Proton transfer: **It's a stringent process**

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Proton transfer into and out of proteins is important, both for many enzyme reaction mechanisms and proton pumping across membranes. Recent work on several proteins has revealed stringent requirements for amino-acid side chains and subtle reorganisation of hydrogen-bond networks involving bound water molecules.

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"Protons? They are everywhere and don't they just find their own way in and out?" Such is a typical reaction from many a biochemist concerning protons as reactants or products in enzyme-catalysed reactions. In fact, proton access routes are not so simple. Often overlooked is the fact that the proton can only move 0.25 Å at a time through a proton-hopping process analogous to electron tunelling which can operate over much larger distances. Thus, for protons to move at rates commensurate with typical turnover times for enzymes, mechanisms are needed to transport them to and from active sites.

The issue of proton movement through proteins becomes more complex when it is recalled that protons are not only participants in enzyme-catalysed reactions but are also pumped across membranes by proteins such as bacteriorhodopsin, cytochrome aa_3 oxidase and ATP synthase. In the case of a pump, one cannot be relaxed about the path of the proton because it is obviously necessary for there to be a molecular switch of some kind that ensures the vectorial movement of the proton across the membrane.

It follows from the above that, in a number of contexts, the pathway of a proton into and out of the interior of a protein is very important for the functioning of a protein. When one starts to think about a proton route then several possibilities come to mind. One is that a suitably oriented array of water molecules, natural proton conductors, might provide such a pathway; another is that either alone, or in combination with such water molecules, suitable amino-acid side chains might provide the way stations for protons.

Tracking protons through proteins is not easy; exchangeable protons are invisible to X-ray diffraction and other methods. In a recent study, Chen *et al.* [1] have used a multidisciplinary approach to understanding how protons access the FeS centre of a protein called ferredoxin I from *Azotobacter vinelandii*. The obvious function of this ferredoxin is as an electron transfer protein, and thus it undergoes cycles of oxidation and reduction. The high resolution structure of the protein shows that the FeS centre is approximately 8 Å below the surface. Studies of the oxidation and reduction of the protein have established that, over certain ranges of pH, the change in oxidation state of the FeS centre is coupled to protonation/deprotonation of the centre. What is not at all clear from the crystal structure is how the proton moves in and out, because there are no water molecules visualised within the structure at appropriate places, and there is no clue from altered side-chain positions between different oxidation states.

The structure of the ferredoxin does, however, show that, lying between the protein surface and the FeS centre, is an aspartate, residue 15. The presence of this type of acidic residue immediately suggests that its side chain might be involved in proton transfer to and from the FeS centre. This proposition has now been examined in great detail through use of four techniques: fast-scan protein voltammetry, high-resolution crystallography, site-directed mutagenesis and molecular dynamics [1]. The first of these will be the least familiar to the general reader. It involves the adsorption, as a film, of a protein such as ferredoxin I on the surface of a carbon electrode. Protein molecules within this film can exchange electrons rapidly with the electrode. By varying the applied voltage the equilibrium oxidation-reduction potential of the FeS centre can be determined. By altering the rate at which the voltage is changed, it is possible to extract information about both the energetics and the rates of oxidation and reduction of the FeS centre.

The kinetic analysis as a function of pH has allowed the establishment of a detailed reaction scheme for electron and proton transfers. In essence, it was found [1] that the oxidised protein receives an electron at a much faster rate than it does the proton, which thus arrives significantly after the electron. Once the proton has transferred to the FeS site, re-oxidation cannot occur before the proton is released. Thus, deprotonation effectively controls the rate of, or gates, the oxidation event. Protein thin film voltammetry is, therefore, a powerful technique for the probing of electron transfer events and their relation to proton transfer.

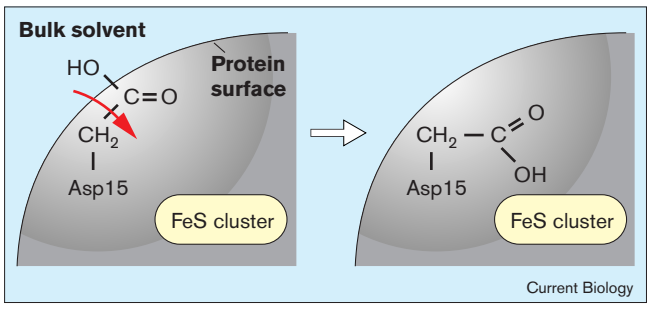
Having established these features for the wild-type protein, it became possible to probe the suspected role of aspartate 15 as a proton carrier from the surface to the interior of the protein using site-directed mutants. The obvious change to make is that of aspartate 15 to asparagine: this

was indeed found to result in a very considerable slowing of protonation/deprotonation and loss of the pH-dependence of the process. It cannot, however, be immediately concluded that the observations with this mutant mean that aspartate 15 is a key player. The high-resolution crystal structure shows that the carboxylate of aspartate 15 makes a salt bridge with the side chain of lysine 84. Any role for this salt bridge in the proton transfer was eliminated by the finding that conversion of the lysine to glutamine had no effect on the proton transfer. Amongst other 'control' mutants examined was one carrying glutamate at position 15; lengthening of the side chain by just one methylene group was found to have a large deleterious effect on the rate of protonation/deprotonation.

Measurements using protein film voltammetry with several carefully selected mutants thus unequivocally implicated the aspartate 15 side chain as a key player in proton movement. The approach revealed that addition of an electron to the FeS cluster increases the pK_a of aspartate 15, facilitating proton capture from the solvent [1]. The pK_a change reverses upon oxidation of the FeS cluster. But it is one thing to identify the importance of an aspartate side chain, quite another thing to understand how it helps the proton move over 8 Å. To address this question, molecular dynamics methodology was used [1]. This showed that, in the state where the aspartate side chain is protonated and the reduced cluster is unprotonated, high-frequency excursions of the aspartate 15 side chain produce both close encounters with the FeS centre and visits to the surface of the protein where protons can be exchanged with solvent water (Figure 1).

An issue in understanding proton transfer is that there might be a range of different internal organisations inside proteins for achieving the same end. Thus, there may be limited scope for extrapolation from one protein to another. In this context it is instructive to revisit one of the oldest problems in proton transfer within an enzyme, that of how

Figure 1



Schematic representation of the movement of the side chain of asparate 15 from the surface of ferredoxin towards the sulfur atom of the FeS cluster. (See text for details.)

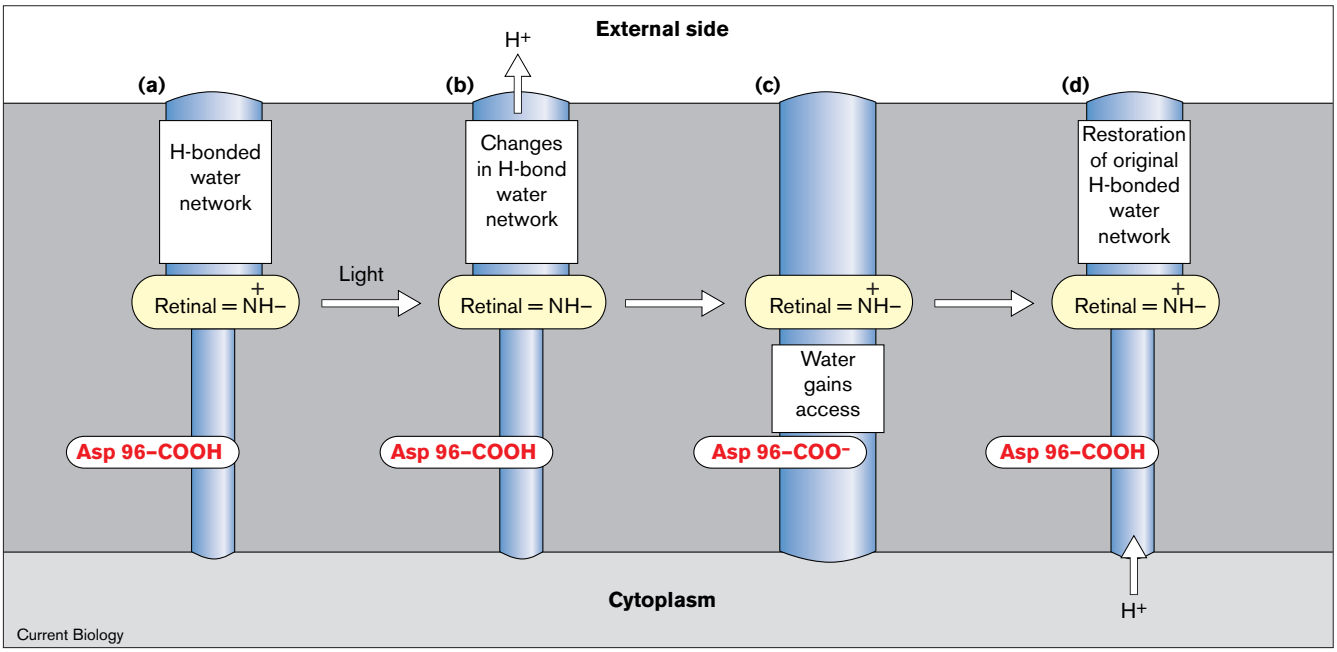
protons are transferred into and out of the active site of carbonic anhydrase. Here the constraint is that the enzyme works at the diffusion limit, so that the proton transfer from the active site that follows hydration of carbon dioxide to give bicarbonate must be very fast indeed.

An enormous number of studies on α-carbonic anhydrase have shown that the zinc catalytic centre is at the bottom of a 15 Å deep conical depression, and that a histidine, residue 64 in the sequence, serves as a way station for the proton. The side chain of this histidine is argued to make excursions, analogous to those discussed above for aspartate 15 of ferredoxin, in order to shuttle protons to a buffer in solution [2]. The rate of proton transfer is so fast that the buffer, rather than water molecules, must be the acceptor [2]. But even in the case of the carbonic anhydrase reaction, biology has found more than one solution to the proton transfer problem. A new structure recently solved [2] of a plant β-carbonic anhydrase shows an active site that is less buried than that in α-carbonic anhydrase. Thus, whilst the buffer is still important there seems to be no need for a proton way station analogous to histidine 64 of α-carbonic anhydrase. The two different solutions to the proton transfer problem in carbonic anhydrase are thought to have been generated by convergent evolution [2].

Clearly, the mechanisms established for aspartate 15 of the ferredoxin and proposed for histidine of α-carbonic anhydrase could also be involved at single steps along the much longer route taken by protons when they are pumped from one side of a membrane to the other. This process is understood in greatest detail in the case of bacteriorhodopsin, the light-driven proton pump of *Halobacterium salinarum* [3]. Inspection of a high-resolution structure of the dark-adapted protein, in combination with a wealth of knowledge about the photochemical events in the protein, has allowed a detailed mechanism to be devised for much of the proton translocation pathway. The structure of the protein in the dark shows that there is a network of hydrogen bonds extending from the retinal Schiff's base nitrogen atom at the core of the protein to the external surface. This network relies extensively upon the strategic positioning of several water molecules (Figure 2).

Upon illumination of bacteriorhodopsin, a proton is released from the protonated Schiff's base and concommitantly another proton is released to the external medium. Much of the molecular basis for this proton movement has been revealed by a crystal structure [3] of an illuminated mutant of bacteriorhodosin which is blocked at a central point of the photocycle. This structure shows that there are critical changes in the number and position of internal water molecules which have altered the connectivity of the hydrogen-bond network (Figure 2). Furthermore, there are small but important changes in the positions of amino-acid side chains. In

Figure 2



Schematic representation of the role of hydrogen-bonded water networks in bacteriorhodopsin. **(a)** Bacteriorhodopsin in the dark showing a wider channel from the Schiff's base nitrogen to the external surface than to the cytoplasmic side. **(b)** Relatively early consequence of illumination, showing proton release to the external phase. **(c)** Subseqent step in which the channel to the cytoplasmic surface opens allowing entry of waters and transfer of a proton from aspartate 96 to the Schiff's base. **(d)** Final closing of the cytoplasmic channel and restoration of the high pK_a of the aspartate 96, thus ensuring proton uptake from the cytoplasm.

summary, this work shows that proton movement over a relatively long distance has been achieved through a set of subtle movements in hydrogen bonds. As Gennis and Ebrey [4] have pointed out, this work on bacteriorhodopsin is likely to have important implications for understanding enzymes in general, where internal proton movements are often a crucial feature of catalysis. It seems, however, that the reorganisation of waters seen for bacteriorhodopsin does not apply to the *A. vinelandii* ferredoxin and arguably not to the carbonic anhydrases.

Thus far, an outline has been provided as to how protons move from the Schiff's base nitrogen of bacteriorhodospin to the external aqueous phase. This leaves two related questions: why does the proton not depart the Schiff's base for the opposite side of the protein (its cytoplasmic surface)? And how does the deprotonated Schiff's base regain a proton specifically from the cytoplasmic side, so as to complete proton transfer right across the membrane? Inspection of the dark structure of wild-type bacteriorhodopsin and of the illuminated mutant structure showed that aspartate 96, implicated as the proton donor to the Schiff's base by many biochemical criteria, was 10 Å distant, and that there are no water molecules or other side chains to facilitate proton movement over this distance. The absence of water would ensure a hydrophobic environment, which in turn would account for the exceptionally high pK_a of aspartate 96. This high pK_a, the distance and the lack of proton way stations satisfyingly answers the first of these two questions: the initial proton on the Schiff's base would have neither kinetic nor energetic reasons to migrate in the cytoplasmic direction towards aspartate 96.

So how can we answer the second question? It has been postulated that, at some stage of the photocycle, the region of protein around aspartate 96 must become more accessible to water; this would both lower the pK_a of the aspartate and provide a pathway for a proton from it to the Schiff's base. Although the details of the locations of these water molecules are not yet known, a very recent structural study [5] on another mutant of bacteriorhodospin that is blocked at a late stage of the photocycle has provided evidence for a conformational change that would allow water into the vicinity of the asparate 96 (Figure 2). A high-resolution structure of this state would, therefore, be of great interest. Indeed it may prove to show that the aspartate side chain has moved in a manner analogous to that deduced for the ferredoxin [1]. And this is not the end of the story; having lost its proton to the Schiff's base, aspartate 96 must be specifically reprotonated from the cytoplasmic surface of the protein. Further conformational change involving the movement of the aspartate 96 side

chain and the expulsion of water is very likely to be crucial (Figure 2).

In summary, high rates of proton transfer require very precisely oriented side chains and bound water molecules. Many biochemists were hoping that the structure of bacteriorhodospin would reveal a simple story about active transport of protons. Their hopes are dashed! Only in a protein such as ferredoxin from *A. vinelandii* does it appear likely that a single well-defined process is at work. Elsewhere, a process such as this will have to operate in combination with other processes of the kind discussed in this dispatch.

References

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