Controlled protonation of iron–molybdenum cofactor by nitrogenase: a structural and theoretical analysis

Marcus C. DURRANT¹

Department of Biological Chemistry, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, U.K.

Qualitative molecular modelling has been used to identify possible routes for transfer of protons from the surface of the nitrogenase protein to the iron–molybdenum cofactor (FeMoco) and to substrates during catalysis. Three proton-transfer routes have been identified; a water-filled channel running from the protein exterior to the homocitrate ligand of FeMoco, and two hydrogen-bonded chains to specific FeMoco sulphur atoms. It is suggested that the water channel is used for multiple proton deliveries to the substrate, as well as in diffusion of products and substrates between FeMoco and the bulk solvent, whereas the two hydrogen-bonded chains each allow a single proton to be

added to, and subsequently depart from, FeMoco during the catalytic cycle. Possible functional differences in the proton-transfer channels are discussed in terms of assessment of the protein environment and specific hydrogen-bonding effects. The implications of these observations are discussed in terms of the suppression of wasteful production of dihydrogen by nitrogenase and the Lowe–Thorneley scheme for dinitrogen reduction.

Key words: dihydrogen, molecular modelling, nitrogenase, proton channels.

INTRODUCTION

Nitrogenases are enzymes that catalyse the reduction of dinitrogen to ammonia. The most extensively studied nitrogenase contains the transition metals Fe and Mo and comprises two components, known as the Fe and MoFe proteins. The Fe protein contains a single Fe₄S₄ cubane, and functions as a specific electron donor to the larger MoFe protein. X-ray crystallographic structures of the MoFe protein from three different organisms are available at various resolutions: *Clostridium pasteurianum* [Cp1; 3.0 Å (1 Å = 0.1 nm)] [1], *Azotobacter vinelandii* (Av1; 2.0 Å) [2] and references cited therein) and, most recently, *Klebsiella pneumoniae* (Kp1; 1.6 Å) [3]. Although all three proteins are structurally similar, Av1 and Kp1 are more closely related to each other than they are to Cp1, as expected from amino-acid-sequence comparisons [3]. The X-ray crystal structures of the Fe protein from *A. vinelandii* and *C. pasteurianum* have also been determined, both in isolation [4,5] and in combination with the MoFe protein as a putative transition-state complex formed with ADP–AlF₄[−] [6]. The MoFe protein is an α₂β₂ tetramer that contains two unique metal–sulphur clusters; the iron–molybdenum cofactor, FeMoco (stoichiometry MoFe₇S₉·homocitrate, Figure

1) and the P-cluster (stoichiometry Fe₈S₇). The catalytic site for reduction of H⁺, N₂ and other substrates is believed to be located somewhere on the FeMoco, whereas the accepted role of the P-clusters is to mediate electron transfer from the Fe protein to FeMoco [7,8]. Although production of H₂ at the P-clusters is also conceivable, mutant MoFe proteins that contain only P-clusters and no FeMoco do not reduce protons [7]. It is possible to extract intact FeMoco from the nitrogenase protein into *N*-methylformamide solution; moreover, the extracts are able to restore the activity of a FeMoco-free mutant apoprotein.

The accepted limiting stoichiometry for reduction of N₂ by molybdenum nitrogenase is:

A detailed scheme for the reduction of N₂ to NH₃ with concomitant formation of H₂ from H⁺ was worked out by Lowe and Thorneley from their stopped-flow kinetic analysis of molybdenum nitrogenase isolated from *K. pneumoniae* [9–12]. In the Lowe–Thorneley scheme (Scheme 1), the stoichiometry of reaction (1) is explained by loss of H₂ from the FeMoco upon binding of N₂ (obligatory hydrogen evolution; OHE [13]). This is consistent with the long-standing experimental observation that H₂ evolution is not completely suppressed during N₂ reduction, even when very high partial pressures of N₂ are employed [14]. It is noteworthy, however, that the available experimental data do not demand a limiting stoichiometry of one H₂ produced per N₂ reduced, and the true limiting value for molybdenum nitrogenase, although greater than zero, may be less than unity [7]. The analogous vanadium nitrogenase is less efficient in suppressing H₂ formation, with a limiting H₂:N₂ stoichiometry of 3.5 [15].

A generally accepted feature of catalysis by nitrogenase is that the arrival of each electron at the FeMoco is intimately coupled

Figure 1 The FeMoco, showing the numbering of the central sulphur atoms and the homocitrate ligand attached to Mo

Abbreviations used: Av1, Cp1, Kp1, molybdenum nitrogenases from *Azotobacter vinelandii*, *Clostridium pasteurianum* and *Klebsiella pneumoniae* respectively; COX, cytochrome c oxidase; DFT, density functional theory; FeMoco, iron–molybdenum cofactor; OHE, obligatory hydrogen evolution.

¹ e-mail marcus.durrant@bbsrc.ac.uk

Scheme 1 The Lowe–Thorneley scheme for the MoFe protein cycle of nitrogenase

Each of the arrows between successively reduced states E_nH_n represents the transfer of an electron from the Fe protein via association/dissociation of the Fe and MoFe proteins.

with the movement of a proton. In the Lowe–Thorneley scheme, this is indicated by the association of H_n with each successive reduced state E_n . In electrochemical studies on the isolated FeMoco, in which protons were added in an uncontrolled manner from solution, it was possible to measure protonation equilibria for two successive reductions, before loss of H_2 returned the system to its initial redox state [16]. Similarly, in the functioning enzyme, the partially reduced states E_2H_2 , E_3H_3 and E_4H_4 are all vulnerable to loss of H_2 , in each case returning the enzyme to an earlier, less reduced state in the catalytic cycle (Scheme 1). These observations emphasize the idea that the protein environment must play an essential role in delivering protons to FeMoco in such a way that their thermodynamically favourable recombination with electrons to give rise to H_2 is kinetically suppressed.

In spite of the wealth of information now available on nitrogenase, there is still no direct experimental evidence for the nature of its interactions with substrates at an atomic level. The Lowe–Thorneley scheme was published several years before the X-ray crystal structures of the nitrogenase proteins were solved, hence the kinetic interpretation had to be made in the absence of the detailed knowledge of the structure of FeMoco and its protein environment now available. Furthermore, continued advances in computational chemistry, most notably the development of density functional theory (DFT), have allowed reliable calculations to be made on the binding characteristics of FeMoco with H atoms, H_2 and N_2 [17]. It therefore seemed appropriate to revisit those aspects of the Lowe–Thorneley scheme concerned with protonation, reduction and H_2 formation. The present study uses molecular modelling to relate published DFT calculations on isolated FeMoco [17] to the wider context of the protein environment. In this paper a mechanism is proposed for production of H_2 at FeMoco consistent with the available experimental and theoretical data. This H_2 production mechanism is characterized, not as a necessary feature of the reduction of dinitrogen to ammonia, but rather as a side-reaction, which is difficult to suppress experimentally.

MATERIALS AND METHODS

Protein coordinates were retrieved from the Brookhaven Protein Data Bank, accession codes 1QGU, 1QH1 and 1QH8 (Kp1), 2MIN and 3MIN (Av1), 1MIO (Cp1) and 1N2C (Av1–Av2 complex). The first five of these structures contain resolved water molecules, which were used in the modelling studies. Throughout the text, distances quoted are taken from the α -subunits of the

following structures: Kp1, 1QGU; Av1, 2MIN; and Cp1, 1MIO. Molecular modelling calculations were performed using Chem-X software [18]. Protein segments were overlaid using the rigid-fit option and four or five selected atoms as fitting pairs. Figures were prepared using RasMol molecular graphics [19].

RESULTS AND DISCUSSION

A crucial question for the analysis of H_2 production on FeMoco concerns the mobility of H atoms on the cluster itself, i.e. once an H atom has been added to a given FeMoco atom, how readily can it migrate to an alternative site? Such H atom scrambling is likely to occur very readily for free FeMoco in solution, where water can participate in simultaneously accepting a proton from the first site and donating a proton to the second site. In the gaseous phase, to which DFT studies are most directly related, topological migration of H from atom to atom would probably allow scrambling of H atoms across the central Fe_6S_3 section of the FeMoco. However, in the protein, both of these H migration mechanisms are likely to be severely circumscribed by the local steric, electrostatic and hydrogen-bonding properties of the protein environment. Thus one should expect to find within the protein structure proton-delivery channels terminating at specific atoms within FeMoco, whereas other potentially basic sites should be insulated from bulk solution by protein residues lacking biologically relevant acid–base functionality.

Recent DFT studies by Rod et al. [17] have examined the binding energies of H atoms placed on the central Fe_6S_3 portion of the FeMoco. The central three sulphurs are found to be considerably more basic than the sulphurs of the $MoFe_3S_3$ sub-cube (and, by inference, the Fe_4S_3 sub-cube). Transfer of a hydrogen atom from the central sulphurs to an iron atom is endothermic, but not prohibitively so, especially for the first hydrogen added. However, such H atom migration would increase the total formal oxidation state of the metals in the FeMoco by two units, and chemical precedents suggest that the formation of metal hydrides competes with, and is deactivating towards, protonation of N_2 [20]. Given that formation of iron hydrides is likely to deactivate the FeMoco, and, furthermore, that metal hydrides generally react readily in protic media to liberate H_2 [21], it may reasonably be concluded that, in order to access the reduced FeMoco states required for reduction of N_2 , the protein must use kinetic control to suppress the thermodynamically favourable, but catalytically wasteful, production of H_2 via metal hydrides. This has long been recognized as a probable feature of nitrogenase catalysis [9]. With these thoughts in mind, I now consider the protein environment around FeMoco.

The interstitial channel

The FeMoco is completely buried in the protein matrix and is approximately 10 Å from the surface [1–3,8]. It has been stated that there are no permanent channels to allow diffusion of H_3O^+ from the bulk solvent to the FeMoco [8]. However, close inspection revealed a promising candidate for just such a channel in both the Kp1 and Av1 structures (Figure 2). The channel is very similar in both proteins, although the higher number of resolved water molecules in the Kp1 structure makes it a little clearer in the latter. Although the lower resolution of the Cp1 crystal structure did not permit location of any water molecules, the structure of the protein in this region is similar to both Av1 and Kp1, especially for the inner section, and it is reasonable to assume that Cp1 has an analogous water-filled channel. It runs from the ‘water pool’ [8] located around the homocitrate ligand of FeMoco to the surface of the protein, and has two distinct regions. The inner region consists of five water molecules

Figure 2 The interstitial channel

(a) Overlay of the channel from the four available Av1 sub-structures (blue) and the six available Kp1 sub-structures (red), i.e. both halves of the homodimers. The waters from all ten sub-structures are shown in green; other waters of the homocitrate water pool have been omitted for clarity. (b) Overlay of the channel from the four available Av1 sub-structures (blue) and the two available Av1/Av2 transition state complex sub-structures (purple). The view is approx. 90° from that in (a). One set of waters only, from 2MIN, α subunit, are shown. The P-cluster is included to indicate the direction of the docking region for Av2. The following Av1 residues (and corresponding Kp1 residues) have been included; α subunit, 94–104 (98, 99 backbone only), 111, 424–425, 440–446 (441, 443–445 backbone only); β subunit, 105, 449–453 (451, 452 backbone only); β' subunit, 513–523 (518 backbone only).

separated by less than 3 Å for each pair, whose positions are highly conserved in all of the available structures containing resolved waters (Figure 2a). The innermost of these is part of the homocitrate water pool and is directly hydrogen-bonded to the homocitrate. The waters of the outer region of the channel are much more variable in position between the different X-ray structures, and even between the different halves of the same structures. In each case, however, they connect to the surface. The channel is formed at the interstice between two of the protein subunits, and its walls are defined primarily by residues from the α and β' subunits. In addition, elements of the β subunit are also involved, most notably Arg- β 103 in Kp1 (Arg- β 105 in Av1), whose side-chain nitrogens are hydrogen-bonded to the first two waters in the inner region. The waters of the inner region seem well suited to act as a 'proton wire', delivering protons from the outer region to the homocitrate water pool via the Grotthuss mechanism ([22] and references cited therein). Moreover, the highly variable positions of the waters in the outer region of the channel, together with the participation of three of the four subunits of the MoFe protein in defining its boundaries, suggest that it could be a very dynamic region of the protein in solution. If so, it should provide an efficient path for diffusion, not only of protons, but also of dinitrogen and ammonia, between the buried FeMoco and the exterior of the protein.

It is interesting to compare this nitrogenase channel with other protein water channels. The classic example is gramicidin, a membrane protein that forms a water-filled pore and so allows diffusion of univalent cations across the cell membrane [23]. Gramicidin encloses a chain of between eight and ten water molecules; although these are free to diffuse across the channel, the restrictions imposed by the protein dictate that they can only

move in single file. In contrast, the nitrogenase channel is much more loosely organized. Thus the waters in the outer region appear to be very mobile, whereas several of the inner-region waters are part of the homocitrate water pool. Hence the narrowest region of the nitrogenase channel consists of only two or three waters, which have highly conserved positions in the Kp1 and Av1 crystal structures and single neighbours on each side. This flexibility would be important in allowing molecules as diverse as N_2 and the NH_4^+ cation to diffuse rapidly through the channel. Like H_2 , N_2 is a small molecule with no permanent dipole that interacts weakly with organic molecules and water, and hence little can intervene to promote (or hinder) its diffusion through a protein channel. On the other hand, NH_4^+ resembles H_3O^+ in size and charge. In order to maximize diffusion of NH_4^+ , the channel walls should not contain acidic groups or aromatic rings [24] with which it could interact. Examination of the 'bottleneck' region of the channel for both Kp1 and Av1 shows that of the eight residues which form the bottleneck (which are completely conserved between the two structures), only one is acidic (Asp- β' 516 in Kp1, Asp- β' 520 in Av1), and one is aromatic (Tyr- α 444 in Kp1, Tyr- α 446 in Av1); moreover, both of these side chains are poorly orientated to interact with NH_4^+ ions in the channel. Of the rest, four are non-polar and two are arginines. Hence there is little in this region that would hinder diffusion of the NH_4^+ cation to the surface.

Reversible proton channels to FeMoco

In addition to the interstitial channel, two other likely proton relays to FeMoco are suggested by the protein structure. As discussed above, the central three sulphurs are the most favour-

Figure 3 Schematic view of the proton delivery channel to S2B of the FeMoco

Residue numbers are taken from the published crystal structure of Kp1 [3]. Inferred H atom positions relate to the E_0 state in the Lowe–Thorneley scheme. Broken lines indicate hydrogen bonds. It is likely that a similar network exists in Cp also, with His- α 186 and Tyr- α 268 as the corresponding conserved residues, although the low resolution of the structure prevents location of the water molecule.

able sites for protonation of FeMoco; these are now considered in turn, using the established crystallographic nomenclature.

The first central sulphur of the FeMoco, S2B, is closely associated with the aromatic nitrogen of a histidine side chain; His- α 194 (3.2 Å) in Kp1, His- α 195 (3.4 Å) in Av1 and His- α 186 (3.2 Å) in Cp1. This highly conserved residue is known to play an essential role in successful nitrogen fixation, and has been an important target in site-directed mutagenesis experiments (see below). Examining this histidine for possible proton-transfer contacts, a water molecule (57 in Kp1, 683A in Av1) is found to bridge the second aromatic nitrogen of the histidine and the side-chain oxygen of a tyrosine (Tyr- α 279 in Kp1, Tyr- α 281 in Av1). The hydroxy oxygen of the tyrosine is plainly visible from the exterior of the protein, and should be able to receive a proton from bulk water. It is flanked by two histidines (His- α 196 and His- α 383 in Av1), which were identified by Howard and Rees [8] as a potential surface patch for initial proton capture. The network is shown schematically in Figure 3. It is reasonable to conclude that redox-gated transfer of a proton from the bulk to S2B of the FeMoco can occur via a hydrogen-bonded chain ([25] and references cited therein) in all three nitrogenases during catalysis.

The second central sulphur, S5, is also connected to the surface by a potential proton channel; however, the details are quite different. This channel is very similar for the Kp and Av structures (Figure 4), and is more complicated than the channel to S2B. In addition to a water molecule, S5 is associated with the side chain of a conserved arginine residue (Arg- α 95 in Kp1, Arg- α 96 in Av1 and Arg- α 87 in Cp1). As noted by Mayer et al. [3], the crystal structures suggest the presence of a weak hydrogen bond between the side chain nitrogen and S5 in Av1, but not in Kp1. However, for all three protein structures, rotations of the side chain can produce much better hydrogen bonding geometries between this arginine and S5. Changes in the redox level of FeMoco are likely

Figure 4 Main elements of the proton-delivery channel to S5 of the FeMoco

Details are as for Figure 3. Although the Cp structure shares the arginine residue adjacent to S5, the other details of the channel in this case must be quite different, since the other amino acids are not conserved. In the absence of waters in the crystal structure, the details of the putative Cp channel cannot be deduced, though there is a void in the vicinity of S5 which could easily accommodate several water molecules.

to modulate the strength of this interaction, since an increase in negative charge on FeMoco during catalysis should result in stronger electrostatic interactions with all neighbouring cationic groups.

Considering its pK_a , the arginine side chain is much less likely to be involved in formal proton transfer to S5 than the water molecule, which is also hydrogen-bonded to this atom. Rather, the function of this arginine could be to modulate the basicity of S5 with respect to S2B. In the absence of protein environmental effects, these two sulphurs should have near-identical basicities, hence transfer of a single H^+/e^- couple to FeMoco would result in a 50:50 distribution between these two sites. However, ion pairing between S5 and the cationic arginine side chain should result in a reduction of the basicity of S5 relative to S2B. It is well known from organic chemistry that specific hydrogen-bonding interactions to a functional group can have significant effects on its pK_a ; for example, the first pK_a of maleic acid is lower than that of the isomeric fumaric acid due to the formation of an intramolecular hydrogen bond in the anion of the former, but not of the latter [26]. A precedent for the use of the same underlying effect in enzymology is furnished by the cysteine proteases, such as papain. This enzyme features an ion pair formed between cysteine and histidine residues at the active site [27]. The ion-pairing results in a change in the pK_a of the cysteine from its normal value of approx. 9 to approx. 4; that is to say, the proton affinity of the cysteinyl sulphur is significantly attenuated by ion-pairing with a protonated histidine, and this effect is an important catalytic feature. It is worth noting here

Figure 5 Protein environment around S3A

Residue numbers pertain to the α subunit of Kp1, as follows (NH–S distances in parentheses): Gly-354 (3.4 Å), Gly-355 (3.4 Å), Leu-356 (3.6 Å) and Arg-357 (3.5 Å). Corresponding residues in Av1 are Gly-356 (3.4 Å), Gly-357 (3.4 Å), Leu-358 (3.6 Å) and Arg-359 (3.7 Å); corresponding residues in Cp1 are Gly-344 (3.2 Å), Gly-345 (3.3 Å), Ser-346 (4.0 Å) and Arg-347 (3.5 Å).

that the nitrogen of a second arginine, Arg- α 357 in Kp1 (Arg- α 359 in Av1), lies within 4.1 Å of S5.

Further evidence that the protein environment is specifically designed to control the order of protonation of S5 and S2B is provided by comparison of the wider hydrogen-bonding networks extending from the two sulphurs. Thus, whereas S2B has a short connection to the surface for proton transfer, S5 has a more convoluted route. In fact, there appears to be no direct connection between S5 and the surface in the Kp1 and Av1 crystal structures. The most likely route leads to a histidine residue (His- α 272 in Kp1, His- α 274 in Av1), which is hydrogen-bonded to a backbone NH group (Figure 4). However, there is a surface histidine in close proximity (His- α 360 in Kp1, His- α 362 in Av1), which could transfer a proton from solution after formation of an alternative hydrogen-bonding network. Again, both of these histidines, along with His- α 451 in Av1, were identified by Howard and Rees [8] as forming a second surface patch in Av1, which could act as an initial proton-acceptor site. Thus, I deduce that, although both S2B and S5 receive H^+/e^- couples during turnover, the protein matrix is set up in such a way that these two sites are distinguished; on average, S2B will receive the first H^+/e^- couple and S5 the second.

The proton-delivery channels to S5 and S2B of FeMoco differ from the interstitial channel described above in that they involve amino acid residues as well as water. Proton transport through water via the Grotthuss mechanism is a two-step process; after the net transfer of a proton across the hydrogen-bonded chain, it is necessary for the water molecules to realign to their original positions before another proton can be transferred. For a proton channel involving histidine side chains as well as water molecules, the analogous 'turn' step will be much more difficult, since it requires realignment of the imidazole rings to form an alternative hydrogen-bonding network. If, however, the proton channel is only used once during the catalytic cycle, this problem does not arise. This suggests that the protons that arrive at S5 and S2B are not ultimately transferred to substrate, but instead are returned to bulk water at a later stage of the catalytic cycle, when the FeMoco has passed beyond its most reduced levels. In this way, the arrival of a single electron at FeMoco at a specific redox level in the Lowe–Thorneley scheme could trigger the addition of more than one proton to the partially reduced substrate while still retaining the overall 1:1 H^+/e^- ratio, by the simultaneous discharge of a proton from S2B or S5. All of the protons that are added to the substrate would then be supplied by the interstitial

channel, which is composed only of waters and can therefore exploit the Grotthuss mechanism for multiple rapid proton transfers.

The third central sulphur of FeMoco, S3A, is extensively hydrogen-bonded, but only to the protein backbone (Figure 5). Thus, in all three nitrogenases, S3A is associated with the backbone NH groups from four consecutive residues, but there appears to be no connection with the exterior of the protein, which could act as a proton relay. Hence, considering the effects of hydrogen-bonding on effective pK_a values discussed above, the local environment around S3A should support the build-up of negative charge on the FeMoco, without the actual transfer of a proton to the cluster.

It is instructive to compare the proton channels discussed above with those found in other enzymes. Although proton-transfer channels are implicated as components of a wide range of proteins, examples that have been defined by X-ray crystallography are still quite rare. One good example is cytochrome *c* oxidase (COX), the terminal oxidase in cell respiration. This protein can be likened to nitrogenase in that it mediates the reduction of a small molecule, in this case oxygen, at a transition-metal site. It has been proposed, on the basis of mutagenic and crystallographic studies [28], that COX has multiple proton channels, as well as a water channel. The putative proton channels are composed of water molecules and a range of amino-acid side chains, including histidine, tyrosine, serine and aspartate. As discussed above for the S5 nitrogenase channel, it seems likely that conformational changes in COX side chains may be required in order to convert the crystal structure into a proton-transmitting form, and these may be induced by changes in redox level or ligand binding.

The probable significance of the proton channels to S2B and S5 with respect to H_2 formation on the FeMoco is now considered. As discussed above, migration of an H atom from sulphur to iron is likely to be deleterious for successful nitrogen fixation. Hence it is proposed that, although S2B and S5 constitute the initial sites for addition of H^+/e^- to the FeMoco, occasional unwanted migration of H from sulphur to iron results in futile H_2 evolution via hydrolysis. This is consistent with the known chemistry. Thus, in a kinetic study of the reduction of protons to dihydrogen by the model cluster $[Fe_4S_4(SC_6H_5)_4]^{3-}$, it was concluded that the initial sites of protonation are at both types of sulphur (i.e. the sulphides of the Fe_4S_4 core and the thiolato sulphurs of the $SC_6H_5^-$ ligands); subsequent loss of a thiophenolate ligand (as C_6H_5SH) allows protonation at iron, and it is the resulting hydride species that is responsible for dihydrogen production [29].

Clearly, any additional restraint from the protein environment on H migration from sulphur to iron will enhance the efficiency of N_2 reduction. In this regard, transfer of a proton to S2B via histidine has the advantage that the histidine is also well placed to hinder subsequent H migration via formation of a reversed hydrogen bond. This is illustrated in Scheme 2. Given that transfer of an H atom from S to Fe is already intrinsically endothermic [17], the additional cost of breaking a hydrogen bond from the histidine should be sufficient to effectively suppress this process. This extra restraint may be compared with the operation of a ratchet, in that it helps FeMoco to access its more reduced states against a thermodynamic drive towards H_2 formation. An additional consideration is that only S2B will benefit from a reversed hydrogen bond after proton transfer, since the proton donor to S5 is water, which will be too mobile to effectively restrain the migration of this second hydrogen. However, since the hydrogen on S5 arrives at a later stage of the catalytic cycle, it necessarily has less opportunity to migrate.

Scheme 2 Proposed mechanism for H₂ formation via H migration

(a) Hydrogen bond between S2B and histidine, as in Figure 4. (b) Reversed hydrogen bond following transfer of a proton to FeMoco. (c) Disruption of hydrogen bond following nitrogenase associated protein association/dissociation. (d) Migration of H to Fe.

Comparison with mutagenesis results

A number of published mutagenesis studies have dealt with amino acid residues that are components of the proton channels discussed above. The results generally fit in well with the model presented in this paper, as discussed below. The mutagenesis work has largely been performed on Av1; however, there are no significant structural differences with Kp1 in this regard.

The proton channel to FeMoco S2B is considered first. The most important data here come from the extensive literature relating to His- α 195 in Av1 (His- α 194 in Kp1; Figure 3) [30–32]. Substitution of histidine with asparagine, tyrosine, leucine, threonine or glycine gave Nif⁻ phenotypes in each case, whereas substitution with glutamine gave rise to a nitrogenase that can reduce N₂, but only at a rate < 2% of that of the wild type [30]. Interestingly, the Gln- α 195 mutant appears to bind N₂ normally, whereas the other mutants examined appeared to be unable to bind N₂ [31,32]. It has been pointed out [33] that Gln- α 195 would be able to hydrogen-bond to S2B via its amide NH₂ group, whereas the Asn- α 195 NH₂ group would not reach S2B. These results can be accommodated in the present model as follows: although it can hydrogen-bond to S2B, Gln- α 195 would not be able to participate in proton transfer in the same way as His- α 195. The production of each successive state in the Lowe–Thorneley scheme can be viewed as a series of alternating equilibria for addition of H⁺ and e⁻, in which each successive equilibrium constant is contingent upon the previous constants. Thus the Gln- α 195 mutant, which retains a hydrogen-bond to S2B, can sustain reduction of FeMoco to a level beyond that which is attainable in the absence of the hydrogen bond. However, a hydrogen bond supplies less charge stabilization at FeMoco than does the formal transfer of a proton to S2B. Hence, although reduction of FeMoco can proceed one step further in the Gln- α 195 mutant than in the Asn- α 195 mutant, allowing access to a state which can bind N₂, the equilibrium for the next

electron transfer is too poor to allow significant access to states in which N₂ becomes committed to reduction.

Another interesting set of mutations deal with the substitution of Arg- α 277 in Av1 (Arg- α 275 in Kp1) [34]. Although not shown in Figure 3, the side chain of this residue is associated with the phenoxy oxygen of Tyr- α 281 (Tyr- α 279 in Kp1; N–O distances of 3.1 and 3.0 Å in Av1 and Kp1 respectively), which is the initial member of the proton channel to FeMoco S2B. The arginine side chain shows different orientations in Kp1 and Av1; in the former, there is an additional contact between the residue's secondary amine NH group and the water of the proton channel, whereas in Av1 the orientation of the -NHC(NH₂)₂ group is reversed. Substitution of Arg- α 277 with lysine, cysteine, threonine, phenylalanine or leucine gave rise to phenotypes that showed diazotrophic growth at reduced rates compared with the wild type, whereas substitution with histidine gave a Nif⁻ phenotype. The His- α 277 nitrogenase was isolated and found to be incapable of binding N₂, although it could still reduce alternative substrates (H⁺, C₂H₂, HN₃ and HCN) [34]. In the present study, molecular modelling on the Kp1 structure suggests three possible ways in which this mutation could disrupt proton transfer to S2B. First, His- α 275 might displace the water molecule (57 in Figure 3) from its normal position. Secondly, His- α 275 could form an alternative hydrogen bond to Tyr- α 279. Thirdly, and perhaps most likely, His- α 275 could form an alternative hydrogen bond with His- α 194. All three possibilities would disrupt communication between S2B and the surface; in addition, the last possibility would probably also result in a loss of the hydrogen bond between His- α 194 and S2B.

Turning to consider mutagenesis work relevant to FeMoco S5, mutation of Arg- α 96 in Av1 to lysine or glutamine gave rise to proteins that were still capable of nitrogen fixation, although at a reduced rate compared with the wild type [35]. This would be expected from the model presented above, since Arg- α 96 is not itself a component of the proton channel to S5, but rather acts to 'fine-tune' the pK_a. In contrast, His- α 274 in Av1 is a component of the proton channel (His- α 272 in Kp1; Figure 4), but mutation of this residue to glutamine again generated a phenotype that was still capable of diazotrophic growth [36]. The effects of this mutation have been checked by molecular modelling on the Kp1 structure. Since a glutamine side chain occupies only approx. 90% of the volume of a histidine, it proved possible to include an extra water molecule between water no. 56 and His- α 360 (see Figure 4) without significantly perturbing the surrounding protein. Hence the proton channel to S5 might be relatively unaffected in this mutant, with an extra water taking the place of His- α 272 in the hydrogen-bonded chain. There is a precedent for this in the proton channels of COX; here, a serine or threonine residue constituting part of one proton channel is replaced by alanine or glycine in some species, and the proton relay function is thought to be carried out by an extra water in these cases [28]. The apparently lower mutagenic sensitivity of the S5 proton channel compared with the S2B channel, suggested by the above discussion, fits in with the view that the S5 channel is used later in the catalytic cycle, i.e. at a point when N₂ is closer to being irreversibly committed to reduction.

Release of dihydrogen from nitrogenase during turnover

The above analysis has important implications for the Lowe–Thorneley scheme. As shown in Scheme 1, the first measurable release of H₂ is associated with the futile return of state E₂H₂ to E₀. Moreover, from their kinetic analysis, Lowe and Thorneley were able to deduce that two dissociations of the Fe protein from the MoFe protein are required before dihydrogen is evolved [9].

In the absence of structural information on the nitrogenase proteins, these authors proposed that Kp1 is unable to bind substrates or inhibitors or release products when complexed with Kp2, i.e. only free Kp1 can release H_2 . However, this proposal can now be re-evaluated in the light of the available X-ray structural information. Figure 2(b) shows an overlay of the water channels, described above, from the free Av1 and the Av1–Av2 transition-state-complex structures. Although no water molecules were refined in the latter, there is an excellent match in the positions of the amino-acid residues, suggesting that the chain of waters from the water pool around homocitrate to the surface remains intact in the transition-state complex. Moreover, the entrance to the channel lies on the opposite side of the MoFe protein to the Fe-protein-docking region. Given that there appears to be uninterrupted contact with the exterior of the protein in both structures, it now seems unlikely that H_2 , the smallest of all molecules, is unable to escape from the vicinity of the FeMoco at any stage in the catalytic cycle.

An alternative explanation for the experimental observation that H_2 is only lost from state E_2H_2 upon dissociation of the two proteins follows from the hydrogen-bonding effects discussed above. Any structural fluctuations that serve to disrupt the $S2B-H \cdots His$ hydrogen bond will promote hydrogen migration to Fe and subsequent H_2 production. The histidine residue lies close to the extensive interface between the two component proteins in the transition-state complex; for example, the side chain of the next residue, His- α 196, approaches to within 4 Å of Glu- γ 68 of the Fe protein. Although the Fe protein has undergone the most dramatic structural changes in the transition-state complex, it is reasonable to suppose that both proteins will suffer transient, but extensive, distortion during the protein complex association/dissociation step of the Lowe–Thorneley cycle. As a consequence, temporary disruption of the $S2B-H \cdots His$ hydrogen bond would reduce the energy barrier for migration of the hydrogen to iron (Scheme 2, part c). According to this view, it is the act of protein dissociation itself that triggers H_2 evolution by promoting H migration from S to Fe. This hypothesis reconciles the available kinetic and structural data. Although it requires no alterations to the Lowe–Thorneley scheme, it would affect the underlying kinetic analysis. This is because, in the Lowe–Thorneley scheme, H_2 evolution is assumed to occur only from free Kp1, hence the rate of H_2 production depends on the concentration of this species. In the present model, the rate of H_2 production would instead be a function of the number of protein association/dissociation events per electron transferred to Kp1. In this regard, ongoing research by several groups into the all-ferrous state of the Fe protein is relevant [37–39]. It has been argued on thermodynamic grounds that this species could be a viable intermediate in nitrogenase catalysis [37]. The all-ferrous Fe protein can transfer two electrons to the MoFe protein [38], although it is not yet known if the dissociation of the complex occurs after each one-electron transfer or only after both electrons have been transferred. It has been noted [38] that a two-electron transfer mechanism would require a ratio of one ATP hydrolysed per electron transferred, rather than the stoichiometry shown in reaction (1). Furthermore, the H_2 production mechanism discussed above would also be suppressed if two electrons were transferred for each protein association/dissociation event. This in turn suggests a specific experimental test of the current model. Measurement of the limiting $H_2:N_2$ stoichiometry under conditions where the all-ferrous Fe protein was used to mediate electron transfer [i.e. if methyl viologen or titanium(III) citrate was used instead of dithionite as the primary reductant] should give a lower $H_2:N_2$ ratio for the two-electron reductant compared with the one-

electron reductant (although one of the experiments by Simpson and Burris [14] included methyl viologen, this was against a much greater concentration of dithionite).

It should be stressed that the H_2 production mechanism described above is unlikely to be the only way in which H_2 can be produced by nitrogenase; other aspects of nitrogenase catalysis, such as H_2 inhibition of N_2 reduction, the HD formation reaction and OHE must involve other, quite separate, processes, which are the subject of ongoing investigations.

Conclusion

Three separate proton-transfer channels to FeMoco have been identified. The channels appear to be present in all crystallographically characterized nitrogenases within the limits of the crystal-structure resolution. It is suggested that two of the channels are used to enable reduction of FeMoco in such a way that wasteful H_2 production is suppressed. Disruption of either of these channels denies the FeMoco access to its more reduced states, resulting in a loss of catalytic function. The third channel is used to supply protons to the partially reduced substrate, and also for diffusion of substrates and products to and from the buried active site. In this model, H_2 is produced in an unwanted side-reaction promoted by association/dissociation of the protein complex during reduction of the MoFe protein. An experimental test of the hypothesis is suggested by the use of the all-ferrous form of the iron protein in re-measurement of the limiting $H_2:N_2$ ratio for molybdenum nitrogenase.

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