Transient interactions between photosynthetic proteins

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Chapter II

Protonation of a histidine copper ligand in fern plastocyanin

Abstract

Plastocyanin is a small (~11 kDa) blue copper protein that shuttles electrons as part of the photosynthetic redox chain. Its redox behavior is changed at low pH as a result of protonation of the solvent exposed copper coordinating histidine. Protonation and subsequent redox inactivation could have a role in the down regulation of photosynthesis. As opposed to plastocyanin from other sources, in fern plastocyanin His90 protonation at low pH has been reported not to occur. Two possible reasons for that have been proposed: π–π stacking between Phe12 and His90 and lack of a hydrogen bond with the backbone oxygen of Gly36. We have produced this fern plastocyanin recombinantly and examined the properties of wt protein and mutants Phe12Leu, Gly36Pro and the double mutant with NMR spectroscopy, X-ray crystallography and cyclic voltammetry. The results demonstrate that, contrary to earlier reports, protonation of His90 in the wt protein does occur in solution with a pK_a of 4.4 (± 0.1). Neither the single mutants nor the double mutant exhibit a change in protonation behavior, indicating that the suggested interactions have no influence. The crystal structure at low pH of the Gly36Pro variant does not show His90 protonation, similar to what was found for the wt protein. The structure suggests that movement of the imidazole ring is hindered by crystal contacts. This study illustrates a significant difference between results obtained in solution by NMR and by crystallography.

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Introduction

Plastocyanin (Pc) is a small (~11 kDa) blue copper protein, which shuttles electrons from cyt*f* in the cytochrome b_6f complex to $P700^+$ of photosystem I (PSI) in oxygenic photosynthesis[138]. The copper in the type I site is coordinated by the N_δ atoms of two histidines, the thiolate sulphur of a cysteine and weakly by the thioether of a methionine[139]. Apart from one histidine the copper ligands are located in a C-terminal loop. The geometry and nature of the copper ligands are important determinants of the redox potential of blue copper proteins[140,141]. The histidine ligand that is solvent exposed is located in the hydrophobic 'northern' patch, which is important in electron transfer to both cyt *f* and PSI[29,30,40,142-144].

In several blue copper proteins the solvent exposed histidine ligand can be protonated in the reduced protein. The accompanying pK_a values vary between proteins and species. For instance, Pc's show a species dependent pK_a range, from 4.7 for French bean Pc to 5.7 for parsley Pc[85,139,145] and values of ~5 have been reported for pseudoazurin[146-148]. Amicyanin shows the highest pK_a value, ranging from a pK_a of 7.2 for *Thiobacillus* (now *Paracoccus*) *versutus*[149] to 7.5 for *Paracoccus denitrificans*[150]. For azurin and rusticyanin the transition is not observed down to a pH of 2-3 [151-153]. Several studies revealed the structural effects of the protonation[154-156]. Crystal structures of poplar Pc at six pH values showed that upon protonation the imidazole ring moves away from the copper and turns 180º around the C_β-C_γ bond[157]. The new position of the histidine is stabilised by a hydrogen bond with the backbone oxygen of Pro36. As a result of the protonation and 'flip' of the imidazole ring, the copper sinks into the plane of the other three ligands. Consequently, the reduction potential rises and the protein becomes redox inactive[158]. It is not entirely clear whether this serves a physiological purpose, but it is known that the lumen acidifies as a result of the light reaction in photosynthesis[159]. Acidification is thought to be involved in the down regulation of the light reaction. Possibly, protonation and subsequent inactivation of Pc plays a role in this process.

To account for differences in reduction potential and protonation behavior between blue copper proteins several factors have been suggested. Among them are the length of the C-terminal ligand loop[160-162], the hydrogen bond network surrounding the metal site[163],

solvent accessibility of the metal site[164] and π–π stacking interactions with the histidine ligand[77,147]. Stacking interactions have been found in an unusual Pc from *Dryopteris crassirhizoma* (a fern), which is the only Pc reported not to undergo histidine protonation[77]. Studies of this fern Pc, which shows only 33-38% sequence conservation with seed plant sequences, led to the conclusion that π–π stacking interaction between Phe12 and the solvent exposed His90 prevented the histidine ligand from being protonated at low pH[77,85].

We have studied the histidine protonation behavior of *D. crassirhizoma* Pc by site directed mutagenesis, two-dimensional NMR, crystallography and cyclic voltammetry. We found that the protein does show protonation although at a pK_a lower than found for other Pc's. No significant differences are observed between wt Pc and the F12L, G36P and F12L/G36P mutants. It indicates that stacking interactions in fern Pc play no role in determining the protonation behavior. The results are discussed in relation to earlier studies on stacking interactions and histidine protonation in cupredoxins.

Materials and Methods

Gene synthesis and mutagenesis

The gene encoding *D. crassirhizoma* Pc was synthesised by Annabelle Mery and Dr. Martin Ph. Verbeet on the basis of back translation of the amino acid sequence[77], using frequent *Escherichia coli* codons (Wisconsin Package Version 10.0 Genetics Computer Group, Madison, WI). The DNA sequence of fern Pc was divided over four pairs of oligonucleotides (Table 2.1). Gene synthesis was performed in four successive steps. In step 1 oligonucleotides dryoa1 (coding) and dryob1 (non-coding) were used in a PCR reaction without template, consisting of one cycle of 5 minutes denaturation at 95°C, 2 minutes of hybridisation at 60°C and 20 minutes of elongation at 70°C. In step 2 primers dryoa2 (coding) and dryob2 (non-coding) hybridised with the product of step 1 in a PCR reaction consisting of 5 minutes denaturation at 95°C, 30 cycles of 1 minute of hybridization at 40°C, 1 minute of elongation at 70°C, 1 minute of denaturation at 95°C, 1 minute of hybridization at 40°C and finally 10 minutes of elongation at 70°C. Step 3

and 4 were identical to step 2 except that the products of step 2 and step 3, were used as template and dryoa3 / b3 and dryoa4 / b4 as primers, respectively. The introduced NcoI and BamHI sites were used to clone the fragment into the pET28c vector (Novagen). The resulting plasmid was transformed to *E. coli* JM109 cells and all clones were checked for the presence of the insert by SmaI digestion. Sequencing of the resulting pETDPc showed no alterations to the back translated sequence. Mutations F12L and G36P were introduced by site-directed mutagenesis following a procedure based on reference [165] and using oligonucleotides shown in Table 2.1 and T7 primers (Isogen Life Science). The presence of the mutations was confirmed by sequencing.

Table 2.1. Oligonucleotides used for gene synthesis and site directed mutagenesis

Oligonucleotide	**Sequence**
dryoa1	5'-GTGAAACCGGTCACAACATCGTTTTCGACATCCCGGC TGGTGCTCCGGGTACCGTTG-3'
dryob1	5'-GCAGGTCGTTTTCGTCCATAGAAGCAGCTTTCAGTTC GGAAGCAACGGTACCCGGAGCACCAG-3'
dryoa2	5'-GTTTCCGCTGGTGAAGCTGTTGAATTCACCCTGGTTGG TGAAACCGGTCACAAC-3'
dryob2	5'-G**CCCGGG**GTGGAAACTTTAGCTTTGAAGGACGGTTC GTCTTCGGACAGCAGGTCGTTTTCGTC-3'
dryoa3	5'-GGTAACTTCAAATTCTACCCGGACTCCATCACCGTTTC CGCTGGTGAAG-3'
dryob3	5'-CATGTTAGCGGATTTGTGCGGGGTGCAGTAGAAGGTG TAGGTG**CCCGGG**GTGGAAAC-3'
dryoa4	5'-ggtata**ccatgg**CTAAAGTTGAAGTTGGTGACGAAGTTGGT AACTTCAAATTC-3'
dryob4	5'-ctggctcga**ggatcc**tcaTTTAACGGTCAGAGTACCTTTCATGT TAGCGGATTTGTG-3'
dryof12l	5'-GGTATACCATGGCTAAAGTTGAAGTTGGTGACGAAGT TGGTAACCTCAAATTCTACC-3'
dryog36pfor	5'-GGTTGGTGAAACCCCTCACAACATCG-3'
dryog36prev	5'-CGATGTTGTGAGGGGTTTCACCAACC-3'

Capital letters: protein encoding sequence
Small letters: extra nucleotides
Bold: restriction sites NcoI, BamHI and SmaI
Underlined: overlapping regions

Protein expression and purification

Native *D. crassirhizoma* Pc was kindly provided by Prof. Takamitsu Kohzuma (Mito, Japan). It was isolated and purified as described before[77]. Recombinant Pc was produced in *E. coli* BL21(DE3) cells. A single colony of bacteria freshly transformed with pETDPc was incubated in 10 mL LB / kanamycin (50 mg/L) and inoculated at 37º overnight. A 100x dilution was made in 0.5 L 2xYT / kanamycin (50 mg/L) with 0.1 mM copper citrate and incubated at 37º, 250 rpm until the OD_{600} reached 0.7. Expression was induced with 1 mM IPTG and incubation was continued at 30º for 4 hours. Cells were harvested by centrifugation and lyzed with a French pressure cell in presence of 1 mM PMSF and 100 μM $CuNO_3$. Cell debris was removed by centrifugation. Reduced protein was purified using ion-exchange column chromatography with DEAE Sepharose (Amersham Biosciences) in 10 mM sodium phosphate, pH 7.0. The protein was eluted with a gradient of 0-250 mM NaCl. Fractions containing Pc were concentrated and salt was removed by ultra filtration. The chromatography was repeated with Pc in the oxidised state. Then, size exclusion chromatography with Superdex G-75 (Amersham Pharmacia Biotech) was performed in 10 mM sodium phosphate, pH 7.0, 100 mM NaCl. The protein was oxidised with potassium ferricyanide and reduced with sodium ascorbate. Pc concentrations were determined using ε_{590} = 4.7 cm^{-1} mM^{-1} [77] for recombinant wt Pc as well as the mutants. The yield of pure protein was 70 mg/L of culture. Pc was considered pure when the ratio $A_{278}/A_{590} \leq 1.3$ and the protein migrated as a single band on Coomassie stained SDS-gel. ^{15}N-labelled recombinant proteins were produced in M9 minimal medium supplemented with 0.3 g/L $^{15}NH_4Cl$. A single colony of freshly transformed *E. coli* BL21(DE3) was incubated in 10 mL LB / kanamycin (50 mg/L) and inoculated at 37º until the OD_{600} reached 0.6. This preculture was diluted 100x into 0.5 L minimal medium / kanamycin (50 mg/L) and incubated at 37º, 250 rpm until the OD_{600} reached 0.6. Expression was induced with 1 mM IPTG and incubation was continued at 30º overnight. Isolation and purification was done as above, yielding 5 mg of pure protein per litre of culture. For additional ^{13}C labelling the minimal medium was supplemented with 2 g/L ^{13}C-glucose.

Stopped flow kinetics

Stopped-flow experiments were performed using a computer-controlled Applied-Photophysics SX18MV stopped-flow system equipped with a PBP 05-109 Spectrakinetic monochromator. Electron transfer rates were determined in 10 mM sodium phosphate, pH 6 at 50 and 100 mM ionic strength (40/90 mM NaCl). For these measurements horse heart cytochrome *c* was reduced with sodium dithionite and kept under argon to prevent oxidation. Recombinant Pc was oxidised with excess ferricyanide. Excess reductant or oxidant was removed by use of a G25 Sephadex column. The experiment was carried out under pseudo first order conditions. The reaction rate was followed by measuring absorption at 420 nm.

Zn-substitution recombinant Pc

Zn-substitution of recombinant Pc was essentially done as described[166] with the following modifications. Of a 200 mM KCN, 500 mM Tris/HCl, pH 7.0 solution 0.5 mL was added to 0.5 mL 1 mM oxidised Pc. The sample was then loaded on a G25 Sephadex column pre-equilibrated with 1 mM $ZnCl_2$, 50 mM MOPS, pH 7.0. Fractions containing protein were washed with water and 10 mM sodium phosphate, pH 6.5.

NMR sample preparation

For ^{1}H NMR Pc Cu(II) and Zn-substituted Pc were concentrated to 1 mM in 10 mM sodium phosphate, pH 6.5. The copper protein was reduced with 2 mM ascorbate and 0.3 mM trimethylsilyl-d_4-propionate (TSP) was added. For the pH titration followed by ^{1}H NMR the H_2O in the protein solutions was exchanged to D_2O and protein was reduced with 2 mM ascorbate. The pD was adjusted with μL aliquots of 0.1/0.5 M DCl. The sample for assignment of the backbone amide resonances consisted of $^{13}C/^{15}N$ labelled protein in 10 mM sodium phosphate, pH 6.5. Samples for 2D NMR pH titrations contained 1 mM ^{15}N labelled Pc in 50 mM potassium phosphate, pH 8.0 and 2 mM

ascorbate. The pH was adjusted with μL aliquots of 0.1/0.5 M HCl. Argon was flushed through all NMR samples to prevent reoxidation.

NMR spectroscopy

All NMR spectra were recorded at 600 MHz on a Bruker DMX spectrometer at 300 K. The ^{1}H spectra used for the assignment of active site histidine protons were recorded as described in[155,167]. Resonances in the heteronuclear single quantum coherence (HSQC) spectrum of Pc were assigned using 3D HNCACB and HN(CA)CO experiments[168-171]. The assigned resonances were submitted to the Biological Magnetic Resonance Data Bank (BMRB) under accession code 7370. The ^{2}J experiments for the detection of the imidazole ring ^{15}N nuclei[172] was performed using a standard $^{15}N,^{1}H$ HSQC experiment with 1/(2J) = 36 ms, an offset of 144 ppm and a spectral width of 172 ppm for the ^{15}N dimension. No ^{15}N decoupling was performed during ^{1}H detection, producing a cleaner background in the spectrum. The number of complex points in the indirect dimension was 256. Chemical shifts acquired in pH titration experiments were fitted to a model which describes a single protonation event: $\delta = (K_a\ \delta_H + [H^+]\ \delta_L) / (K_a + [H^+])$, where δ_H and δ_L are chemical shifts at high and low pH, respectively.

Electrochemical measurements

Electrochemical measurements and analysis were performed by Dr. Antonio Ranieri and Prof. Marco Sola (Modena, Italy). Cyclic voltammetry (CV) experiments and square-wave voltammetry experiments (SWV, using a frequency 5 Hz and a pulse amplitude of 0.025 V) were performed with a Potentiostat/Galvanostat EG&G PAR model 273 A. A 1 mm-diameter pyrolitic graphite disc (PGE) was used as working electrode, and a saturated calomel electrode (SCE) and a Pt ring as a reference and counter electrode, respectively. Potentials were calibrated against the MV^{2+}/MV^{+} couple (E°'= -0.446 V vs. SHE)(MV = methyl viologen). All the redox potentials reported are referred to the standard hydrogen electrode (SHE). The electric contact between the reference electrode and the working solution was obtained with a Vycor® (PAR) junction. All measurements

were carried out under argon using a cell for small volume samples (V = 0.5 ml) under thermostatic control at 25±0.1 °C. Scan rates varied from 0.02 to 0.2 V s^{-1}. The cleaning procedure of the working electrode is crucial to the voltammetric response. The PGE was first treated with anhydrous ethanol for 5 min, and then polished with alumina (BDH, particle size of about 0.015 μm) water slurry on cotton rug for 3 min; finally the electrode was treated in an ultrasonic pool for about 5 min and used without further treatment. Modification of the electrode surface was performed by dipping the polished electrode into a 1 mM solution of polylysine and morpholine for 30 s, then rinsing it with nanopure water. 15 mM HEPES and 40 mM NaCl were used as base electrolytes. Protein samples were freshly prepared before use and their concentration, in general about 0.1 mM, was checked spectrophotometrically. The pH was changed by adding small amounts of concentrated NaOH or HCl under fast stirring. The experiments were performed several times and the reduction potentials were found to be reproducible within ± 3 mV.

Crystallisation

For crystallisation of the G36P Pc essentially the same conditions were used as for the wt protein[77]. The sitting-drop method was used with ammonium sulphate as precipitant. A drop of 4 μL containing 2.6 M ammonium sulphate, 0.1 M sodium acetate pH 4.5 and oxidised G36P in a final concentration of 0.5 mM was placed above a 1000 μL reservoir containing the same buffer. Single crystals were obtained at 20º C. To reduce the protein, a crystal was placed in mother liquid containing 10 mM sodium ascorbate. The distinct blue colour disappeared after 10 minutes. Data collection and processing as well as analysis of the crystallographic data were performed by Dr. Ellen Thomassen (Leiden, the Netherlands). Data were collected under cryo conditions in 12% glycerol on beam-line BM 14 at a wavelength of 0.95372 Å at the European Synchrotron Facility (ESRF). Data-collection and processing parameters as well as refinement statistics are listed in Table 2.2 and 2.3. The crystal structures were submitted to the Protein Data Bank (codes 2bz7 and 2bzc).

Table 2.2. Data-collection and processing parameters

Data collection	**Oxidised (2BZ7)**	**Reduced (2BZC)**
Crystal dimensions (mm)	0.1 x 0.1 x 0.05	0.1 x 0.1 x 0.05
Wavelength (Å)	0.95372	0.95372
Resolution range (Å)	21.57-1.76	23.90 – 1.79
Crystal system	Hexagonal	hexagonal
Space group	$P6_1$	$P6_1$
Unit cell parameters (Å)	a = 72.92, b = 72.92, c = 29.53	a = 72.98, b = 72.98, c = 29.70
Total No. reflections	99038	187009
No. unique reflections	9095	8720
multiplicity	10.89 (10.98) [a]	21.45 (21.22)[b]
Rsym[c]	0.053 (0.16)	0.059 (0.23)
Completeness (%)	99.8 (100)	100 (100)
Average I/σ (I)	7.62 (4.30)	7.43 (3.25)
solvent content (%)	40.1	40.6
V_M (Å^3 / Da)	2.1	2.1

[a] Data statistics of the outer resolution shell (1.86 – 1.76 Å) are given in parentheses, where applicable.
[b] Data statistics of the outer resolution shell (1.89 – 1.79 Å) are given in parentheses, where applicable.
[c] Rsym = $\Sigma_h \Sigma_i |I_{hi} - \langle I_h \rangle| / \Sigma_h \Sigma_i |I_{hi}|$, where I_{hi} is the intensity of the *i*th measurement of the same reflection and $\langle I_h \rangle$ is the mean observed intensity for that reflection.

Table 2.3. Refinement statistics

Refinement	**Oxidised (2BZ7)**	**Reduced (2BZC)**
Resolution range (Å)	21.57 – 1.76	23.90 – 1.79
number of reflections	8652 (428)[a]	8704 (413)[a]
Rfactor[b]	0.19 (0.24)	0.19 (0.25)
protein atoms / water molecules	761 / 87	761 / 87
r.m.s. deviations bonds (Å)	0.013	0.014
r.m.s. deviations angles (°)	1.505	1.539
Average B value protein / solvent (Å^2)	30.15 / 45.89	28.98 / 40.82
Average B value Cu (Å^2)	26.9	23.7
Ramachandran statistics[c] (%)	91.8 / 8.2 / 0 / 0	91.8 / 8.2 / 0 / 0
ESU[d] based on R value (Å)	0.137 (0.135)	0.142 (0.145)

[a] Data statistics of Rfree are given in parentheses, where applicable.
[b] $R = \sum||F_{obs}(hkl)| - |F_{calc}(hkl)|| / \sum |F_{obs}(hkl)|$
[c] According to the program PROCHECK [173]. The percentages are indicated of residues in the most favoured, additionally allowed, generously allowed and disallowed regions of the Ramachandran plot, respectively.
[d] Estimated overall coordinate error.

Results

Characterisation of recombinant wild type fern Pc

To obtain sufficient amounts of protein and enable mutagenesis a gene encoding *D. crassirhizoma* Pc was synthesised. The nucleotide sequence was obtained by back translation of the amino acid sequence[77] and divided over 4 sets of oligonucleotides (Table 2.1). The gene was then synthesised by polymerase chain reaction (PCR) in 4 subsequent steps. The product was cloned into the pET28c vector and the recombinant Pc was produced in *E. coli*, purified and characterised by mass spectrometry, UV-visible and NMR spectroscopy and stopped flow kinetics. The mass of both native and recombinant Pc is 10774 ± 1 Da. This is close to the theoretical mass of apo fern Pc, of which Met-1 is removed (10776.02 Da). The UV-visible spectrum of recombinant Pc shows the same maxima as the spectrum of native Pc. Reduction by horse heart cytochrome *c* was measured by stopped flow kinetics under pseudo first order conditions. The reduction rate constant at pH 6.0 is 1.3 (± 0.1) × 10^7 M^{-1} s^{-1} at 50 mM ionic strength and 3.9 (± 0.1) × 10^6 M^{-1} s^{-1} and 100 mM ionic strength. The reduction rate constants are comparable to the rate of 4.1 × 10^7 M^{-1} s^{-1}, reported at pH 7.0, ~30 mM ionic strength[174]. They are also similar to those reported for angiosperm Pc. For instance, a reduction rate constant of 3.6 × 10^6 M^{-1} s^{-1} has been reported[175] at 100 mM ionic strength, pH 6.0. The ^{1}H NMR spectrum of recombinant Pc is essentially identical to the native counterpart (Fig. 2.1A).

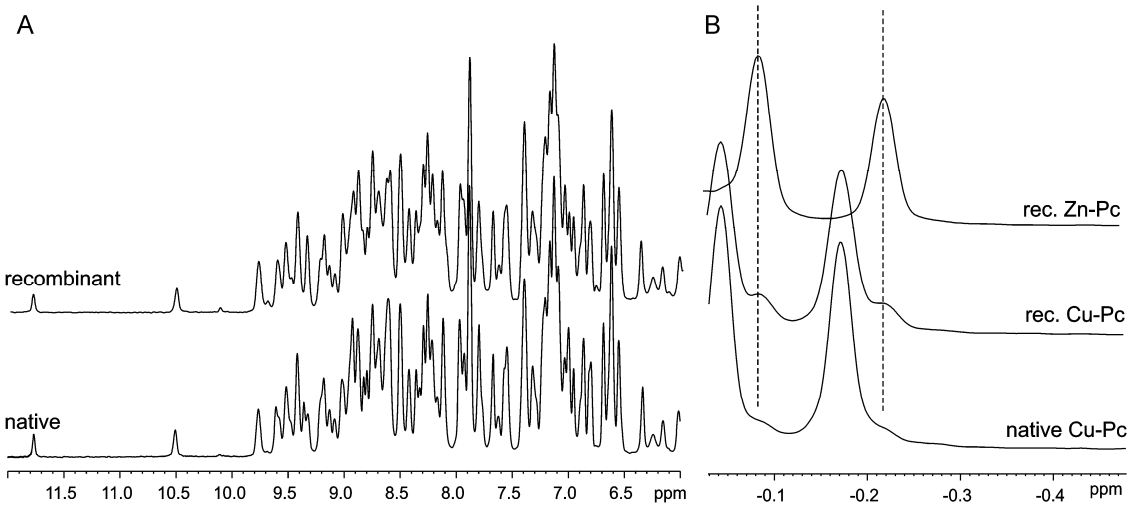

Figure 2.1. A) Amide region of the ^{1}H NMR spectra of native and recombinant Cu(I)-Pc. B) Comparison of upfield part of ^{1}H NMR spectra of native Cu(I)-Pc, recombinant Cu(I)-Pc and recombinant Zn(II)-Pc.

Some low intensity peaks were observed in both native and recombinant spectra, but more strongly for the recombinant species (Fig.2.1B). After comparison with the ^{1}H spectrum of Zn-substituted fern Pc these peaks were identified as corresponding to Zn-substituted Pc. It can be concluded that expression of recombinant Pc in *E. coli* results in the presence of a small fraction of Zn-substituted Pc (5-10%). Zn-Pc is apparently also present in native Pc but in smaller amounts (1-3%). The presence of zinc in blue copper proteins is a well-known phenomenon[176]. It is unclear whether the Zn-Pc is produced in the fern or is a consequence of the purification procedure.

Protonation behavior of His90 ring protons studied by ^{1}H NMR

In order to study the protonation behavior of His90 in fern Pc, mutations were made at Phe12 and Gly36, which were changed to Leu12 and Pro36, the residues most widely found at these positions in Pc's. ^{1}H NMR spectra of wt Pc and the F12L, G36P and F12L/G36P Pc mutants in D_2O were acquired. The singlet signals of histidine ring protons were identified by Hartman Hahn and CPMG experiments[155,167]. We could identify four signals of His37 and His90 protons (see below) in the recombinant wt Pc

spectrum (Fig. 2.2), in agreement with similar experiments done on native fern Pc[85]. As the pD is lowered to 5.8 signals of the His37 ring protons shift no more than 0.02 ppm, while the largest shift of the His90 protons is only 0.05 ppm. However, the resonances of the His90 protons (B and D) show significant broadening at pD 5.8, in line with exchange broadening due to protonation as observed in two-dimensional experiment (see below). At pD=5.4 and below, fern Pc shows significant aggregation, which impairs further ^{1}H NMR experiments.

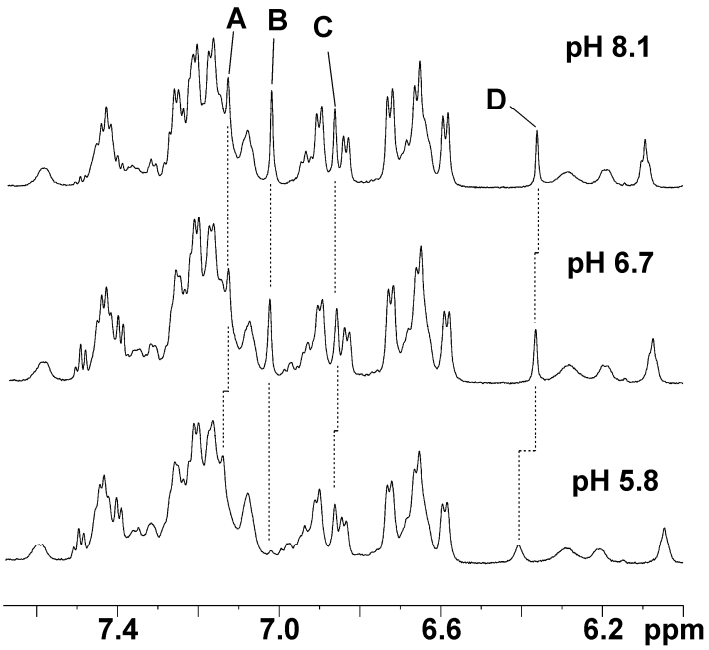

Figure 2.2. Part of ^{1}H NMR spectra of wild type Cu(I)-Pc in D_2O at pD 8.1, 6.7 and 5.8. Histidine peaks are assigned to A: $H_{\varepsilon 1}$(His37), B: $H_{\varepsilon 1}$(His90), C: $H_{\delta 2}$(His37), D: $H_{\delta 2}$(His90).

The histidine ring proton signals in the G36P mutant are all at similar positions as in the wt spectrum (Fig. 2.3). The two signals of His90 show a downfield shift of about 0.4 ppm in the F12L and F12L/G36P mutants (Fig. 2.3). The ring current shift caused by the aromatic ring of Phe12 is not present in these mutants. This confirms that the signals can be assigned to the ring protons of His90, which will be influenced most by the presence of the phenyl ring. Upon lowering of the pH the shifts of the histidine ring protons in the mutants are not significantly different from the wt shifts. This suggests a pH behavior of the mutants that is similar to that of the wt protein.

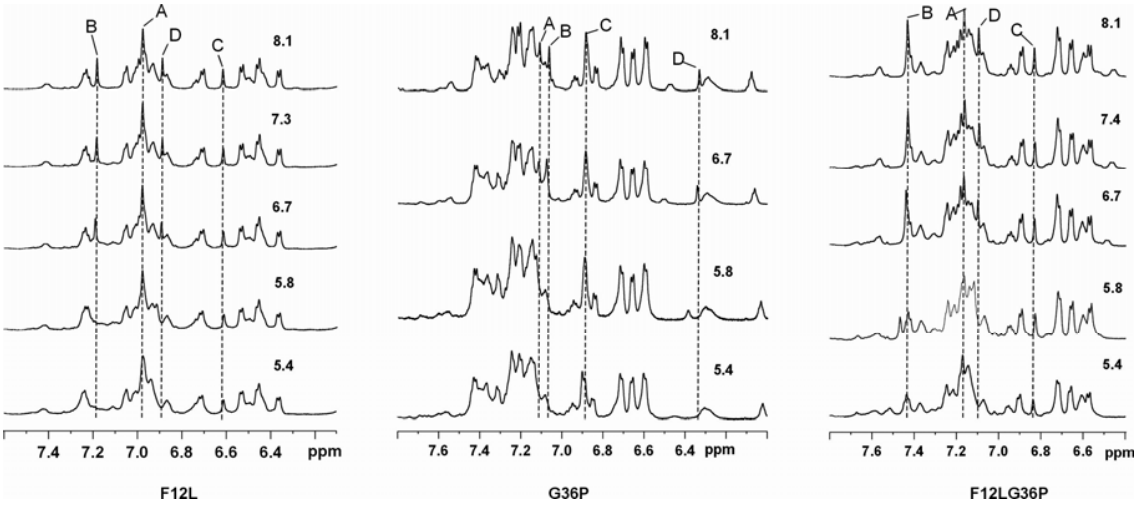

Figure 2.3. ^{1}H NMR spectra of Cu(I)-Pc mutants in D_2O. The numbers next to the spectra are the pD values at which the spectra were obtained. Histidine peaks could be assigned to A: $H_{\varepsilon 1}$ (His37), B: $H_{\varepsilon 1}$ (His90), C: $H_{\delta 2}$ (His37), D: $H_{\delta 2}$ (His90).

His90 backbone amide protonation

To gain a more detailed view of the protonation behavior a pH titration was performed with ^{15}N-labelled fern Pc. The resonances in the ^{15}N, ^{1}H HSQC spectrum were assigned on the basis of HNCACB and HN(CA)CO experiments done on a ^{15}N, ^{13}C-labelled Pc sample. During the pH titration, aggregation, which is a slow process on the NMR time scale, led to loss of peak intensities of the folded form and appearance of new peaks of the denatured form. Because of the high signal-noise ratio acquired by the HSQC experiment, we were able to record spectra of soluble, folded protein down to a pH of 4.0. Residues in the wt Pc spectrum that experience a change in chemical shift were identified. Nearly 73% of the resonances in the spectrum shift significantly upon lowering of the pH. Their chemical shifts in ^{1}H and ^{15}N dimensions were fitted to a pK_a. Surprisingly, the chemical shift of the backbone amide of His90 experiences a large ^{15}N chemical shift change which could be fitted to a pK_a of 4.5 (± 0.1) at 300 K, 50 mM ionic strength (Fig. 2.4A). The chemical shift change in the ^{1}H direction could be fitted to a pK_a of 4.4 (± 0.1). The amide resonances of the other copper ligands and several neighboring residues also experience chemical shift changes with pH. A global fit of these perturbations yields a pK_a of 4.4 (± 0.1) (Fig. 2.4B). This suggests that His90

indeed undergoes protonation and the effect is propagated towards other backbone amides in the vicinity as observed in other cases[148,177,178]. To investigate the effect of removing the phenyl ring that is close enough to have a π–π stacking interaction with His90, spectra were acquired during a pH titration of the F12L mutant. The chemical shift changes of the His90 backbone amide resonance of F12L could be fitted to a pK_a of 4.4 (± 0.1), which is equal to the value derived from a global fit (Fig. 2.4C). It can be concluded that the F12L mutation does not influence the pK_a of His90. The G36P mutation does have a small effect on the pK_a. The value derived from chemical shift changes in the amide backbone of His90 and from a global fit (Fig. 2.4D) is 4.7 (± 0.1). Finally, this small effect is not observed in the double mutant, F12LG36P, where His90 has a pK_a of 4.4 (± 0.1) (Fig. 2.4E).

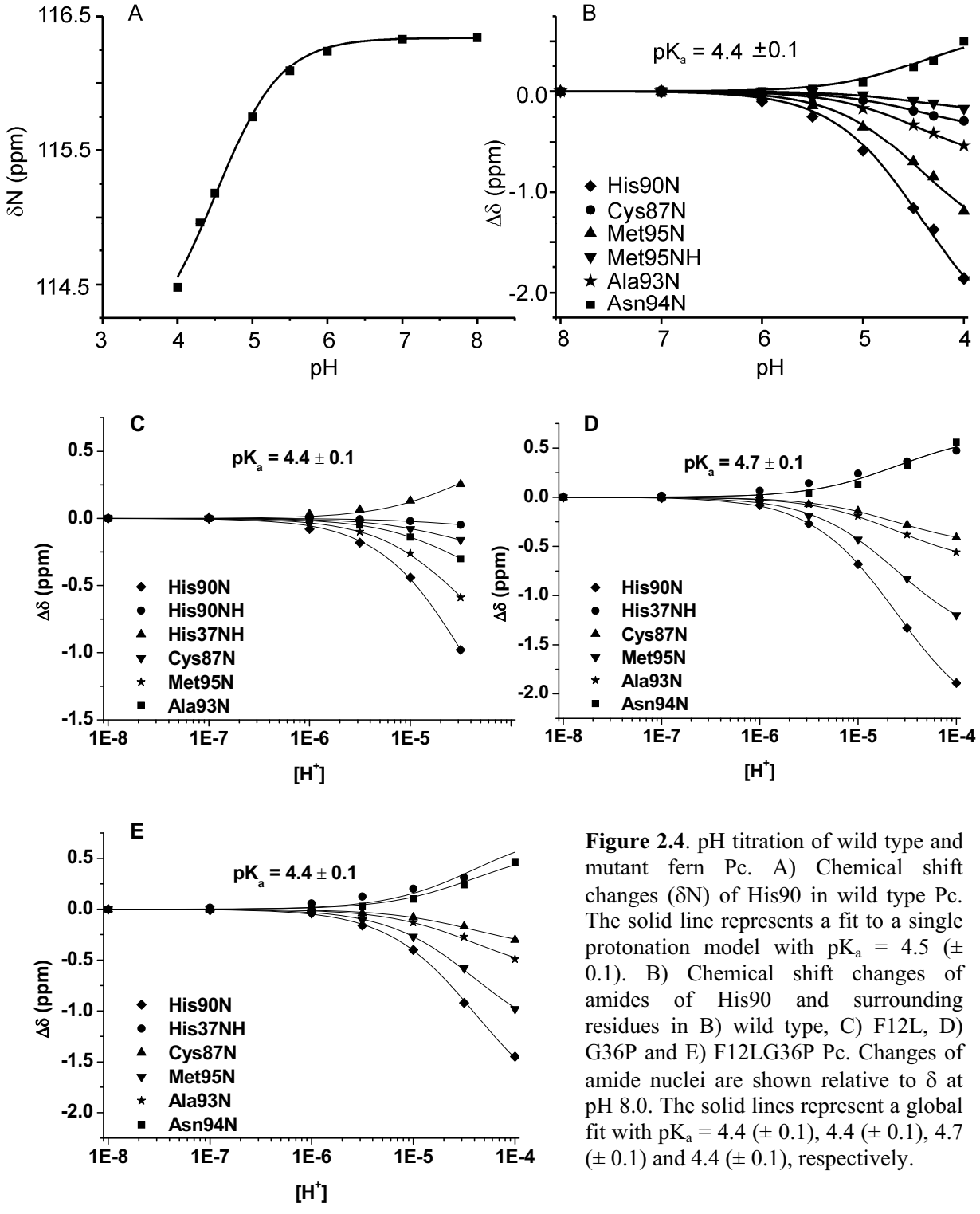

Figure 2.4. pH titration of wild type and mutant fern Pc. A) Chemical shift changes (δN) of His90 in wild type Pc. The solid line represents a fit to a single protonation model with pK_a = 4.5 (± 0.1). B) Chemical shift changes of amides of His90 and surrounding residues in B) wild type, C) F12L, D) G36P and E) F12LG36P Pc. Changes of amide nuclei are shown relative to δ at pH 8.0. The solid lines represent a global fit with pK_a = 4.4 (± 0.1), 4.4 (± 0.1), 4.7 (± 0.1) and 4.4 (± 0.1), respectively.

Protonation behavior of His90 ring protons studied by 2D NMR

It is possible that the arc of acidic side chains, including Glu8, Glu34, Glu68, Asp69 and Glu70 in the proximity of the active site could cause the pK_a of 4.4 for the amide of His90, which is lower than found for other Pc's. To confirm that the imidazole ring of His90 becomes protonated, we performed ^2J coupled 2D NMR experiments on the same samples used for the HSQC experiments. The ^2J coupled experiment allows direct detection of the $N_{\delta1}$ and $N_{\varepsilon2}$ nuclei of His residues, via coupling to the $H_{\delta2}$ and $H_{\varepsilon1}$. Upon protonation the histidine $N_{\delta1}$ resonance shifts approximately -56 ppm, while the $N_{\varepsilon2}$ resonance shift is smaller[172].

The histidines that coordinate the copper are the only histidines present in fern Pc. The resonances of their ring protons could be assigned in the wt and mutant ^2J spectra (Fig. 2.5A). Note that an $H_{\varepsilon2}$ resonance is also observed in this experiment at 11.62 ppm. The resonance is split by ^1J-coupling in the ^1H dimension because the spectra were acquired without ^{15}N decoupling. In other blue copper proteins, the analogous resonance has been assigned to the $H_{\varepsilon2}$ of the N-terminal His of the copper ligands, because this proton always forms a strong internal hydrogen bond, for instance to Thr35 in poplar Pc[179]. Analogously, this resonance can be assigned to His37 of fern Pc. In the crystal structure this $H_{\varepsilon2}$ is hydrogen bonded to a buried water molecule, which would prevent its fast exchange with the solvent in solution. This leads to the assignment of the other His37 ring protons and consequently, those of His90. The assignments agree with the resonances found in the 1D experiments. Upon lowering of the pH, the signals of the His37 ligand ring protons shift slightly in both proton and nitrogen dimensions. This suggests that His37 does not become protonated, but experiences a protonation event in the neighborhood. The signals of the His90 ring protons, however, behave differently. Below pH 5.5 the signals of $H_{\varepsilon1}$ and $H_{\delta2}$ coupled to $N_{\varepsilon2}$ (~165 ppm) can no longer be detected, in accordance with the broadening observed in the 1D spectra (Fig. 2.2). The signal of $H_{\varepsilon1}$ coupled to $N_{\delta1}$ (~237 ppm) disappears from the spectrum at pH 6.0. This behavior is the same as observed for pseudoazurin (kindly provided by Dr. Antoinetta Impagliazzo and Dr. Monica Vlasie), upon titration of its exposed histidine ligand (Fig. 2.6). The large chemical shift change for the ^{15}N ring nuclei results in exchange broadening, most strongly for $N_{\delta1}$.

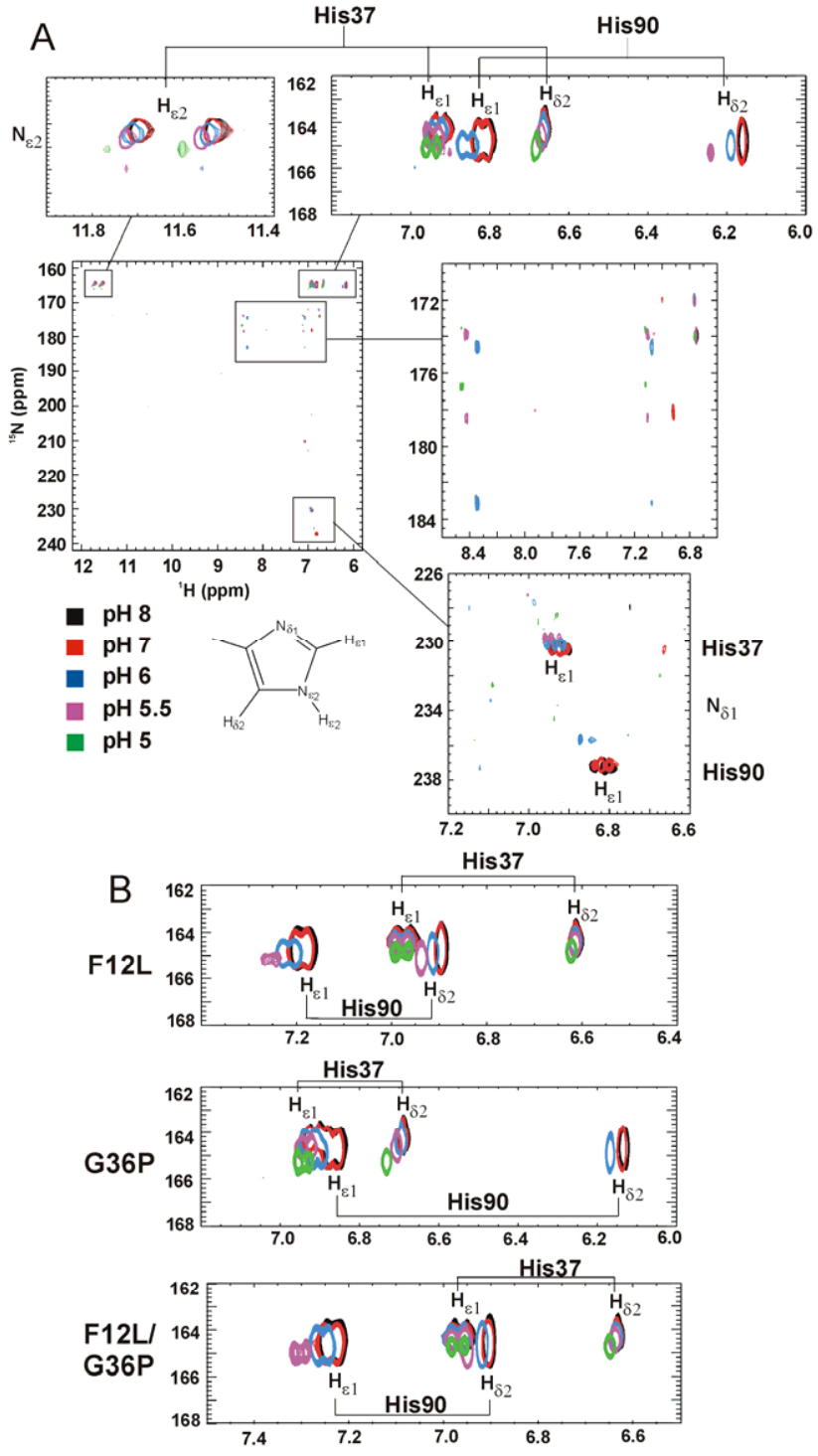

Figure 2.5. Histidine ring protonation. Overlay of ^{2}J coupled NMR spectra of wild type Pc (A) and F12L, G36P and F12LG36P Pc (B) at pH 8 (black), pH 7 (red), pH 6 (blue), pH 5.5 (magenta), pH 5 (green). Resonances of histidine protons are labelled. Areas of interest are enlarged for clarity.

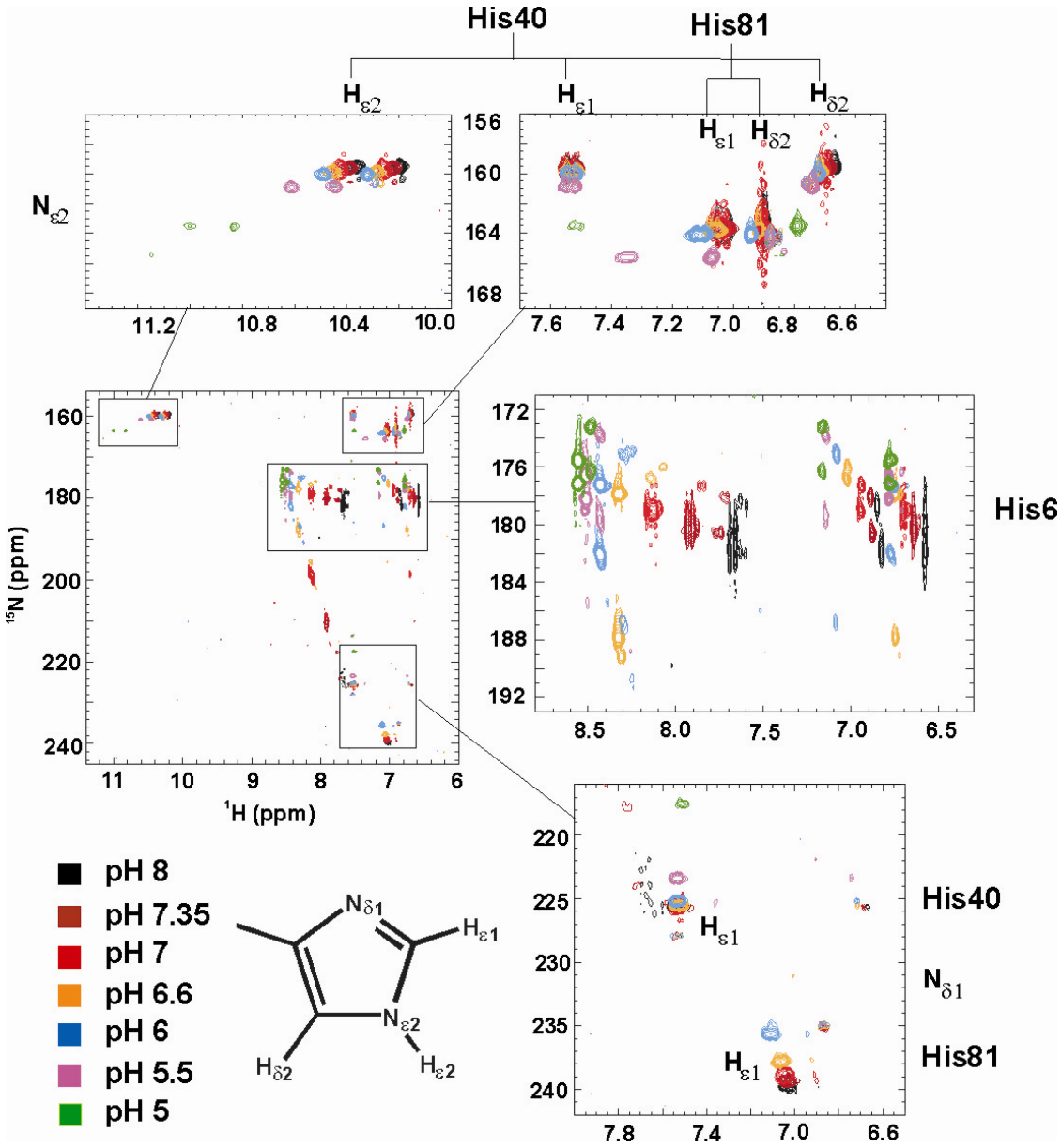

Figure 2.6. Overlay of ^{2}J coupled NMR spectra of wild type pseudoazurin, at pH 8 (black), pH 7.35 (brown), pH 7 (red), pH 6.6 (orange), pH 6.0 (blue), pH 5.5 (magenta), pH 5 (green). Resonances of histidine protons are labelled. Area's of interest are enlarged for clarity.

pH dependence of the reduction potential of fern Pc

Fern Pc shows a CV signal in the pH range of 7-2.5 at 25 °C, due to an electrochemically quasi-reversible monoelectronic reduction/oxidation of the Cu ion. Peak-to-peak separations vary between 60 to 90 mV within the range of scan rates investigated. Anodic and cathodic peak currents were found to be identical, and both were proportional to protein concentration and $\nu^{1/2}$ (ν= scan rate), indicating a diffusion controlled electrochemical process. Under these conditions, the $E_{1/2}$ values can be assumed to represent the E°' values. Potential values (E°') of +376 mV, +363 mV, +364 mV and +352 mV (± 3mV) have been determined at pH 7 for the wt, G36P, F12L and F12LG36P species, respectively. These values are in the range expected for blue copper proteins[82,164,180,181]. The reduction potential of recombinant wt fern Pc is similar to that for the native protein[85] (+376 vs. +382 mV). The small decrease in E°' for the F12L and G36P mutations of 10 mV appears to be additive as the change is 20 mV in the double mutant. When the pH is lowered below 7, the reduction potential increases (Fig. 2.7). This behavior has been observed elsewhere for Pc's[82,85,146,182], and other blue copper proteins[183], and has been attributed to a coupling of copper(II) reduction to histidine ligand protonation and detachment from the metal. Down to pH 4.5, the change in E°' of the wt protein is consistent with the behavior observed previously for native fern Pc[85]. At lower pH values, a pronounced potential increase is observed. The slope of the E°'/pH profile is 27 mV/pH, lower than the theoretical value of 59 mV/pH. This could be the result of protein unfolding occurring at these low pH values. The slope is similar for the F12L and G36P mutant, but smaller (15 mV/pH) for the double mutant. This could be due to more pronounced unfolding at low pH values.

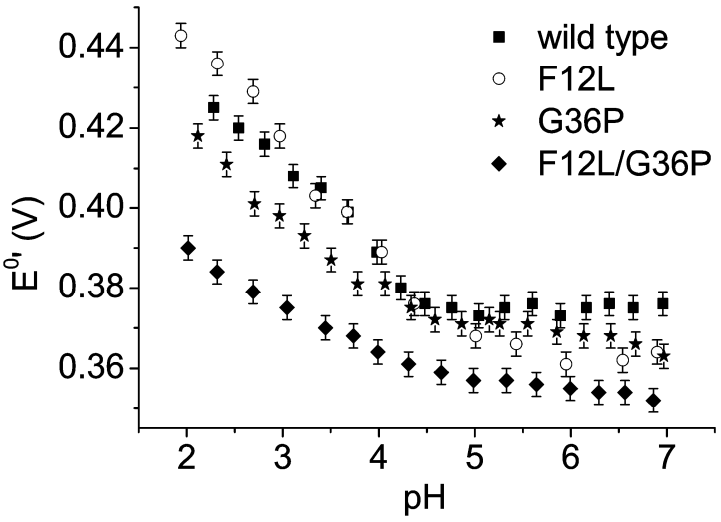

Figure 2.7. pH dependence of the reduction potential for wild type and mutant fern Pc.

Crystal structure of G36P plastocyanin

Crystals of the G36P mutant were grown at pH 4.5 and diffracted to a resolution of 1.7 and 1.8 Å for the oxidised and reduced protein, respectively. It was not possible to grow crystals of F12L or F12LG36P mutants even after extensive screening. The structure of the G36P protein was determined by molecular replacement with MOLREP[184], using the oxidised and reduced wt structures as models[185]. The average root-mean-square deviation (rmsd) between the wt and G36P Pc structures is 0.37 Å and between oxidised and reduced G36P Pc 0.24 Å. The differences observed between oxidised and reduced G36P Pc have also been reported for the oxidised and reduced wt Pc[185]. As in the reduced wt Pc at this pH, His90 in the G36P mutant does not flip and move away from the copper. The distances from the copper to all four ligands do not change significantly between the oxidised and reduced structures (Table 2.4) or from the wt structure. The apparent discrepancy between NMR and crystallographic data may be explained by the presence of crystal contacts around His90. Glu4 of the neighboring unit and a water molecule are found close to His90 (Fig. 2.8). The distance between the $O_{\varepsilon 1}$ of Glu4 and the water is 2.69 Å and that between $N_{\varepsilon 2}$(His90) and the water molecule is 2.92 Å. Apparently, the proximity of the neighboring asymmetric unit favors crystallization of the deprotonated fern Pc.

Table 2.4. Dimensions of the copper site in oxidised and reduced G36P fern Pc at pH 4.5.

	Oxidised Pc[a]	**Reduced Pc**[a]
Bond lengths (Å)		
Cu-N (His 37)	2.09	2.02
Cu-N (His 90)	2.14	2.14
Cu-S (Cys 87)	2.20	2.23
Cu-S (Met 95)	2.89	2.93
Bond angles (degrees)		
N (His 37)-Cu-N (His 90)	100.8	102.7
N (His 37)-Cu-S (Cys 87)	130.1	130.4
N (His 37)-Cu-S (Met 95)	81.7	81.2
N (His 90)-Cu-S (Cys 87)	123.2	121.8
N (His 90)-Cu-S (Met 95)	105.7	106.2
S (Cys 87)-Cu-S (Met 95)	104.8	104.2

[a] The ESU (estimated overall coordinate error) value for both structures is 0.14 Å.

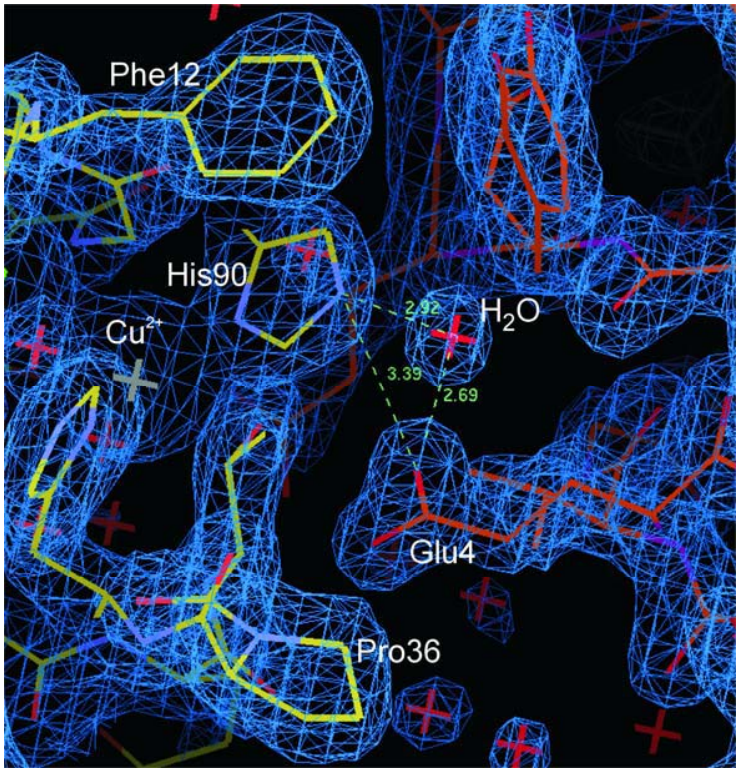

Figure 2.8. His90 in the crystal structure. $2F_0$ -F_c electron density map contoured at 1.5 σ of the area around the Cu-site of reduced G36P fern Pc (yellow), with nitrogen atoms in blue and oxygen atoms in red. Distances (Å) between $N_{\varepsilon 2}$ of His90, crystalline water and $O_{\varepsilon 1}$ of Glu4 in the neighbouring asymmetric unit (red and purple) are shown in green.

Discussion

Recombinant fern Pc was used to study the reported unusual pH dependent behavior of the solvent exposed histidine Cu-ligand. From our spectroscopic and mass spectrometry characterization as well as from comparison of the 1D ^{1}H NMR experiments[85] it can be concluded that the native and recombinant proteins are the same. These experiments show small chemical shift changes of the histidine ring protons in the ^{1}H dimension (Fig. 2.2) down to pH 5.4. At lower pH, aggregation impairs further experiments. This problem is less pronounced in the 2D HSQC experiments, because these are more sensitive. Another advantage is that more residues can be observed, gaining a more detailed picture. Chemical shift changes in the backbone amide of His90 of the wt protein could be fitted to a pK_a of 4.5 (± 0.1) (Fig. 2.4A). This corresponds well to the value 4.4 (± 0.1) from a global fit of chemical shift changes in the other copper ligands and surrounding amides (Fig. 2.4B). The ^{2}J coupled experiment shows that indeed the imidazole ring of His90 is protonated. Therefore it can be concluded that, contrary to previous reports, protonation of His90 in fern Pc does occur.

The relatively low pK_a value in combination with aggregation at low pH explains why it was difficult to determine this value before. 1D ^{1}H NMR experiments can only be done down to pH 5.4, which explains why protonation in fern Pc is difficult to detect with that method. Furthermore, it is well established that protonation in Pc is accompanied by a large increase in the midpoint potential[158,186]. As reported previously[85] the reduction potential for fern Pc does increase at lower pH, consistent with protonation. Although potentials could be measured at pH values below 4.3, it is not possible to fit this increase to a single acid-base equilibrium equation, probably due to the presence of unfolded protein.

Crystal structures have been a crucial method in studying the protonation behavior of blue copper proteins. The 'flipping' histidine was first shown in poplar Pc[187] and later for *A. faecalis* pseudoazurin[156]. Also, it was shown that histidine protonation is prevented in amicyanin when bound to MADH[150]. The crystals of poplar Pc showing a flipped histidine ligand have a space group different from that of fern Pc, leaving ample space for the histidine to make a 180º rotation. In the case of fern Pc, crystal packing forces appear

to favor the crystallization of the deprotonated form. Our work shows that sometimes differences can be found between results obtained in the solution and the crystalline states.

Mutations were made around the active site to test whether π–π stacking with Phe12 or hydrogen bond formation with the backbone oxygen of Gly36 influences the protonation behavior. The G36P crystal structure at pH 4.5 does not show significant differences from the wt structure. No crystals could be obtained for either of the Phe12 mutants, so it cannot be excluded that this mutation has a subtle effect on the structure. The NMR spectra indicate that no major change occurs. To our surprise both the F12L and G36P mutations and the double mutation do not cause a large change in the pK_a of His90. This suggests that Phe12 and Gly36 do not contribute significantly to the protonation behavior of the His90 copper ligand in fern Pc, contrary to what has been reported for pseudoazurin. An increased reduction potential and decreased pK_a were observed in a pseudoazurin mutant in which a π–π interaction was introduced[147].

The explanation for the relatively low pK_a value found for fern Pc remains as yet unclear. It may be related to the unique position of the acidic patch surrounding the hydrophobic patch in fern Pc, which will create an electrostatic environment different from that in other Pc's.