DOI: 10.1002/cbic.200700603

NMR Spectroscopy Reveals Cytochrome *c*–Poly(ethylene glycol) Interactions

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In vitro protein studies are typically performed on samples that are composed almost entirely of water. However, the cell interior is a heterogeneous "crowded" solution of small molecules, proteins, nucleic acids and membranes. At a concentration of 300–400 g L^{-1}, the macromolecular content of the cell influences the kinetics and thermodynamics of protein folding, ligand binding and protein–protein interactions through excluded volume effects.[1–3] Therefore, in order to build realistic models of protein structure and function, it is necessary to study proteins in vivo or under "crowded" conditions that mimic the cellular environment.

The necessity for in vivo protein characterisation is being addressed by the development of in-cell NMR spectroscopy.[4–6] While "biologically inert" proteins are largely unaffected by the crowded cell interior,[6] the disordered protein FlgM was shown to gain structure inside *Escherichia coli* cells.[5] A similar gain in structure occurred in vitro in the presence of crowding agents.[5] Artificially crowded environments can be created by using sugars, proteins or polymers such as Ficoll, dextran and poly(ethylene glycol) (PEG).[1–3] Such sample conditions are accessible by NMR spectroscopy, and the effects of macromolecular crowding on protein structure and dynamics have been investigated.[4–11] Related NMR studies of macromolecular confinement have been performed by using polyacrylamide gels,[12] reverse micelles,[13] sol–gels[14] and agarose gels.[15] Generally, crowding/confinement tends to accelerate protein folding, promotes self-association and stabilises protein structure.[3,5,8–13]

Given that macromolecular crowding can enhance protein association, the use of crowding agents is likely to facilitate the structural characterisation of weak protein interactions. We are interested in using NMR spectroscopy to study the effects of macromolecular crowding on the transient interactions[16] of redox proteins. *Saccharomyces cerevisiae* cytochrome *c* (cyt *c*) in PEG-containing solutions was chosen for initial studies. PEG–protein interactions are usually repulsive, and volume exclusion results in preferential hydration of the protein surface.[17,18] The repulsive interactions can be reduced by minimising (through conformation changes, precipitation or crystallisation) the protein surface area exposed to the solvent.[18] The effect of PEG on protein solutions is not limited to volume exclusion. Although highly water soluble, PEG is hydrophobic in nature and can interact with hydrophobic proteins.[19] An interesting example of this type of interaction is found in the crystal structure of the odorant-binding protein from *Anopheles*, in which a hydrophobic channel is occupied by a PEG molecule (PDB code: 2erb).[20] The study of protein–PEG mixtures is further underlined by the growing importance of PEGylated-protein therapeutics.[21] When modified by the covalent attachment of a PEG chain, proteins are less susceptible to proteolysis and have reduced immunogenicity.

We report here the interaction of cyt *c* with PEG as revealed by ^{1}H,^{15}N correlation spectroscopy. For comparison, experiments were performed on cyt *c* embedded in agarose gels. Similar PEG-induced effects were observed for both reduced and oxidised cyt *c*, and therefore this report focuses on the results for reduced cyt *c*. ^{15}N-labelled cyt *c* was studied in the presence of different sizes and concentrations of PEG. Samples containing up to 300 g L^{-1} of PEG were used to mimic the intracellular macromolecular content. Figure 1A illustrates a region of the ^{1}H,^{15}N correlation spectrum of cyt *c* and the spectral changes associated with the presence of increasing concentrations of PEG 8000. The majority of cyt *c* resonances demonstrated small changes in line width, increasing on average by 25–35% at 200 and 300 g L^{-1} PEG. Compared to the approximately twofold line-width increases for cyt *c* bound to cyt *c* peroxidase,[22] and cyt b_5 encapsulated in sol–gel,[14] this indicates that the rotational correlation time (τ_c) of cyt *c* is weakly influenced by PEG. Resonance broadening was greater for a number of amides found in flexible loops, including Gly34,[23] which was broadened beyond detection. Considering that loops are prone to conformation changes, the resonance broadening suggests that, in the presence of PEG, the exchange between different conformations is slow on the NMR timescale.

In addition to line broadening, concentration-dependent chemical-shift perturbations of the order of 0.1 ($^{1}H^{N}$) and 0.3 (^{15}N) ppm were observed. Figure S1 in the Supporting Information gives a plot of the averaged $^{1}H^{N}$ and ^{15}N shifts for each backbone amide. Mapping these perturbations onto the crystal structure of cyt *c*[24] reveals that the majority of the shifts surround the exposed haem edge (Figure 1B) with Gln16 and Lys79 standing out as most strongly affected. Note that Lys79 lies flat on the protein surface and thus contributes to the hydrophobic patch around the haem (Figure S2). Similar results were found for PEG 3350, 8000 and 20000; this indicates that the molecular weight of PEG does not affect its propensity to bind cyt *c*. Surprisingly, the chemical-shift-perturbation map of cyt *c* in the presence of PEG is qualitatively similar to the binding maps for cyt *c* in complex with cyt *c* peroxidase,[22] and the nonphysiological partner cyt *f*.[25] In particular, the down-field

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Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

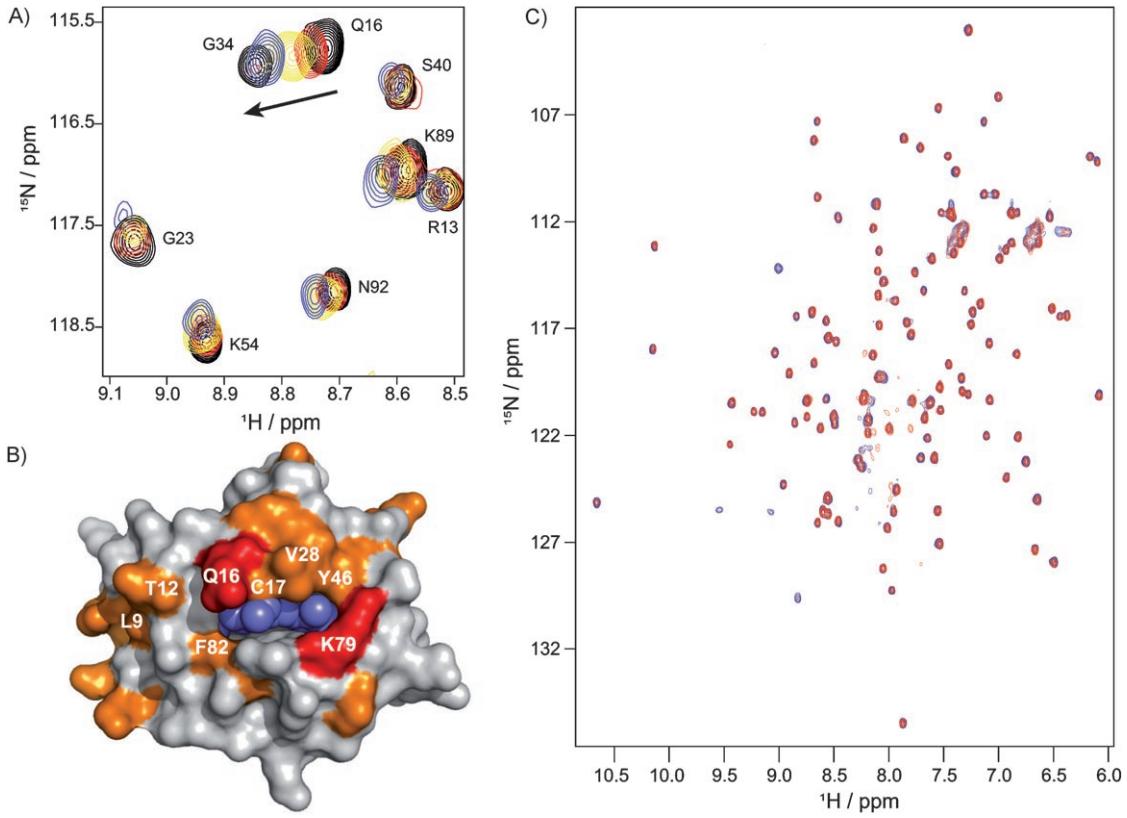

Figure 1. A) A region from the overlaid ^{1}H,^{15}N HSQC spectra of reduced cyt *c* (black) in the presence of 100 (red), 200 (yellow) and 300 (blue) g L^{-1}of PEG 8000. Labels indicate the resonance assignments at 25 mM potassium phosphate, 100 mM NaCl, pH 6.4 and 313 K; the arrow emphasises the large shift for the Gln16 resonance. The resonance of Gly34 broadens beyond detection at high PEG concentrations. Within error, identical perturbations were observed for the Gln16 resonance in oxidised cyt *c* (data not shown). B) PEG-induced chemical shift perturbations mapped onto the structure of cyt *c*.[24] The molecular surface was generated in Pymol, and residues for which the amide resonance experienced significant ($\Delta\delta_{PEG} > 0.03$ ppm) or large ($\Delta\delta_{PEG} \geq 0.06$ ppm) shifts are coloured orange and red, respectively. The haem is shown as blue spheres. C) Overlaid ^{1}H,^{15}N HSQC spectra of pure, reduced cyt *c* (blue) and cyt *c* embedded in a 1.5% agarose gel (orange). The buffer composition was identical to that used in the PEG experiments.

perturbation of the $^{1}H^{N}$ resonance of Gln16 bears a striking resemblance to that observed in the cyt *c*–cyt *c* peroxidase complex (compare Figure 1B with Figure 3B in ref. [22]). This shift is associated with a conformation change of the Gln16 side chain,[22] which increases the hydrophobicity around the haem (Figure S2). Taken together, these data suggest that PEG prompts a similar conformation change that facilitates the protein–PEG interaction.

Besides complex formation, there are other factors that could give rise to the chemical-shift changes observed in the presence of PEG. For instance, the amide resonances might be sensitive to PEG-induced conformation changes and to reorganisation of water molecules at the protein surface (preferential hydration). The latter might be responsible for the small perturbations experienced by many of the resonances at the highest PEG concentrations studied. The resonances of charged residues are likely to be particularly prone to such effects. For instance, Asp60 and Glu61, which adjoin Trp59 and Phe36, form a small patch that experiences PEG-induced shifts. Self-association of cyt c molecules could also give rise to the observed chemical-shift perturbations. However, the relatively dilute sample concentration (0.1 mM) and the high charge on the protein preclude a stable cyt *c* association. Note that crystallisation of cyt *c* requires saturated solutions of $(NH_4)_2SO_4$. Further evidence for the absence of cyt *c* self-association is provided by the fact that the HSQC spectrum of pure cyt *c* was identical at protein concentrations of 1.0 and 0.1 mM. The concentration of cyt *c* had no significant effect on the resonance line widths or chemical shifts (data not shown). Finally, small pH differences could contribute to the chemical-shift perturbations. To test for pH effects, spectra of cyt *c* were acquired over the pH range 4.6–7.8. While the chemical shifts of several resonances demonstrated large pH dependences, there was no correlation between pH- and PEG-induced perturbations. Moreover, the resonances of Gln16 and Lys79 were not affected by pH.

Despite the increased sample viscosity, PEG had a marginal effect on the line widths of cyt *c* resonances. Similarly small line-width increases, due to viscosity changes, were observed for the GB1 protein in cell extracts.[6] Highly concentrated PEG solutions are best described as a "sea" of glycol monomers.[26] Thus, cyt *c*–PEG interactions do not affect the τ_c of cyt *c* in the same way as a protein–protein binding event. Remarkably, the hydrophobic face (surrounding and including the exposed haem edge) of cyt *c*, which plays a central role in protein complex formation,[16,22,25] is also utilised in cyt *c*–PEG interactions

(Figure 1B). This result suggested that we study cyt *c* in the presence of a hydrophilic crowding agent. Samples of cyt *c* embedded in 1.5% agarose gels yielded $^{1}H,^{15}N$ HSQC spectra essentially identical to those of the free protein (Figure 1C). While the resonances of Gly45 and Asp60 were broadened (probably due to conformation changes, see Gly34 above), there were only minor chemical shift changes, of the order of ~0.01 ppm ($^{1}H^{N}$). This indicates that the hydrophilic polysaccharide surface does not favour cyt *c* binding at this concentration. In contrast, when encapsulated in sol–gels, strong electrostatic interactions between cyt *c* and the matrix result in severely broadened and shifted resonances.[14] While it would be informative to investigate cyt *c*–PEG interactions within the confines of the agarose gel, such studies are currently unsuited to NMR, as PEG precipitates agarose to give heterogeneous gels.[27]

Protein–protein interactions usually involve hydrophobic surface patches, the sizes of which contribute to the binding affinity of complex formation. Redox proteins such as cyt *c* utilise small hydrophobic patches to participate in transient interactions with partner proteins.[16,28] The NMR data presented here demonstrate the capacity of PEG to bind to the hydrophobic surface of cyt *c*. Therefore, this study highlights that caution is necessary when interpreting the effect of PEG on protein–protein interactions. While macromolecular crowding/volume exclusion effects might predominate, it is important to rule out the contribution of PEG–protein binding. Moreover, the propensity for PEG binding is expected to be enhanced with the increasing magnitude of the hydrophobic surface borne by a given protein.[7,19,20] In terms of studying crowding effects, the criterion of "inertness" might be better satisfied by crowding agents such as polysaccharides, which are predominantly hydrophilic. Finally, the observation that PEG interacts with hydrophobic protein surfaces suggests that PEGylated proteins might be shielded against interactions with proteins of the immune system.

Experimental Section

^{15}N-labelled cyt c was prepared and characterised according to published methods.[22,29] For NMR studies, the typical sample composition was reduced cyt *c* (0.1 mM), potassium phosphate (25 mM), NaCl (100 mM), sodium ascorbate (0.1 mM, as a reductant) and 10% D_2O (pH 6.4). Ferricyanide was used to prepare oxidised cyt *c*, which was transferred into fresh buffer by ultrafiltration methods. PEG 3350, 8000 and 20000 were purchased from Sigma. The PEG concentration was varied from 50 to 300 g L^{-1} in increments of 50 g L^{-1}. The sample pH was verified, and if necessary corrected, after the addition of PEG. Samples of cyt *c* embedded in agarose gels were prepared by using a modification of the method of Pastore et al.[15] The required amount of protein was placed in an NMR tube immersed in a water bath at 60 °C. Subsequently, low-melting agarose dissolved in buffer (see above) at approximately 80 °C was transferred to the NMR tube and rapidly mixed with the protein solution before the solution was cooled to room temperature. The high thermal stability of cyt *c* supports this method of sample preparation.

$^{1}H,^{15}N$ HSQC spectra were acquired at 313 K with spectral widths of 14.1 ppm (^{1}H) and 40.0 ppm (^{15}N) on a Varian 600 MHz NMR System spectrometer. Presaturation was used to eliminate the PEG signal. The analysis of the PEG-induced chemical-shift perturbations ($\Delta\delta_{PEG}$, with respect to the spectrum of pure cyt *c*) was performed in CARA (http://www.nmr.ch). The perturbations were averaged for each backbone amide resonance by using Equation (1):

$$\Delta\delta_{PEG} = \left(\frac{\Delta\delta_{N}^{2}/25 + \Delta\delta_{HN}^{2}}{2}\right)^{1/2} \quad (1)$$

Here $\Delta\delta_{N}$ and $\Delta\delta_{H^N}$ correspond to the change in the ^{15}N and the $^{1}H^{N}$ chemical shifts, respectively.

Acknowledgements

The authors thank Peter Sandor (Varian Inc.) for assistance with VnmrJ BioPack, and University College Dublin for funding. The 600 MHz spectrometer was funded by the Higher Education Authority through PRTLI cycle 3.

Keywords: hydrophobicity · macromolecular crowding · NMR spectroscopy · proteins · recognition

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Received: October 9, 2007
Published online on February 7, 2008

