Transient interactions between photosynthetic proteins

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Chapter VI

Effect of macromolecular crowders on the transient *Ph. laminosum* plastocyanin - cytochrome *f* complex

Abstract

The presence of large amounts of macromolecules in the cellular environment, described by the term macromolecular crowding affects proteins and protein-protein complexes. The effect of macromolecular crowding on transient protein-protein association was investigated by NMR. Initially, the effect of four high molecular weight crowders on the 2D NMR spectrum of ^{15}N-Src-SH2, a small globular protein was tested. It was found that addition of up to 20% Ficoll70 leads to minimal line broadening and the least amount of signal loss. For this reason, *Ph. laminosum* ^{15}N-Pc was titrated with increasing amounts of cyt*f* in the presence of 20% Ficoll70. The resulting binding constant of the Pc- cyt*f* complex is not significantly different from the binding constant in the absence of crowder. The similarity between the chemical shift perturbation maps in the absence and presence of crowding agent also indicates that the binding is not detectably affected by the presence of Ficoll70. Both observations support the idea that high molecular weight crowders show low 'microviscosity' and are not uniformly distributed. The question remains whether this is a appropriate representation of *in vivo* crowding conditions.

Introduction

The dilute solutions in which proteins and protein complexes are often studied are different from the cellular environment in which these proteins normally function[226]. Inside an *E. coli* cell, for example, the total concentration of proteins and RNA is approximately 300-400 g/l^{-1} [227]. The presence of a large amount of other macromolecules in high concentrations, described by the term macromolecular crowding can have effects on both diffusion and the effective volume available to proteins and protein complexes[228-234]. Generally, reaction rates can decrease due to diffusion limitations or increase because the activity of reactants rises through a decline in effective volume. Alternatively, both effects can cancel each other out. The effective volume available depends on the size of the proteins with respect to the other molecules crowding the solution. In order to mimic this situation *in vitro* macromolecular crowders are added to the reactants. The effects of these crowders on protein-protein association have been studied by a multitude of approaches including stopped-flow kinetics, fluorescence spectroscopy and isothermal titration calorimetry[235-239]. All results indicated crowders have detectable effects on protein-protein association. It was also suggested that the properties of high molecular weight crowders better mimic the intracellular environment then low molecular weight crowders[237]. NMR studies have shown that the presence of crowders stabilises intrinsically disordered proteins, such as FlgM[240] and α-synuclein[241]. Also, a ^{13}C-NMR off-resonance rotating frame spin-lattice relaxation study has shown that high concentrations of γ-crystallin in the ocular mammalian lens leads to self-association[242]. However, the effects of macromolecular crowding on the binding interface and structure of protein-protein complexes have not been studied. Here, we investigated the transient complex of Pc and cyt*f* under crowding conditions with NMR. The complex plays an important role in photosynthetic electron transport in all oxygenic organisms. Cyt*f* is part of a membrane protein complex (cytochrome b_6f) and the interactions with Pc takes place in the lumen of chloroplasts. Therefore, the *in vivo* conditions in which the complex functions might be different to those in the *E. coli* cytoplasm. Still, it was shown that effects of molecular crowding can provide an insight in the well-studied reaction kinetics of this complex[238]. The SH2 domain of Src from mouse was used as a test system for the

effect of molecular crowders on the 2D NMR spectrum of small globular proteins (~13 kDa). Four different crowders were compared to assess the influence of their different physical properties.

Materials and Methods

Removal of leader peptide from Ph. laminosum Pc

A new construct for cytoplasmic expression of the *Ph. laminosum* Pc gene (*petE*) was created. In order to remove the endogenous leader sequence from *petE*, an oligonucleotide encoding the translation initiation site and 13 subsequent bases was designed as forward primer (5'-pCGTATACCATGGAAACCTTCACCG-3'). A standard T7 primer (Isogen Life Science) was taken as backward primer. The pET11Pc plasmid was used as a template in a PCR reaction consisting of one cycle of 5 minute denaturation at 95°C, 30 cycles of 1 minute of hybridization at 53°C, 1 minute of elongation at 70°C, 1 minute of denaturation at 95°C and finally 10 minutes of elongation at 70°C. The resulting fragment was cloned back into the pET11d vector (Novagen) using the NcoI and BamHI restriction sites. Sequencing of the resulting plasmid pET11Pcnl showed the removal of the leader sequence and an unaltered coding sequence.

Protein production and purification

For the production of *Ph. laminosum* ^{15}N-Pc competent BL21 *E. coli* cells were transformed with the pET11Pcnl plasmid and incubated overnight at 37° on a LB/Amp (0.1 g/L ampicillin) plate. A single colony was inoculated in 10 mL LB/Amp medium and incubated overnight at 37°, shaking at 250 rpm. Five mL of the pre-culture was inoculated in 0.5 L M9 minimal medium[222] supplemented with 0.3 g/L $^{15}NH_4Cl$ and 0.1 g/L ampicillin in a 2 L flask. The cultures were incubated at 37°, shaking at 250 rpm before induction with 1 mM IPTG at an OD_{600} of 0.7. At the same time the temperature was lowered to 30° and growth was continued for five hours before harvesting. Cells were resuspended in the minimal volume of buffer (5 mM TRIS pH 7.5), followed by

addition of 5 mg DNase and lysozyme, 1 mM PMSF and 5 mM $CuNO_3$. Cytoplasmic extracts of the harvested cells were obtained through use of the French press cell. Cell debris was removed by ultra-centrifugation at 30.000 rpm after which the supernatant was dialysed against 5 L of 5 mM TRIS pH 7.5 for 3 hours and overnight at 4°. Reduced Pc was purified using ion exchange chromatography with DEAE sepharose (Amersham Biosciences) in 5 mM TRIS pH 7.5. Pc was eluted with a gradient of 5-50 mM TRIS pH 7.5. The fractions containing Pc were concentrated and the ion exchange chromatography was repeated with oxidised Pc. Size exclusion chromatography was performed with Superdex-G75 (Amersham Pharmacia Biotech) in 50 mM TRIS pH 7.5, 100 mM NaCl. Pc was oxidised with potassium ferricyanide and reduced with sodium ascorbate. The Pc concentration was determined by optical spectroscopy using $\varepsilon_{597} = 4.3\ mM^{-1}$ for oxidised Pc[243]. Pc was considered pure when $A_{278}/A_{597} \leq 2.5$ [202]. The yield of pure ^{15}N-Pc after purification was 5 mg/L. Comparison with periplasmically expressed Pc was made with optical spectroscopy, mass spectroscopy and 2D NMR.

Ph. laminosum cyt*f* was produced and purified as described before[40], with the exception that growth under semi-anaerobic conditions was achieved by rotation of 1.7 L culture in 2L flasks at 180 rpm, instead of 100 rpm. The cyt*f* concentration was determined by optical spectroscopy using $\varepsilon_{556} = 31.5\ mM^{-1}$ for reduced cyt*f* [202]. Yields of up to 10 mg/L of cyt*f* after purification were obtained.

^{15}N-Src-SH2 was produced from plasmid pSRC_mouse in BL21 *E. coli* cells in M9 minimal medium[222] supplemented with 0.3 g/L $^{15}NH_4Cl$ and 50 mg/L kanamycin. Gene expression was induced with 0.5 mM IPTG when OD_{600} reached 0.6 and incubation at 37° and 250 rpm was continued for three hours. Cells were lysed as described above. The protein was purified using affinity chromatography with Ni-NTA (Qiagen) in 20 mM TRIS pH 8.0, 0.5 M NaCl, 10 mM imidazole. The protein was eluted with 100 mM imidazole. Protein concentration was determined with the theoretical[244] $\varepsilon_{280} = 14.4\ mM^{-1}\ cm^{-1}$.

NMR experiments

All protein samples were concentrated by ultrafiltration (Amicon, MW cut-off 10 kDa for cyt*f* and 5 kDa for Pc and Src-SH2). The pH was adjusted with µL aliquots of 0.1 or 0.5 M HCl.^{15}N-Src-SH2 samples contained 1 mM of the protein in 20 mM sodium phosphate pH 6.5, 6% D_2O. Pc samples contained 10 mM sodium phosphate, pH 6.0, 6% D_2O and 2 mM sodium ascorbate. Argon was flushed through Pc samples to prevent reoxidation. Aliquots of a 1.33 mM cyt*f* stock solution were added to samples of 0.6 mM ^{15}N-Pc. Ficoll70 (Sigma-Aldrich), dextran T70 (Fluka), poly-ethylene glycol 6000 (PEG-6000, Merck) and bovine serum albumin (BSA, Sigma-Aldrich) were purchased and used without further purification. Stock solutions of 40% (w/v) crowder in 20 mM sodium phosphate, pH 6.5, 6% D_2O were prepared.

NMR spectroscopy

All NMR spectra were recorded at 300 K on a Bruker DMX600 spectrometer. ^{15}N,^{1}H HSQC were obtained with spectral widths of 27 ppm (^{15}N) and 13.5 ppm (^{1}H) for ^{15}N-Src-SH2 and 31.3 ppm (^{15}N) and 12 ppm (^{1}H) for ^{15}N-Pc. Data were processed with AZARA 2.7 [203] and analysed in ANSIG for Windows[204].

Binding curves and chemical shift mapping

Chemical shift titration curves were analysed with a two-parameter non-linear least-squares global fit to a 1:1 binding model, which corrects for dilution effects [16,38]:

$$\Delta\delta_{bind} = \frac{1}{2}\Delta\delta_{\max}\left(A - \sqrt{(A^2 - 4R)}\right) \qquad (3b)$$

$$A = 1 + R + \frac{PR + C}{PCK_a} \qquad (3c)$$

where R is the [cyt*f*] : [^{15}N-Pc] ratio, $\Delta\delta_{bind}$ is the chemical shift perturbation at a given R, $\Delta\delta_{max}$ is the chemical shift perturbation at 100% bound ^{15}N-Pc, P is the initial [^{15}N-Pc], C is the stock concentration of cyt*f* and K_a is the association constant of the complex.

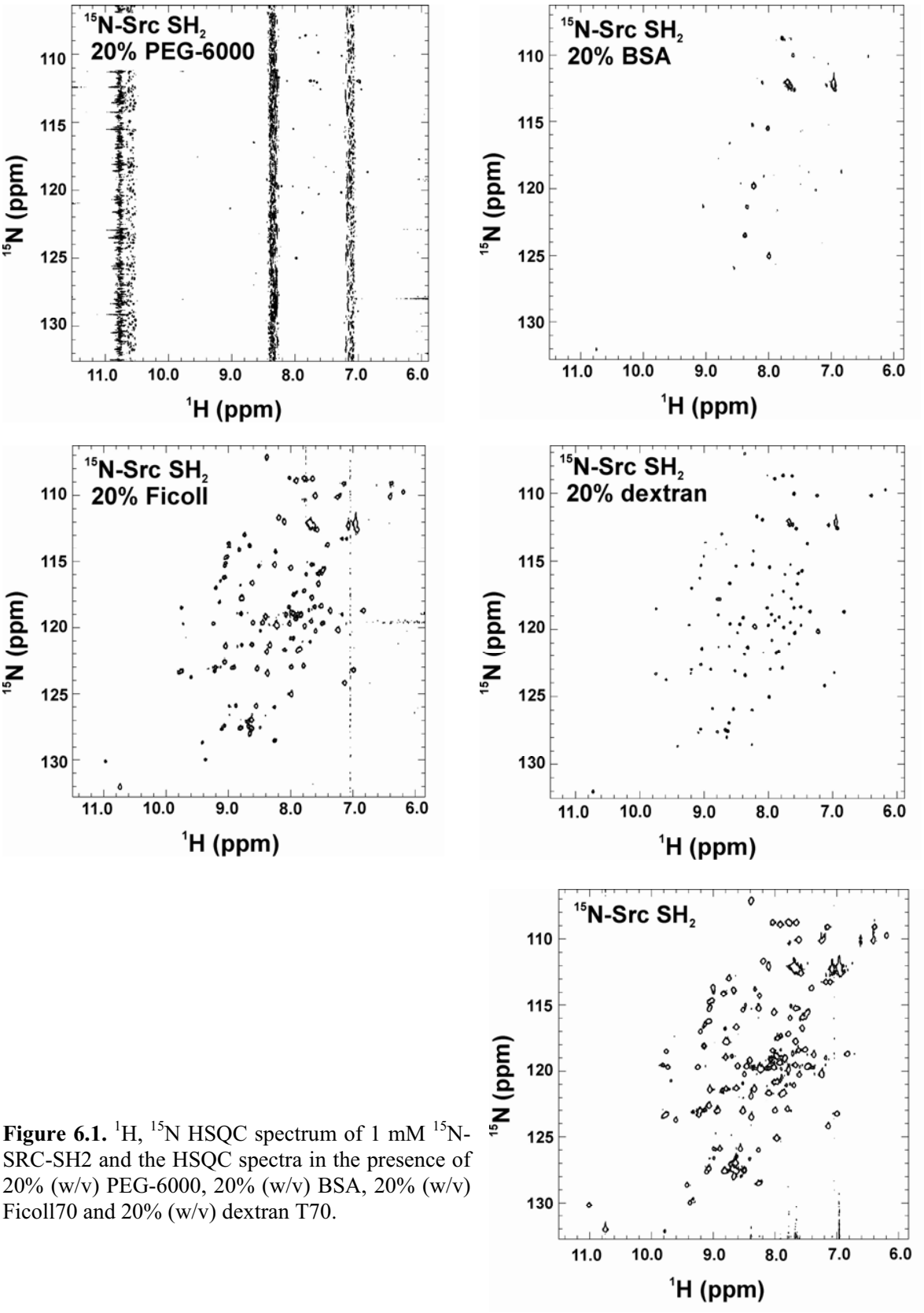

Figure 6.1. ^{1}H, ^{15}N HSQC spectrum of 1 mM ^{15}N-SRC-SH2 and the HSQC spectra in the presence of 20% (w/v) PEG-6000, 20% (w/v) BSA, 20% (w/v) Ficoll70 and 20% (w/v) dextran T70.

Results and discussion

Effects of various high molecular weight crowders on ^{15}N-Src-SH2

In order to determine the effect of four high molecular weight crowders on the ^{15}N,^{1}H HSQC spectrum of a small globular protein, ^{15}N-labelled Src-SH2 (~13 kDa) was titrated with dextran T70, Ficoll70, PEG-6000 and BSA. Dextran T70 (average MW 70 kDa) is a non-ionic, linear flexible, polysaccharide with few and short branches[245,246]. Ficoll70 (average MW 70 kDa) is a non-ionic, highly branched, synthetic polymer of sucrose and epichlorohydrin which approximates a sphere more closely than dextran[245,246]. PEG-6000 (average MW 60 kDa) is a non-ionic, linear, non-branched polymer of ethylene glycol. Bovine serum albumin is the only charged crowder with pI of 4.7 and a molecular weight of 66 kDa. It has been shown for several crowders that the macroscopic viscosity is not equal to the microscopic viscosity experienced by proteins or probes. In fact, diffusion of molecules was much faster than expected from the macroscopic viscosity of these crowders[237,247-250]. Spectra were recorded at 1%, 5%, 10% and 20 % (w/v) of each crowder. Comparison of the resulting spectra at 20% of each crowder (Fig. 6.1) shows a clear difference between these crowders. The presence of charged BSA leads to an inhomogeneous decrease in the intensity of the resonances, indicating a large increase in the rotational correlation time, which may be attributed to interactions between BSA and SH2. However, the same effect is observed for uncharged PEG-6000, which in addition disturbs the spectrum in the ^{1}H dimension. It can therefore not be ruled out that microscopic viscosity influences the rotational correlation time in the presence of both PEG-6000 and BSA. Both dextran T70 and Ficoll70 have only small effects on the SH2 signal. The presence of 20% Ficoll70 leads to the least decrease in intensity of the SH2 resonances. From these results it was expected that a reasonable signal to noise ratio can also be recorded for ^{15}N-Pc in a solution with up to 20% Ficoll70. Higher concentrations of crowders could not be investigated due to experimental limitations. This is still well in the range of physiological significance because, for instance, 5% Ficoll70 represents the approximate volume occupancy found *in vivo*[251].

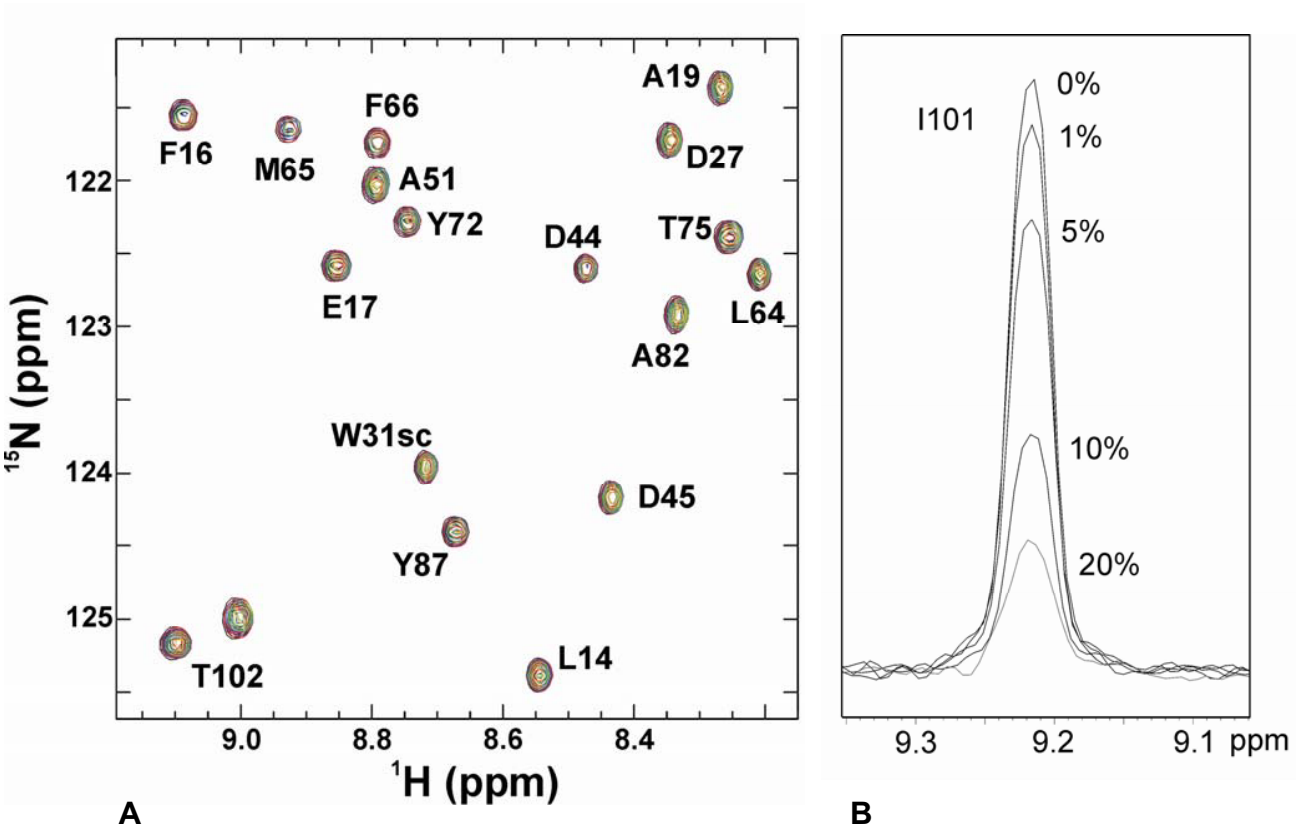

Figure 6.2. A) Overlay of part of ^{1}H-^{15}N HSQC of *Ph. laminosum* Pc in the presence of 0% (black), 1% (red), 5% (blue), 10% (green), 20% (orange) Ficoll70. B) Cross-sections through $^{1}H^{N}$ Pc resonance of Ile101 at various concentrations of Ficoll70.

Effects of Ficoll70 on the NMR spectrum of ^{15}N- Pc

Resonances of *Ph. laminosum* ^{15}N-Pc produced in the cytoplasm have similar chemical shifts to those reported for *Ph. laminosum* Pc from the periplasm[40], with the exception of one extra peak, most likely corresponding to the amide of Glu1. Mass spectroscopy on cytoplasmic Pc revealed that initial Met is not cleaved off from Pc produced in the cytoplasm. In the case of periplasmic expression it is removed together with the leader peptide. This means that Glu1 in cytoplasmically expressed Pc is no longer the N-terminal residue and the Glu1 amide is detectable in the 2D NMR spectrum.

In order to assess the effects of Ficoll70 on ^{15}N-Pc, a titration with 1%, 5%, 10% and 20% Ficoll70 was performed. An overlay of part of the recorded spectra is shown in Figure 6.2A. A representative cross-section of one of the resonances (Fig. 6.2B) indicates

that line-broadening is only limited. This shows that the rotational correlation of Pc is not dramatically changed by the addition of 20% Ficoll70. The decrease in intensity can partly be explained by the decrease in protein concentration due to the addition of crowder stock solution as well as a little protein precipitation at higher crowder concentration. As has been reported before[237], semi-dilute solutions of high molecular mass crowders can be inhomogeneous at the microscopic level. This could lead to a fraction of immobilised and therefore NMR-undetectable protein and a fraction of protein which is affected only by a small increase in (microscopic) viscosity. This would explain the observed decrease in intensity and lack of significant change in rotational correlation time. Apparently Ficoll70 can be used as macromolecular crowder without interfering too much with the 2D NMR spectrum of ^{15}N-Pc.

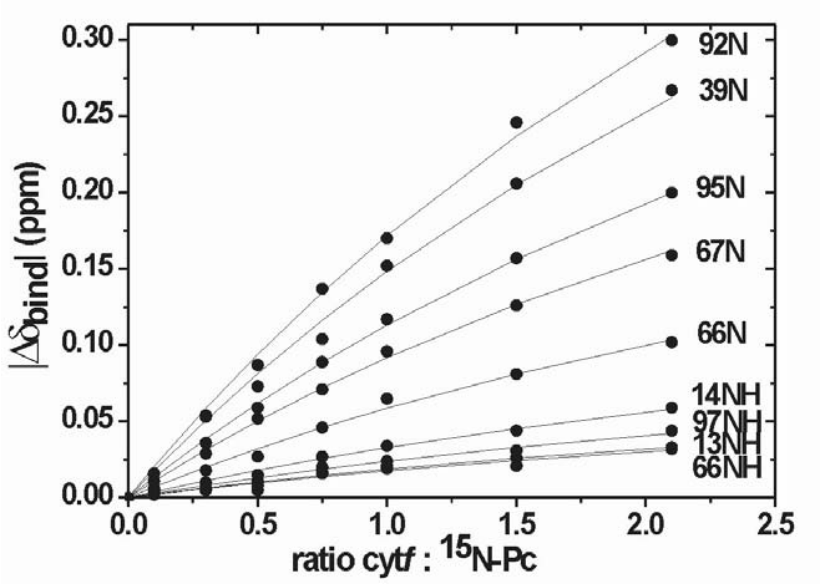

Figure 6.3. Binding curves for complex formation between *Ph. laminosum* ^{15}N-Pc and cyt*f* in presence of 20% Ficoll70. The $|\Delta\delta_{bind}|$ of individual residues is plotted as a function of the cyt*f* : Pc ratio. Global non-linear least squares fits (solid lines) to a 1:1 binding model[16] yielded a K_a of ~10^2 M^{-1}.

Effects of Ficoll70 on Ph. laminosum Pc - cytf complex

To study the effect of crowding on the *Ph. laminosum* Pc- cyt*f* complex cyt*f* was titrated into Pc in the presence of 20% Ficoll70. As in the absence of crowder, the presence of cyt*f* gives rise to changes in the ^{1}H-^{15}N HSQC spectrum of ^{15}N-Pc. A single averaged resonance was observed for each amide indicating that exchange between free and bound Pc is fast on the NMR-timescale. The observed chemical shift changes ($\Delta\delta_{bind}$) of the most affected residues were plotted against the molar ratio of cyt*f*: ^{15}N-PCu (Fig. 6.3) and fitted to a 1:1 binding model[16]. This yields a very low K_a, of ~10^2 M^{-1}, which is similar to the K_a in the absence of Ficoll70 [40]. It has to be noted that due to weak binding the K_a cannot be determined with high accuracy. Only large differences would be detectable. The ^{15}N chemical shift changes were colour-coded and plotted on a surface representation of the crystal structure of Pc[74] to create a chemical shift perturbation map (Fig. 6.4). Compared to the chemical shift perturbation map previously determined for the complex[40], no major differences are observed. In both cases the hydrophobic patch, located at the northern end of the protein is most involved in complex formation. In the presence of 5% Ficoll70 similar results are obtained.

It can be concluded the presence of crowder does not detectably change the binding in the *Ph. laminosum* Pc - cyt*f* complex. This is in agreement with the report that the overall electron transfer rate (k_2) is not affected by the presence of high molecular weight crowders[238]. It supports the idea that high molecular weight crowders such as Ficoll70 are not uniformly distributed in solution and form a 'porous' medium[237] in which relatively small proteins can move as they would in dilute solution. The question remains whether this is an appropriate model for the physiological conditions the proteins encounter and which crowder or combination of crowders is most successful in mimicking the cellular environment.

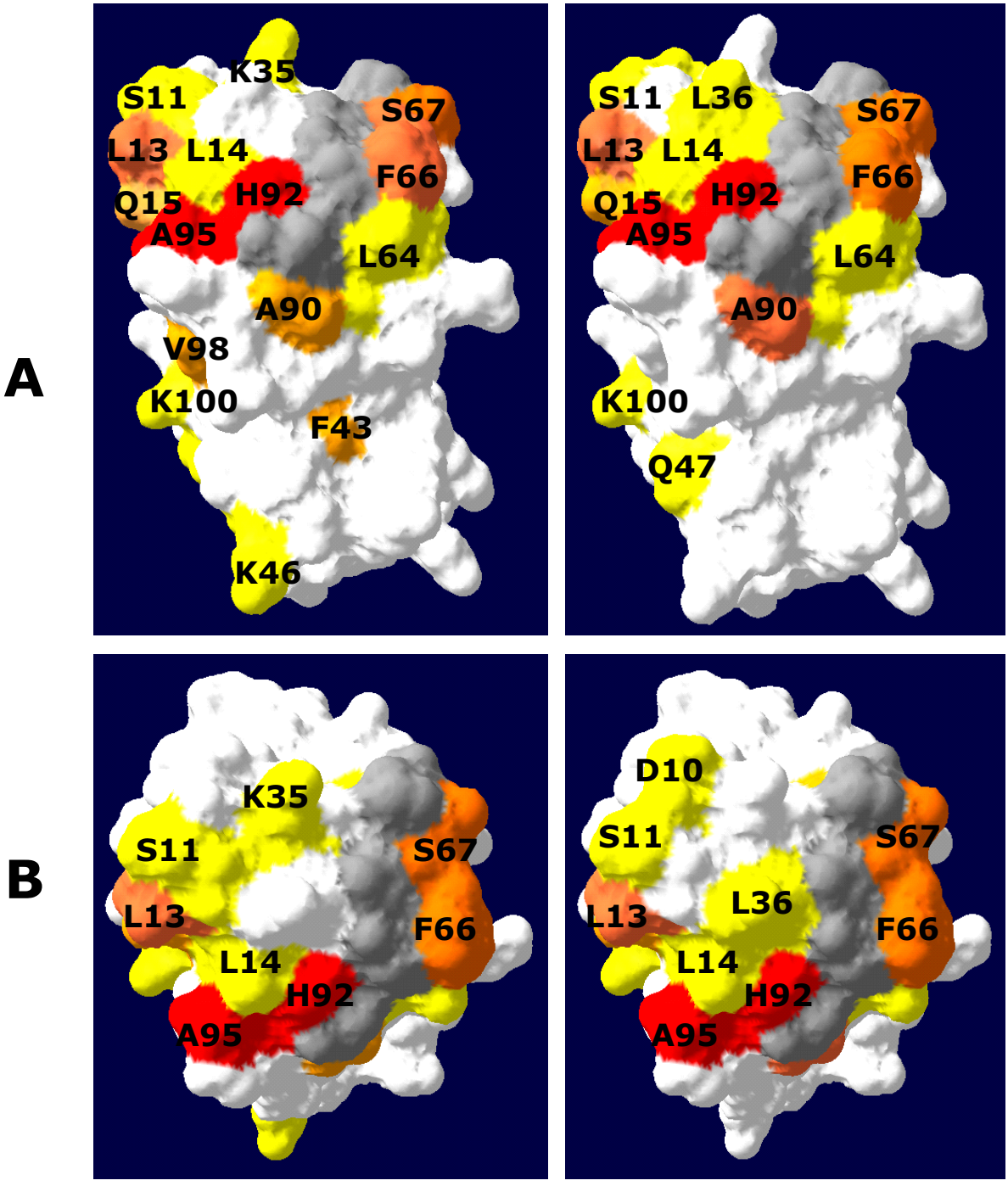

Figure 6.4. Surface representations of Pc in the presence of 0% (left) and 20% (right) Ficoll70. Chemical shift changes ($\Delta\delta_{Bind}^{15N}$) in the presence of ferrous cyt*f*. Residues with $\Delta\delta_{Bind}^{15N} \leq 0.05$ ppm in white, $\Delta\delta_{Bind} \geq 0.05$ ppm in yellow, $\Delta\delta_{Bind} \geq 0.1$ ppm in orange, $\Delta\delta_{Bind} \geq 0.3$ ppm in red and proline residues in gray. A) side view B) top view.