. The EPR spectra in figure 7 are reminiscent of the spectra normally observed for type 2 copper sites. This is confirmed by the broadened hyperfine splitting, $A_{||}$, as is listed in table 2 in section 5.5. Addition of external ligands to the His87Gly mutant did not result in any restoration of the type 1 properties as was observed for the corresponding mutant, His117Gly, in azurin [134, 140].

My general observation is that the copper site in Pc is more sensitive to mutations than in azurin. This is indicated by the lower expression yields and that the electron transfer from His87 mutants to photosystem 1 is abolished [Ejdebäck and Sigfridsson, unpublished].

5.3.8 Remodelling of plastocyanin

In a protein engineering project, the negative residues in the acidic patches 42–45 and 59–61 were mutated into their corresponding neutral counterparts. A new acidic patch was subsequently introduced on the opposite "south-western" side of Pc by site-directed mutagenesis, taking advantage of existing negative charges in this region. The remodelling was done in several steps: The mutations in the small acidic patch were introduced on the existing double-mutant Glu43Gln, Asp44Asn. The resulting mutant, here called ME5, could be expressed and purified. The gene for ME5 was then used as a template for the mutagenesis of the remaining acidic residues (42 and 45) in the large acidic patch and the ME7 mutant with neutralised acidic patches and a total charge of –1 was created. Purification of this mutant was difficult since it attached very weakly to the anion exchange column. Instead negative charges were re-introduced in a step-wise manner. The modifications introduced were Val1Asp, Ser20Asp, Ala22Asp, Lys30Gln, Lys77Gln, Lys95Glu and Asn99Asp resulting in a charge change of –8. Although the mutated residues are solvent exposed and the size of the substituting residues were as similar as possible, none of these mutants expressed. My observation from the initial neutralisation of Pc was that the expression yield dropped for every new mutation and an accumulated effect of the mutations on protein stability is therefore the most probable explanation for the unsuccessful production of these mutants.

5.4 Expression levels for plastocyanin mutants

The expression level is influenced by every process in the cell, from the initiation of transcription to the stability of the processed holo-enzyme. At the level of transcription the nature of the promoter determines the amount of mRNA that is produced. The translation of this transcript is further regulated by the mRNA stability, possibility for and strength of secondary structures, consensus of the ribosome binding site, codon usage as well as termination of translation. Folding and post-translational processing (incorporation of copper in this case) also have effects on the expression yield.

Single mutations generally result in small structural changes within the closest vicinity of the site in question and they are expected to have only small effects on packing and thereby on protein stability. A distortion in the packing can often be accommodated by a small structural reorganisation of the overall structure upon mutation. Mutation of residues on the protein surface is often straightforward whereas mutation of buried residues is more problematic. Fersht et al. have determined the effect on protein stability upon removing a hydrogen bond [141]. The contribution of such a bond to the stability of tyrosyl-tRNA synthetase was determined to about 2 kJ/mol and 4–6 kJ/mol for an exposed and buried residue, respectively. Baldwin et al. have calculated the change in reorganisation energy for a large to small or small to large mutation to 8.4 and 10.5 kJ/mol [142]. These reported values should be compared with the low free energy of folding of apo–Pc (9 kJ/mol) [92].

The equilibrium unfolding curves of two mutants, Val40Met and Val40Leu, are shown in figure 12. Both mutants were destabilised as compared to the wild type protein. The larger side chain of the leucine is not well accommodated by the structure and neither is that of the methionine. The lower stability of these mutants can be compared with data from the first domain of CD2, which is similar

to the cupredoxin fold. The protein was destabilised by 4.6 ± 2.9 kJ/mol per methylene group removed [143]. An even higher value, 6.3 ± 2.0 kJ/mol per methyl/methylene group, has been reported for barnase and the reduction in stability was proportional to the number of methyl and methylene groups within 6 Å [144]. It has been proposed that the destabilising effect upon removal of these groups is due to loss of van der Waals interactions in the native state and to a reduced exposure of non-polar groups in the denatured state [143]. It is evident that even very small changes in the protein can have large effects on the protein stability and thereby on the expression level.

The expression levels for the His87 mutants were very low, 0 to 15 % of the wild type yield (table 1). The yields of the mutant proteins where the histidine was replaced by an uncharged and similar sized amino acid were highest, while an introduced charge further reduced the yield. To check for changes in the folded structure, a CD spectrum of some His87 mutants was recorded and normalised to equal protein concentrations (figure 13)[Ejdebäck and Sigfridsson, unpublished]. A lower absorbance in the β–sheet region (195–210 nm) was observed for the His87Gln and His87Asp proteins while His87Asn was similar to wild type. A theoretical study predicts that the CD spectrum of two closely packed β–sheets is strongly dependent on the inter-sheet separation and their relative orientation [145]. This could explain the lower absorbance, however it could also be interpreted as a higher amount of random coil in the protein as induced by the larger size of the glutamine residue or the negative charge of the aspartate. Caution is needed when the Pc concentration is calculated from the absorbance at 278 nm. An increased absorption in the UV region of up to 56 % of that of the oxidised protein has been reported for reduced Pc [116]. The protein concentration was therefore also determined with the BCA protein assay [146]. The concentrations obtained with this method were generally 15–45 % lower than the values determined from the absorbance at 278 nm, however the relative magnitudes of the CD absorbances were similar.

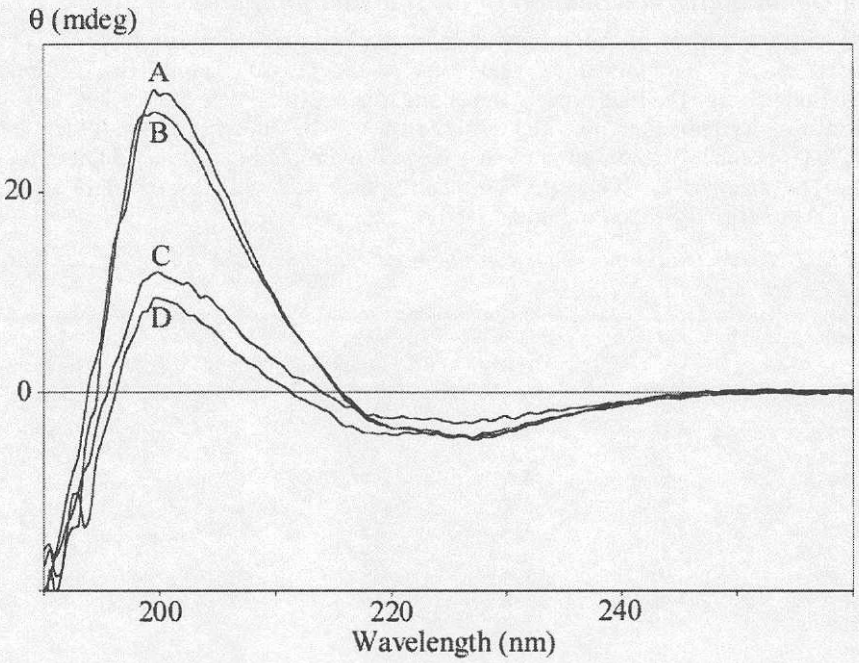

Figure 13. CD spectra of wild type Pc(B) and the mutants His87Asn (A), His87Gln (C) and His87Asp (D).

The expression levels of the mutants created in this work can be divided into categories where the amounts produced in the very low category are only enough for a basic characterisation whereas more protein is needed for a thorough characterisation, see table 1.

Table 1. Expression levels of plastocyanin mutants.

Wt-like >20 mg/l	Medium 10-19 mg/l	Low 2-9 mg/l	Very low 1-2 mg/l	Not detectable <1 mg/l
Glu59Lys	Leu15His	Val40Ala	Tyr83Ala	Phe14Trp
Glu60Lys	Val40Leu	Tyr79His	Tyr83Gln	Pro16Gly
Glu60Gln	Val40Met	Gln88Lys	His87Gly	Phe29Trp
Asp61Lys	Glu59Gln	Ala90Trp	His87Asn	His37Asp
Tyr70Trp	Lys81His		His87Asp	His37Gly
	Asp61Asn		ME7	His37Asn
	ME3			Val40Gly
	ME5			Phe41Trp
				Tyr80Phe
				Tyr80Trp
				Tyr80Leu
				Tyr83Gly
				His87Arg
				His87Glu
				His87Ile
				ME8-14

5.5 General characterisation of plastocyanin mutants

The characterisation of the expressed mutants is summarised in table 2. The largest effects are observed for mutations of the copper ligand His87. These mutations change the blue type 1 copper site to a non-blue type 2 site. The largest effects on the reduction potential are shown by the mutations Val40Met and Ala90Trp, probably as an effect of an increased hydrophobicity around the copper site. The changes in isoelectric point correspond well with the changes in the proteins total charge upon mutation.

Table 2. Isoelectric point, reduction potential and spectral parameters for wild type Pc and mutants.

Protein	p*I*	E°pH 7.5 (mV)	λmax (nm)	$g_{\parallel}$	$A_{\parallel}$ (mT)
Wild type	3.82	384	597	2.24	6.4
Leu15His	4.10	379	597	2.24	6.4
Phe35Cys	3.84	367	597	n.d.	n.d.
Pro36Gly*	3.81	398	597	2.20	5.2
Val40Leu	3.84	392	597	2.24	6.4
Val40Met	3.81	411	597	2.24	6.4
Val40Ala	3.81	n.d.	597	n.d.	n.d.
Glu59Gln	3.90	394	597	2.24	6.4
Glu59Lys	4.08	388	597	2.24	6.4
Glu60Gln	3.90	388	597	2.24	6.4
Glu60Lys	4.15	384	597	2.24	6.4
Asp61Asn	3.95	391	597	2.24	6.4
Asp61Lys	4.09	393	597	2.24	6.4
Tyr70Trp	3.86	386	597	2.23	6.6
Thr79His	4.03	381	597	2.24	6.4
Lys81His	3.86	369	597	2.24	6.4
Tyr83Ala	3.81	364	597	2.24	6.3
Gln88Lys	4.03	377	597	2.24	6.4
Ala90Trp	3.58	406	597	2.24	6.4
His87Asn	3.77	n.d.	non-blue	2.22	18.2
His87Asp	3.63	n.d.	non-blue	2.22	17.8
His87Gly	3.80	n.d.	non-blue	2.21	17.8
His87Gln	3.76	n.d.	non-blue	2.22/2.24	17.5

** The values for this mutant are taken from [147].*

Chapter 6. Protein-protein interactions

Characteristic for electron-transfer complexes is their dynamic nature where the stability of the complex is balanced against a rapid turnover. This involves the same kind of interactions that maintain the structure of a protein, namely hydrogen bonding as well as electrostatic and van der Waals interactions. Electrostatic interactions are long-range and dependent on pH and ionic strength. The overall net charge of the proteins influences their association rate [19]. As the distance between the proteins decreases, water is excluded from the interaction area and additional interprotein forces become important. These short-range interactions are influenced by local charge and they determine the alignment and orientation of the proteins [148]. At this distance hydrogen bonds, van der Waals contacts and the hydrophobic effect also contribute to the binding free energy as does the reduction in the number of conformations in the complex.

A protein-protein complex is characterised by favourable interactions. In the case of redox complexes a good electronic coupling between the donor and acceptor sites is also required as well as a rapid turnover. A rearrangement between a high-affinity conformation with low electron-transfer rate and a low-affinity conformation with high electron-transfer rate is often necessary to achieve all these criteria [149]. Factors that influence the electron-transfer rate are for example the thermodynamic driving force (difference in reduction potential), the distance between the donor and acceptor and the nature of the intervening medium. Since the protein complexes studied in this work are non-covalent, association and dissociation rates as well as rearrangements within the complex also have effects on the electron transfer.

The conditions *in vivo* are often hard to simulate *in vitro* due to the complexity of the cell and limitations in the equipment. The diffusion in the cell can be hindered by membranes and the ionic strength is often as high as 0.3 M. The viscosity is also quite high, for example the thylakoid lumen contains about 25 different proteins and the total protein concentration is about 20 mg/ml [78]. At very low ionic strength non-native protein interactions may form. There is therefore often first an increase in electron transfer upon increasing the ionic strength. At higher ionic strengths counter ions from the buffer start to shield the charges so that electrostatic interactions are weakened and other interactions may become dominant. An intermediate ionic strength of 0.1 M is therefore often used *in vitro*. *In vivo* experiments on the Pc–cyt *f* interaction in the alga *Chlamydomonas reinhardtii* suggest that electrostatic interactions may not be relevant in the thylakoids because of the high Pc concentration, high ionic strength, limited volume and possibility for lateral diffusion of Pc on the membrane surface [23, 24].

6.1 Interaction with photosystem 1

The interaction between Pc and photosystem 1 is proposed to occur by binding of the hydrophobic patch on Pc to a shallow hydrophobic pocket on the lumenal side of photosystem 1 [4]. This places the copper ion close to P700 and provides a short electron-transfer pathway. Electrostatic interactions between the acidic patches on Pc and positive charges on PsaF have also been suggested to influence the association and to stabilise the complex [7-9]. Cross-linking, chemical modification and site-directed mutagenesis have been used to study these interactions [6, 8, 9, 64, 67, 70]. Evidence for the involvement of both the acidic and hydrophobic patches in the electron transfer to photosystem 1 has been published in earlier work from the department [10, 68, 69].

The interaction between Pc and PsaF is probably weak or absent in cyanobacteria. The PsaF subunit in these organisms does not protrude enough from the membrane surface because of a shortening of the N–terminal end [2]. The PsaF subunit of many cyanobacteria also lack the positively charged residues in the N–terminal helix and the negative residues are often missing in Pc from the same organism. The electron transfer in these cyanobacteria seems to follow a simple collisional mechanism [14, 150]. The charged N–terminal part of PsaF from the alga *Chlamydomonas reinhardtii* has been fused with the C–terminal portion of PsaF from the cyanobacterium *Synechococcus elongatus* [151]. This resulted in a 2–3 fold increase in the electron-transfer rate from *Chlamydomonas reinhardtii* Pc and cyt c_6 to the modified photosystem 1. A fast phase was observed in the electron transfer from cyt c_6 but not from Pc, which indicates that additional interactions may be necessary for an efficient electron transfer from Pc.

In this work the focus has been on Glu59, Glu60 and Asp61 in the small acidic patch. Each residue has been converted to its neutral counterpart and into a lysine. In addition, Gln88 has been converted to a lysine. These residues and some other key residues important for the interaction between Pc and its redox partners are shown in the figures below.

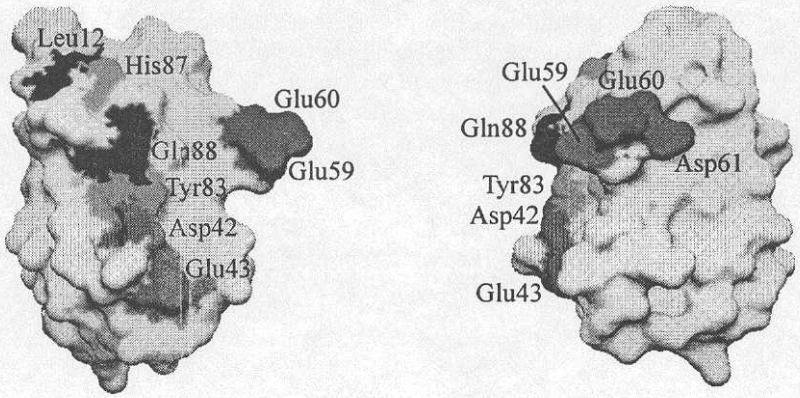

Figure 14. Surface pictures of Pc as seen from "front" and rotated by 90°.

6.1.1 Model

The early models for the electron transfer from Pc to the reaction centre P700 (denoted P in the formulas below) were relatively simple [58]. The biphasic kinetics were interpreted according to a model where the electron is transferred directly after complex formation:

$$Pc^{red} + P^{ox} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} Pc^{red}\cdot P^{ox} \xrightarrow{k_{et}} Pc^{ox}\cdot P^{red}$$

This would result in biphasic and monophasic kinetics at low and high Pc concentrations respectively, where the rate constant of the slow phase would saturate at k_{et} at high Pc concentrations. The amplitude of the fast phase is a measure of the amount of complex formed during the equilibrium before the flash whereas its rate approximates the rate of intracomplex electron transfer. This model is too simple to describe the results since two kinetic phases are observed at high Pc concentrations and the rate constant of the slow phase saturates at a value lower than k_{et} [152, 153]. To explain this, a model that includes a rate-limiting rearrangement has been proposed [152, 153]. First, Pc and photosystem 1 associate to form a "distant" complex that rearranges in a rate-limiting step to a "close" complex, which is competent for electron transfer. An electron is then transferred and the complex dissociates.

$$Pc^{red} + P^{ox} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \underset{distant}{Pc^{red}\cdots P^{ox}} \underset{k_c}{\overset{k_d}{\rightleftharpoons}} \underset{close}{Pc^{red}\cdot P^{ox}} \xrightarrow{k_{et}} Pc^{ox}\cdot P^{red}$$

This model assumes irreversible electron transfer because of the large difference (~120 mV) in reduction potential between free P700 and Pc. The reduction potential of Pc has been shown to be essentially the same in the complex with photosystem 1 [152] and it was believed that the same is true for P700. In a recent experiment, Drepper et al. determined the reduction potentials of associated Pc and P700 [59]. The difference between them decreased to about 55–70 mV upon complex formation. The rather small difference in reduction potential questions whether the back electron transfer can be neglected. Therefore, a new model that includes both the reversible electron transfer and the dissociation of the complex has been proposed [59].

$$Pc^{red} + P^{ox} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} Pc^{red}\cdot P^{ox} \underset{k_{-et}}{\overset{k_{et}}{\rightleftharpoons}} Pc^{ox}\cdot P^{red} \underset{k'_{on}}{\overset{k'_{off}}{\rightleftharpoons}} Pc^{ox} + P^{red}$$

The release of oxidised Pc was found to be 2.5 times faster than that of reduced Pc and binding of oxidised Pc was found to be lowered by the same amount [59]. If one neglects the binding of oxidised Pc to photosystem 1, kinetic data can be interpreted with the same differential equations irrespective of the model used, a rearrangement or a reversible electron transfer [paper V].

6.1.2 Experiments

The electron transfer from Pc to photosystem 1 was followed by flash-photolysis absorption spectroscopy. A mixture of Pc and photosystem 1 was excited by a

10 ns laser flash at 532 nm and the absorbance of P700 monitored at 830 nm with a continuous-wave diode laser. The instant absorbance increase due to photo-oxidation of P700 was followed by a decrease due to the reduction of $P700^{ox}$ by Pc. The reaction mixture contained ascorbate and methyl viologen as electron donors and acceptors respectively and $MgCl_2$ to control the ionic strength. Several exponential components were present in the absorbance decay. The curve was fitted to three kinetic components, k_f to k_{vs}, where k_f is the concentration independent rate constant of the fast decay, k_s is the concentration-dependent rate constant of the slow decay, and k_{vs} represents the very slow reduction of Pc by ascorbate. The fraction of fast phase, a_f, and k_s show a saturating behaviour that reach maximal values of a_{fmax} and k_{smax}, respectively. The rate constant for the electron transfer, k_{et}, and other mechanistic parameters can be extracted from k_f, k_{smax} and a_{fmax} and from the behaviour of the kinetics at low Pc concentration [paper V].

6.1.3 Experimental findings

The mechanistic parameters of both the conformational change model and the reversible electron-transfer model have been calculated from the observed kinetic parameters as described in paper V. It is concluded that the model that includes a conformational change can best describe the reaction. First, the calculated effective dissociation constant in the conformational-change model is similar to the one observed [154]. Second, a plot of ln K against the square root of the ionic strength (where K is either the equilibrium constant for the conformational change or the electron transfer) results in a negative slope as is expected for a model that involves a conformational change but at variance with the other model. The data are therefore interpreted according to the conformational change model in the rest of this section.

The rates of the electron transfer to photosystem 1 from the mutants in the small acidic patch were similar to wild type, whereas a slight increase was observed for the Gln88Lys mutation. The binding to photosystem 1 was strengthened by the Glu60Gln, Glu60Lys and Asp61Lys mutations but weakened by the Glu59Lys mutation as shown by their decreased or increased dissociation constants, respectively. The rearrangement to the "close" conformation was hindered in the Glu60Gln, Glu60Lys and Asp61Lys mutants while it was promoted by the Gln88Lys mutation [paper V].

The rate of the fast phase of wild type Pc and some mutants increases slightly with increasing amounts of Mg^{2+} as a result of an increased k_d at high ionic strength. k_{smax} and a_{fmax} decrease with increasing ionic strength. The decrease was much less pronounced for the Glu60Gln and Glu60Lys mutants [paper V].

6.1.4 Conclusions

Site-directed mutagenesis studies of the electron transfer in this and earlier work show that the interaction between Pc and photosystem 1 is of both electrostatic and hydrophobic nature. Mutations in the large acidic patch have been shown to influence the electron-transfer reaction more than mutations in the small acidic patch [8-10, 67]. However, mutations in the small acidic patch have larger effects on the reduction potential because of the shorter distance to the copper site and this could have effects on the driving force for electron transfer. Glu60 is important for the electrostatic interaction with photosystem 1 since mutation of this residue

resulted in a reduced dependence on Mg^{2+} and a stronger binding, where the largest effect on the electron transfer and ionic-strength dependence was observed for the Glu60Lys mutant [paper V]. This residue is negatively charged in most plants, while it is positively charged or carries a hydroxyl group in cyanobacteria.

The kinetics for the Gln88Lys mutant are faster than wild type. Wild-type like kinetics are exhibited by two other mutations in this position, Gln88Glu and Gln88Asn [68, 69]. Obviously, this position is not important for the interaction with photosystem 1.

The fast phase disappeared or was severely reduced upon mutation of Leu12, Pro36 and His87 in the hydrophobic patch of Pc [68, 69, 137]. Similar effects were observed for the Gly10Leu and Ala90Leu mutations in the same area [70]. These results show the importance of specific hydrophobic interactions in the electron transfer to photosystem 1. Electron transfer from mutants in peripheral regions of this patch (residues 7, 8, 11, 35 and 85) is less disturbed [155]. The electron transfer from mutants constructed in positions outside the acidic and hydrophobic patches (residues 15, 40, 70, 79, 81 and 83) were similar to wild type [Ejdebäck, unpublished].

6.2 Interaction with cytochrome f

The interaction between cyt *f* and Pc has been studied using cross-linking, chemical modification and site-directed mutagenesis and the results suggest interactions between basic residues on cyt *f* and acidic residues on Pc [22-24, 156, 157]. The electron-transfer pathway is still of some controversy, however the most recent studies propose His87 on Pc to be the site of electron transfer from cyt *f*. A static structure of protein complexes can be solved by co-crystallisation, but a more dynamic view can be determined by chemical-shift mapping with NMR. Protein complexes in solution can be studied and the interaction sites identified by monitoring the chemical-shift change of a protein's resonances as a function of added interacting protein.

In this work the area on Pc that interacts with cyt *f* has been determined by NMR. The results have been used as input into restrained rigid-body molecular dynamics to obtain a minimised structure of the complex between Pc and cyt *f* [paper II]. The chemical-shift change of side-chain protons of Cu(I) Pc has also been studied [paper IV].

6.2.1 Model

The model used in this work involves as a first step an association of the free proteins to loose and flexible electrostatic complexes in rapid equilibrium. In a second step, these complexes form a single, tighter complex where both electrostatic interactions between opposite charges on Pc and cyt *f* and short-range interactions are important.

The proposed model is based on two observations. First, charged residues are involved in the interaction as have been shown by chemical modification and site-directed mutagenesis [8, 20, 22, 64, 158]. Second, cross-linked complexes between cyt *f* and Pc are inactive or almost inactive in electron transfer, which indicates a need for a conformational change from the initial electrostatic complex to a conformation more competent for electron transfer [21, 149, 156]. The rearranged complex could provide a shorter copper to heme distance, a smaller dielectric constant or a better electronic coupling.

6.2.2 Experiments

Cyt *f* from turnip was used in the experiments since this protein can be purified from cruciferous plants. These plants contain a protease that cleaves the two soluble domains from the membrane anchor, which otherwise would cause oligomerisation of the protein. experiments ^{15}N–labelled Cd(II)-substituted Pc was used in 2D–HMQC NMR experiments to obtain backbone information. Cd(II) Pc was used to mimic the Cu(II) Pc, which cannot be studied because of the paramagnetic effects of Cu(II). The complex between Cd(II) Pc and cyt *f* should be similar to the "association complex" between Pc and cyt *f*. In the subsequent experiments, unlabelled, reduced Cu(I) Pc was used to obtain side-chain information. This complex should be more similar to the "dissociation complex".

NMR–samples containing 1 mM Pc were titrated with reduced cyt *f* and the chemical-shift changes of the resonances observed. These chemical-shift changes originate from two different effects. First, chemical groups in the interaction area experience a difference in their environment upon complex formation that makes

their NMR peaks shift. By identifying these shifted peaks, the contact area can be determined. Secondly, the heme group in oxidised cyt *f* induces pseudocontact shifts on Pc, which are dependent on the relative orientation and distance to the heme group. From this information the distance from the shifted peak to the heme group can be calculated. NMR data were then used as input in restrained rigid-body molecular dynamics and energy minimisation calculations. Unlabelled, reduced Pc was used in the 2D–NOESY/TOCSY NMR experiments to provide side-chain information.

6.2.3 Experimental findings

The complex between ^{15}N–labelled, Cd(II)–substituted Pc and cyt f. A small number of residues (~25 %) showed chemical-shift changes in their amide and/or amide proton resonances upon complex formation (see table 1 in paper II). These residues were located in the hydrophobic patch or in the acidic patches. The largest pseudocontact shifts were observed for residues in the hydrophobic patch, which suggests a model where this patch binds close to the heme group of cyt *f*.

The complex between unlabelled, Cu(I) Pc and reduced cyt f. In this experiment ^{1}H–NMR was used obtain side-chain information. A larger number of residues (~40 %) showed chemical-shift changes, probably as an effect of the larger amount of information provided by the side-chain protons. The affected residues were essentially the same as in the experiment with ^{15}N–labelled Pc, however chemical-shift changes of additional residues were also observed (see table 1 in paper IV). These residues showed small chemical-shift changes and they were predominantly located in peripheral regions of the hydrophobic patch. The largest chemical-shift changes were observed for the side-chain protons of residues 12, 37, 62, 87 and 92 and the backbone protons of residues 6, 10–13, 34 and 92. Smaller chemical-shift changes were exhibited by protons of residues in the acidic patches. Residues 10–13 showed larger chemical-shift changes than expected from the lower amount of cyt *f* used. A model of the complex is shown below in figure 15. Another representation is shown on the front cover of this thesis.

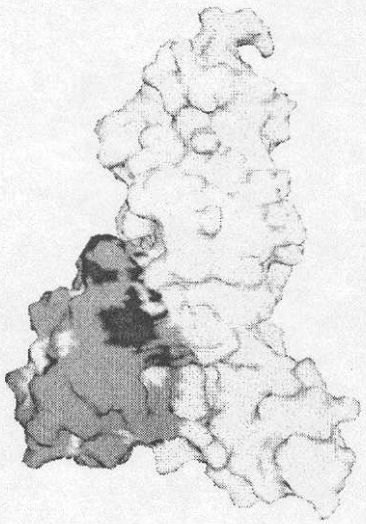

Figure 15.

Surface representation of the Cu(I)Pc-cyt f complex. Cyt f and Pc are shown in bright grey and dark grey, respectively. The chemical-shift changes on Pc as induced by complex formation have been mapped onto the Pc surface and are shown in black.

6.2.4 Conclusions

The chemical-shift changes induced by complex formation has been monitored and both studies reported here show that the acidic patches and the hydrophobic patch on Pc interact with cyt *f*. The distance dependence of the pseudocontact shifts suggest an interaction where the hydrophobic patch on Pc binds to the heme region on cyt *f*. The short distance between the heme-ligand Tyr1 on cyt *f* and the copper ligand His87 on Pc makes this pathway highly probable for electron transfer. The differences observed between the two studies can be explained by differences in the structure around the copper site as induced by the substitution of the copper ion for a Cd^{2+} or by the different ionic strengths used. The change in the identity and the charge of the metal ion could induce small structural changes in the Pc structure and thereby in the diprotein complex. A charge change followed by a conformational change could be one possible mechanism that facilitates the dissociation of reduced Pc after electron transfer *in vivo*.

The determined structure fits well with theoretical models of the complex [19, 159-161]. However, Ullmann et al. did not suggest any electron transfer through Tyr1 [161] and site-directed mutagenesis has shown that the tyrosine can be replaced by other aromatic amino acids in *Chlamydomonas reinhardtii* without any large effect on the growth and photooxidation *in vivo* [162]. A slower re-reduction but no large effect on the growth was observed for a serine mutant while a proline mutant was unable to grow photoautotrophically. An alternate electron-transfer pathway from a heme propionate [161] or through Phe4 on cyt *f* [paper II] can explain these observations.

The complex between Pc and cyt *f* in this study has some similarities with the Pc–cyt *c* complex determined previously with the same technique [163]. Both involve interactions between the acidic patches on Pc and the area around the heme on the cyt, however the complex with cyt *f* is tighter and consists of a small number of highly similar conformations. In addition the hydrophobic surface on Pc interacts with cyt *f* and pseudo-contact shifts can be observed. This can be explained by the difference in shape and size between cyt *f* and cyt *c* as well as by a larger binding constant between the physiological redox partners.

6.3 Interaction with cytochrome c, a non-physiological redox partner.

Cyt *c* is a redox protein located in mitochondria, where it transfers electrons from the cyt bc_1 complex to cyt *c* oxidase. Although not a natural redox partner to Pc, cyt *c* has similarities to cyt *f*. It can interact with Pc through charge interactions between the heme and surrounding basic residues on cyt *c* and the acidic patches on Pc and the complex is competent for electron transfer. This reaction has been studied extensively by Kostic and co-workers. Cross-linked, chemically modified, and Pc subjected to site-directed mutagenesis has been used in the electron-transfer experiments [164-167][paper III]. The complex between the proteins has also been studied by theoretical calculations and NMR [163, 168, 169].

In this work, 12 single mutants and one double mutant were used to determine the effects of the size and orientation of the Pc dipole on the reaction with cyt *c* [paper III]. The mutants were distributed over regions on Pc that are important for the interaction with its physiological redox partners. The mutants were Leu12Asn, Leu12Glu and Leu12Lys in the hydrophobic patch, Asp42Asn, Asp42Lys and Glu43Asn in the large acidic patch, Glu59Gln, Glu59Lys, Glu60Gln, Glu60Lys and Glu59Lys/Glu60Gln in the small acidic patch as well as Gln88Glu and Gln88Lys, which are located between the small acidic patch and the hydrophobic patch (see figure 14).

6.3.1 Model

Cyt *c* and Cu(II) Pc first associate to form an electrostatic complex where the dipole moments of cyt *c* and Pc are aligned head-to-tail. This first encounter complex resembles the maximum overlap (*max ov*) conformation described by Roberts et al., with the heme and basic residues of cyt *c* located close to the acidic patches and Tyr83 of Pc [169]. The complex rearranges to the maximum overlap rotated (*max ov rot*) or the northern/equatorial (*n/eq*) conformation by a 180° rotation or a 90° rotation accompanied by a translation respectively. While the position of cyt *c* on Pc in the *max ov rot* conformation is essentially the same as in *max ov*, a rearrangement to the *n/eq* conformation involves a movement of the small acidic patch on Pc over the basic patch of cyt *c*. In the latter conformation residues 59, 60 and 88 are close to the heme, the heme to copper distance is relatively short and an electron can be transferred. The electron transfer is thus treated according to a model similar to the one presented for the electron transfer from Pc to photosystem 1 involving a rearrangement. At the low ionic strength used in the experiments the complex is pre-formed in the inactive conformation and the reaction is limited by the rearrangement. The reaction scheme can therefore be simplified to:

$$Pc^{ox} \cdot cyt^{red} \xrightarrow{k_F} Pc^{red} \cdot cyt^{ox}$$

6.3.2 Experiments

The electron transfer between pre-formed complexes of horse heart Zn–cyt *c* and spinach Pc was measured by monitoring the decay of the flash-induced triplet state of the zinc-porphyrin at 460 nm. The reaction is unimolecular at low ionic strength and since the rate of this rearrangement is slow compared to the electron

transfer, k_F describes the structural rearrangement in the complex. The rearrangement within the complex and its dynamics were studied by changing the solution viscosity and the temperature. The effect of these physical parameters and of the mutations of interacting amino acids on the electron-transfer rate has been determined.

6.3.3 Experimental findings

Smaller association constants were found for all mutants in the acidic patches whereas the values for mutations at the other positions (except for Gln88Lys) were similar to wild type. This implies that both acidic patches are important for complex formation. The rate constant for the unimolecular reaction decreases as the charge of the small acidic patch is made less negative and the largest effects were observed for the Glu59Lys and Glu60Lys mutants. The orientation and magnitude of the dipole moments were also most affected by these mutations. The unimolecular reaction of these mutants is more dependent on the solution viscosity, which can be explained by the enhanced friction due to electrostatic and/or steric effects caused by the lysines. By raising the temperature protein friction decreases. The temperature dependence of the proteins mutated in positions 59 and 60 is severely reduced as compared to wild type. The largest effect can be seen for Glu59Lys, which has a reaction rate that is almost constant in the temperature range used. Obviously, it is hard for this diprotein complex to overcome its "locked" conformation in this temperature interval.

6.3.4 Conclusions

Soluble, well characterised and easily accessible, cyt *c* and Pc are well suited for electron-transfer studies. The results suggest that the proteins first associate to a docking conformation that involves both acidic patches. A rearrangement then occurs to a conformation involving the small acidic patch and the area near Gln88. This rearrangement is hindered by lysines introduced in positions 59 and 60. The different results for the lysine mutants in positions 59 and 60 can be explained by that the former lies in the protein-protein interface whereas the latter lies on the border of this interface. In contrast to the large acidic patch, the small acidic patch and the area between it and the hydrophobic patch can provide efficient electron-tunnelling pathways to the copper [paper III].

These data support previously published results [166] and suggest the *n/eq* conformation to be active in electron transfer. The *n/eq* conformation has a good electronic coupling due to a short heme to copper distance and essentially the same acidic residues are involved as in the complex between cyt *f* and Pc. The larger size of cyt *f* allows additional contacts with the hydrophobic patch on Pc [paper II, IV]. Caution is needed in the comparison with cyt *f* since the size and shape of the two proteins differ, cyt *f* being an elongated molecule whereas cyt *c* is globular. This may affect the binding constant and the electron-transfer rate. The interaction area between Pc and cyt *c* has been determined by NMR [163]. The small shifts observed and the absence of pseudo-contact shifts indicated an ensemble of dynamic structures that involves both acidic patches as well as Asn64 and Val93 on Pc.

Chapter 7. Future perspectives

During recent years a large number of papers concerning the structure and function of Pc have been published. Structures of Pc from a variety of organisms have been solved and site-directed mutagenesis studies have provided evidence for regions on Pc that are important for the interaction with its redox partners. Despite this, some questions still remain to be answered.

What limits the expression of Pc in Escherichia coli*?*

The expression levels of Pc are low in comparison with the expression levels of azurin when using the same expression system. Since the Pc and azurin genes were isolated from eukaryotic and prokaryotic organisms respectively, a different codon usage in the Pc gene and in the expression host can partly explain the different yields. Upon removal of a codon cluster, the expression levels of Pc increased but not to the level of azurin [paper I]. The different expression levels can also be a result of a higher protein stability of azurin due to the presence of a disulphide bridge and a longer copper-binding loop. It would therefore be of interest to see if an introduction of these features into Pc could raise its stability. Larger amounts of Pc could possibly also be obtained by expression in yeast.

How does Pc bind to photosystem 1?

The structure of photosystem 1 from the cyanobacterium *Synechococcus elongatus* has been solved [2], however the cyanobacterial PsaF subunit is shortened and the electrostatic interactions with charged residues on Pc are probably absent. It would therefore be of great interest to know the structure of plant photosystem 1. The structure of spinach photosystem 1 has recently been solved by crystallography but at a resolution too low to distinguish anything but the subunits, and none of the subunits were identified as PsaF [170]. Ultimately, a structure of the complex between Pc and photosystem 1 would considerably facilitate the interpretation of kinetic data. A large number of mutants in the acidic patches have been created, and the study of the ionic-strength dependence of the reaction between Pc mutated in the small acidic patch and photosystem 1 could be expanded to include single mutations of residues in the large acidic patch.

Do the different redox forms of Pc bind to cyt f in the same way?

NMR studies on unlabelled Cu(I) Pc and ^{15}N–labelled Cd(II) Pc show that both proteins bind to cyt *f* essentially in the same way. Some differences are observed in the chemical-shift changes of residues 10–13, which are more affected in Cu(I) Pc than expected from the lower amounts of cyt *f* used. This could be explained by a different complex conformation as induced by the different charge and identity of the metal. A NMR study where unlabelled Cd(II) Pc is used could clarify the situation. It would also be of interest to study the complex between cyt *f* and Pc mutated in the interaction areas.

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