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Watershed for the structure of cytochrome *c*

Redox-dependent changes in the position of bound water inside cytochrome *c* in solution may be important for its function as an electron transfer protein.

THE importance of water to the structure and function of biological molecules is well established; however, the view of water as a lumpy continuum surrounding, or surrounded by, biological molecules is not only inaccurate but may also hinder our comprehension of the inner workings of the molecular machinery. Now, thanks to increasingly sophisticated techniques for structure determination, the nature of water's contribution is becoming clearer. In a paper in this month's *Nature Structural Biology*[1], Qi *et al.* exploit the ability of NMR to measure specific classes of atomic interaction in order to observe the position of structurally important water molecules in the haem-containing electron transport protein cytochrome *c*. What is more, they identify changes in the position of some water molecules in the oxidized and reduced forms of the protein, which may well have a bearing on its function.

The ability to detect and position water molecules within protein structures is not new. Electron density maps derived from X-ray diffraction of protein crystals invariably show a layer of highly ordered water molecules coating the surface of the protein and filling any available holes or crevices (reviewed in ref. 2). Owing to the nature of the crystallographic experiment, however, it is hard to assign biological significance to these water molecules, even when X-ray diffraction is complemented by neutron diffraction to reveal the positions of the hydrogen as well as the oxygen atoms of water. The structures derived from X-ray crystallography are averaged over times measured in hours, so the presence of well-defined water implies that there is a water molecule at that position for most of the time in most of the protein molecules, and not that the water is necessarily tightly held there. This can evoke an impression of water networks that are more highly ordered than the *in vivo* arrangement.

Also in this month's *Nature Structural Biology*: alteration of the substrate specificity of a serine proteinase; peptide helices in membrane environments; determination of the structure of two new zinc-finger domains; long-range effects of single amino-acid substitutions on the conformations of peptides; and an empirical algorithm for the determination of helical propensity.

Investigation of the structure of water in proteins by NMR has been hampered by problems in resolving signals due to bound water molecules from those arising from bulk solvent. The development of multidimensional NMR techniques has allowed the detection of long-lived water in proteins such as pancreatic trypsin inhibitor[3], interleukin-1β[4] and FK506-binding protein[5]. In general, the positioning of these water molecules is the same as in the X-ray crystallographic structures. Qi *et al.*[1] have used a combination of nuclear Overhauser enhancement (NOE) and total correlation spectroscopy, in what is essentially a filtered two-dimensional experiment, to identify six water molecules in ferrocytochrome *c* and five in ferricytochrome *c* that have residence times longer than 300 ps.

Early X-ray crystallographic studies of cytochrome *c* from tuna[6,7] revealed four deeply buried water molecules, one of which was implicated in setting the redox potential of the haem group. But as unnaturally high salt concentrations were used to facilitate crystallization, the relevance to the situation *in vivo* was uncertain. Qi and colleagues also detected these four water molecules, but could not fully interpret their NMR spectra without including other long-lived water molecules (two in ferrocytochrome *c* and one in ferricytochrome *c*).

Four of the water molecules in cytochrome *c* appear to be classic examples of structural water filling holes inside the protein. As such they are unlikely to fulfil a functional role, although they must be important to the stability and integrity of the protein. It is in fact one of these waters that escapes detection in the oxidized form of the protein: it sits in a loop region close to the surface of the protein, so this elusiveness may reflect a decreased residence time in ferricytochrome *c* rather than its absence from this site.

The other two water molecules lie in the haem-binding cleft of cytochrome *c* and are thus of greater functional interest. One of these had previously not been spotted by X-ray crystallography and could now help resolve a long-standing inconsistency between theory and experiment. Earlier estimates of the solvent reorganization energy based on models of cytochrome *c* with only one water molecule bound in the haem cleft were too low: the extra water should correct the discrepancy.

The second water molecule in the haem cleft, originally postulated to influence the redox potential, lies against the haem ring. It changes position according to the oxidation state of the protein: upon oxidation the water moves 3.7 Å further away from the haem iron (from 5.6 Å to 9.3 Å). This movement alters the entire hydrogen-bonding structure within the cleft. It is important that the NOE distance restraints defining the water's position involve quite different residues in the oxidized and reduced protein. Changes in the electronic structure so close to the haem iron will profoundly affect the redox potential of the haem.

The resurgence of interest in biological electron transfer is being fuelled by studies like this. With each new piece of data it is becoming increasingly clear that the former pre-eminence of X-ray crystallography in structure determination, focusing as it does on the semi-rigid regions of the peptide scaffold, may well have adversely constrained our thinking in this area.

Improvements in molecular dynamics simulations and the flourishing of NMR spectroscopy offer a more mobile picture of biological molecules. For example, the idea of a fixed complex between cytochrome *c* and cytochrome *c* oxidase, inferred from X-ray crystallography, has recently been called into question[8]. Consequently the popular concept of 'molecular wires', or predetermined covalent pathways that effect electron transfer between redox donors and acceptors in the respiratory chain, is now open to debate.

Cytochrome *c* has shown us that a protein's water can be used in more than a purely structural role. Evidently we must consider the structure and dynamics of the whole protein, non-peptide as well as peptide. **Christopher Surridge**

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