An Atomic-Level Mechanism for Molybdenum Nitrogenase. Part 2. Proton Reduction, Inhibition of Dinitrogen Reduction by Dihydrogen, and the HD Formation Reaction†

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Received February 4, 2002; Revised Manuscript Received August 6, 2002

ABSTRACT: Quantum calculations have been used to examine the energetics of the reactions of diazene and isodiazene with H_2 and the properties of the Fe and Mo sites of the nitrogenase iron−molybdenum cofactor with respect to the binding of H and H_2. The results have been used to extend the model for N_2 reduction by nitrogenase given in the preceding paper to describe the formation of HD from D_2. The proposed mechanism for HD formation invokes a combination of two well-established chemical reactions, namely, competitive protonation of metal N_2 species at either the metal or at N_2, followed by scrambling of D_2 at a metal hydride. The model is evaluated against the available biochemical data for the nitrogenase HD formation reaction and extended to account for H_2 inhibition of N_2 reduction and the reduction of H^+ in the absence of other substrates.

In the preceding paper, a detailed mechanism for the reduction of dinitrogen by Mo nitrogenase was proposed on the basis of theoretical calculations, published biochemical data, and established chemical precedents. In addition to their essential biological function in the reduction of dinitrogen to ammonia, all known nitrogenases catalyze the production of dihydrogen from protons. Under turnover conditions but in the absence of any other reducible substrate, nitrogenase catalyses the reduction of H^+ to H_2, a process which has been referred to as general hydrogen evolution (*1*). When N_2 is introduced, the rate of H_2 evolution decreases until a limiting ratio of N_2 reduced to H_2 produced is reached; this nonsuppressible H_2 evolution is known as obligatory hydrogen evolution (OHE)[1] (*1*). The exact N_2:H_2 ratio varies with different nitrogenases; for the Mo enzyme, the accepted limiting H_2/N_2 ratio is ∼1 (*2*), but for V nitrogenase, it is 3.5 (*3*). Several other facets of the chemistry of Mo nitrogenase with protons, H_2, and N_2 have been revealed by experiment. Thus, H_2 is a competitive inhibitor for the reduction of N_2, but of no other substrate (*4*, *5*). One of the most intriguing properties of the enzyme is its ability to produce HD from D_2, the so-called HD formation reaction (*2*, *4*, *5*). Although hydrogenases also produce HD when operating under an atmosphere of D_2, the nitrogenase-catalyzed reaction has a number of striking differences. Most notably, HD formation by nitrogenase requires the presence of N_2 as well as D_2 (*6*). Since the steady-state kinetics of HD production and of H_2 inhibition of N_2 reduction by nitrogenase were found to be the same, it has generally been assumed that these two phenomena are different manifestations of the same underlying molecular process (*2*). Recent experimental studies on the α-Asn[195] mutant nitrogenase from *A. vinelandii* have qualified this view (*5*). This nitrogenase binds N_2, apparently in the same way as the wild-type enzyme, but does not reduce it. H_2 was found to inhibit N_2 binding, but the mutant gave no HD when turning over under a D_2/N_2 atmosphere. Hence, H_2 inhibition of N_2 binding (distinguished from N_2 reduction) appears not to be directly related to HD formation. The authors suggested that initial binding of N_2 at the FeMoco suffers from direct competition by H_2, whereas a reduced form of N_2 may be required for HD formation. The idea that a reduced N_2 intermediate is somehow involved in HD formation is consistent with the observations that one electron is consumed for each molecule of HD produced and that these electrons are diverted exclusively from N_2 reduction, i.e., OHE is unaffected by HD formation (*7*).

Another key difference in HD formation by nitrogenase compared to hydrogenase concerns the exchange of H^+/D^+ with the solvent. Hydrogenases generally react with D_2 in water to give HD, H_2, and HOD. This has been interpreted in terms of the reversible heterolytic cleavage of D_2 to give a metal hydride and a protonated amino acid residue (*8*−*12*). Both of the D atoms can then exchange with H_2O, but the hydride exchanges more slowly than the protonated amino acid (*8*−*10*). In contrast, tritium labeling experiments on nitrogenase showed that the rate of incorporation of T^+ into the aqueous phase was much slower than the rate of HD formation under the same conditions (*13*). This suggests that unlike the hydrogenase-catalyzed reaction, cleavage of D_2 by nitrogenase does not produce readily exchangeable protons.

† Support was provided by the Biotechnology and Biological Sciences Research Council, U.K.

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[1] Abbreviations: Av1, Kp1, molybdenum nitrogenases from *A. vinelandii* and *K. pneumoniae*, respectively; BE, binding energy; DFT, density functional theory; FeMoco, iron−molybdenum cofactor; MP2, MP4, second- and fourth-order Møller−Plesset perturbation theory, respectively; OHE, obligatory hydrogen evolution; zpe, zero-point energy.

10.1021/bi025624r CCC: $22.00
Published on Web 10/30/2002

A central point of controversy in models for the nitrogenase HD formation reaction centers on the chemical nature of the species involved in this process. As noted above, some features of the reaction are best explained in terms of HD formation mediated by a partially reduced N_2 intermediate (*5*), such as diazene (*13*). However, the D−D bond is strong (106 kcal mol^{-1}), and chemical systems for H_2 activation most commonly involve metal hydrides. This was interpreted by Lowe and Thorneley (*2*, *14*) in terms of homolytic cleavage of D_2 at the nitrogenase active site, followed by attack of H^+ on the resulting dideuteride to give HD. This is consistent with known chemistry (*1*) and fits in with the Lowe−Thorneley scheme for reduction of N_2 (*2*, *14*−*17*). In the Lowe−Thorneley scheme, N_2 binding can only occur by the reversible displacement of H_2; HD formation and H_2 inhibition of N_2 reduction then arise from displacement of N_2 by D_2 and H_2, respectively. Although this model provides a chemically reasonable pathway for D−D bond cleavage via metal deuterides, it does not invoke any reduced N_2 intermediate; hence, it cannot easily be reconciled with the new observation from the α-Asn^{195} mutant studies (*5*) that binding of N_2 to the FeMoco is of itself not sufficient to give HD.

An important requirement for any atomic model for nitrogenase action is that it should describe not just N_2 reduction, but also the H^+, H_2 and D_2 chemistry of the enzyme. This paper deals with the extension of the model described in the preceding paper to accommodate this chemistry. A new model for the HD formation reaction is proposed on the basis of competition for protons between N_2 and the FeMoco core. As noted above, some aspects of the HD formation reaction are best explained in terms of a reduced N_2 intermediate; others suggest that metal hydrides are required. Much of the discussion in the literature contrasts these two aspects of the reaction, which are generally viewed as mutually exclusive possibilities. A key feature of the model presented in this paper is that it reconciles these two facets of the reaction by invoking both a reduced N_2 species and a metal hydride as intermediates.

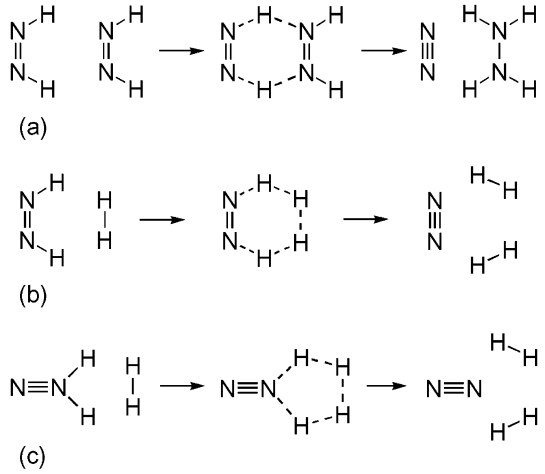
MATERIALS AND METHODS

All quantum calculations were performed using GAUSSIAN 98W (*18*). DFT calculations were carried out exactly as described in the preceding paper. Calculations on the reactions of free N_2H_2 isomers with H_2 were done following the methodology of McKee et al. (*19*). Full geometry optimizations (calculated without any symmetry constraints) and frequency calculations were carried out at the MP2/6-31G* level. Final single-point calculations at the calculated geometries were done at the MP4/6-31+G(2d, p) level of theory. Protein coordinates were retrieved from the Brookhaven Protein Data Bank (*20*), accession codes 1QGU (Kp1) and 2MIN (Av1). Figures were prepared using RASMOL molecular graphics software (*21*).

RESULTS

Direct Reaction of N_2H_2 Species with H_2. As mentioned in the Introduction, the nitrogenase HD formation reaction has been widely interpreted in terms of the direct reaction of an N_2H_2 intermediate with D_2. In principle, this could involve either diazene or isodazene (Scheme 1), either bound to the cofactor or having dissociated into solution. Free diazene is very reactive and, in the absence of other reagents, undergoes rapid disproportionation in solution to give N_2 plus hydrazine. Note that *trans*-diazene is the more stable isomer; however, the cis form required for disproportionation or reaction with H_2 is only ∼6 kcal mol^{-1} higher in energy. The disproportionation reaction (Scheme 1a) was investigated quantum mechanically by McKee et al. (*19*) using Møller−Plesset perturbation theory to account for the effects of electron correlation. The results were in very good agreement with experiment and showed that there is essentially no potential energy barrier for this reaction. The same methodology has been applied in the present study to the hypothetical reactions of free diazene and isodiazene with H_2 (panels b and c, respectively, of Scheme 1). As expected, the diazene reaction b was calculated to be strongly exothermic (MP4 energy −50.5 kcal mol^{-1}, or −58.3 including zpe's). However, the transition state had a calculated MP4 energy of +26.6 kcal mol^{-1} (+25.6 kcal mol^{-1} including zpe's). The transition state structure was identified as a genuine saddle point by virtue of its single imaginary frequency (−2021 cm^{-1}). For the sake of comparison, the reaction was also examined by DFT (B3LYP/LanL2DZ), giving a barrier of +24 kcal mol^{-1}. The isodiazene reaction c is even more exothermic (MP4 energy −71.4 kcal mol^{-1}, or −79.1 kcal mol^{-1} including zpe's), but the energy barrier was found to be higher; in this case the calculated MP4 transition state energy was +33.0 kcal mol^{-1} (+29.6 kcal mol^{-1} including zpe's; single imaginary frequency of −2208 cm^{-1}), compared to a DFT value of +35 kcal mol^{-1}.

Scheme 1: (a) Disproportionation of *cis*-Diazene; (b) Hypothetical Reaction of *cis*-Diazene with H_2; (c) Hypothetical Reaction of Isodiazene with H_2[a]



[a] Dashed bonds are used to indicate the transition state structures.

In order for the free N_2H_2 species to react with D_2 as described above, it would first need to dissociate from the FeMoco. The calculated DFT energies for the dissociation of *cis*-diazene and isodiazene from the FeMoco were calculated by comparison of structures **1** and **2** with structure **5** in Figure 1, as +44 and +56 kcal mol^{-1}, respectively; combining these figures with the DFT energies for the reactions discussed above gives overall energy barriers for the dissociation of diazene and isodiazene from the FeMoco followed by reaction with H_2 of +69 and +90 kcal mol^{-1}, respectively.

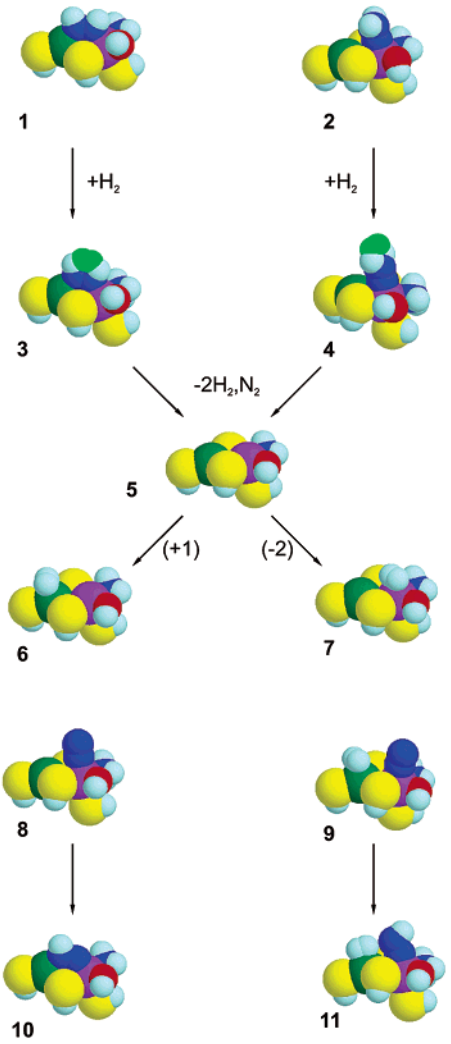


FIGURE 1: Reactions of bound diazene and isodiazene intermediates with H_2, binding characteristics of H_2 at the trigonal Fe and homocitrate ring-opened Mo sites of the FeMoco, and destabilization of the NNH intermediate by H_2. Color code: C, gray; H, pale blue; N, dark blue; O, red; Fe, green; Mo, purple; S, yellow. The incoming H_2 molecules in structures **3** and **4** are highlighted in green.

An alternative possibility is that H_2 reacts directly with the N_2H_2 intermediate without dissociation of the latter from the FeMoco. The calculated overall energies for reaction of the bound diazene and isodiazene species, **1** and **2**, respectively, in Figure 1, with H_2 to give N_2 (bound at Mo) plus $2H_2$ were -4 and $+1$ kcal mol^{-1}, respectively. Therefore, both reactions are thermodynamically allowed. However, the transition state structures **3** and **4** had energies of $+69$ and $+76$ kcal mol^{-1} compared to **1** and **2**, respectively. The geometries of these bound-N_2H_2 transition state structures are very similar to those for the free species discussed above.

Binding of H_2 at FeMoco Sites. The model complexes used to evaluate the H_2 binding characteristics at different FeMoco sites, namely, the trigonal Fe^{II} atoms and the five-coordinate Mo^{III} atom generated by opening of the homocitrate ring, are shown in Figure 1. Comparison of complexes **5**, **6**, and **7** in Figure 1 gave H_2 binding energies (BE's) of $+1$ and -2 kcal mol^{-1} for the Fe^{II} and Mo^{III} sites, respectively. Hence, binding of H_2 at both sites is extremely weak and the energies involved are of the same order as van der Waals interactions. The result for the Fe site is in good agreement with the calculations of Rod and Nørskov (*22*), which gave H_2 BE's of $+2$ to $+10$ kcal mol^{-1}, depending on the number of H atoms added to the cluster core (as with N_2 binding, the simpler model employed in the current work does not take into account the cost of distortion of the FeMoco on binding ligands at Fe). The calculated H−H bond lengths of 0.751 and 0.752 Å in **6** and **7**, respectively, compared to 0.744 Å calculated for free H_2 (experimental value 0.741 Å), suggest essentially no weakening of the H−H bond upon coordination to either metal. Hence, the FeMoco seems to be incapable of strong binding and activation of H_2 with respect to oxidative addition to give a dihydride at either its Fe or Mo sites. Structure **9** in Figure 1 indicates that the presence of bound N_2 on Mo has no detectable effect on H_2 binding at Fe. Indeed, initial geometry optimization on this complex resulted in complete dissociation of the H_2, and structure **9** could only be generated by constraining one Fe−H distance to 2.30 Å; the H_2 BE for **9** is $+1$ kcal mol^{-1}.

Perturbation of N_2 Protonation by H_2. In the preceding paper, it was found that addition of H to N_2 terminally coordinated at Mo (as in Figure 1, **8**) resulted in spontaneous rearrangement to a bridging diazenido(1−) ligand, structure **10**. This suggests a possible role for H_2 in inhibiting the reduction of N_2; although H_2 binds very weakly at Fe, the presence of H_2 on Fe as in **9** could destabilize the diazenido-(1−) state by preventing access to this bridging mode. The structure of the resulting terminally bound, H_2-blocked complex is shown in Figure 1, **11**. Assuming that the H_2 BE in this complex is the same as that for complex **9**, the diazenido(1−) ligand in **11** is destabilized by 5 kcal mol^{-1} compared to **10**. It is important to note immediately that this destabilization is a kinetic effect, since dissociation of H_2 to allow bridging of the NNH ligand is thermodynamically favorable. Nevertheless, kinetic stabilization of thermodynamically disfavored intermediates is the central principle of enzyme catalysis.

Destabilization of the initial protonated form of N_2 could have important consequences, since this state is well-known to be the most energetically unfavorable in the course of reduction of N_2 to NH_3. Specifically, it has been known for many years from chemical studies on dinitrogen complexes that protonation of N_2 competes with protonation at the metal (*23*−*28*). This possibility was investigated in the present work. First of all, the effects of adding a single H atom to either the Fe or Mo site of the model to give a metal hydride were evaluated. The Mo hydride proved to be more stable than the Fe hydride; indeed, for some spin states, the H atom spontaneously migrated from Fe to Mo during the geometry optimization. The final minimum-energy structure is shown in Figure 2, **12**. The Mo−H bond energy in this structure is 45 kcal mol^{-1}. Although the H atom appears to be bridging between the metal atoms, the Fe−H and Mo−H bond lengths (2.21 and 1.79 Å, respectively) show that it is bound primarily to Mo. Next, an H atom was added to the MoN_2 complex at the Mo atom rather than at the terminal N. This resulted in complete dissociation of the N_2 ligand (Figure 2, **13**).

H_2 Scrambling and H_2 Production Properties of the Hydride Ligand. Formation of a metal hydride by the process outlined above raises the possibility of scrambling of the hydride with H_2 or D_2. This type of reaction is well established in complexes containing both hydride and H_2

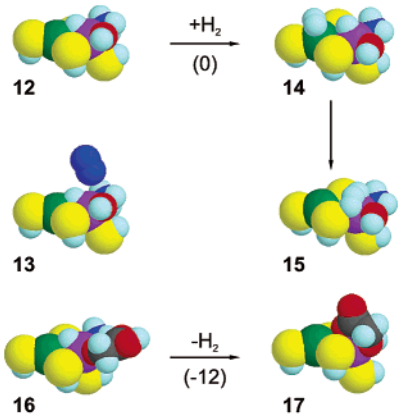


FIGURE 2: Binding characteristics of H, effect on N_2 binding, H_2/H scrambling, and H_2 formation at the Mo site. Color code as in Figure 1.

ligands (*29*). Binding of H_2 at the Fe atom of the Mo hydride **12** to give **14** was found to be thermoneutral (as with **9**, one Fe−H distance had to be fixed at 2.30 Å to prevent dissociation of the H_2). In the case of D_2, transfer of the D_2 to Mo, giving a $MoHD_2$ species, would then allow scrambling to a molybdenum deuteride plus HD. Such a scrambling reaction would necessarily be approximately thermoneutral; the transition state for this process is approximated by structure **15**, in which the H−H distances between the central and peripheral H atoms of the H_3 ligand were constrained to be equal in the geometry optimization. Comparison of structures **14** and **15** gave a barrier of approximately +23 kcal mol^{-1} for the scrambling reaction.[2]

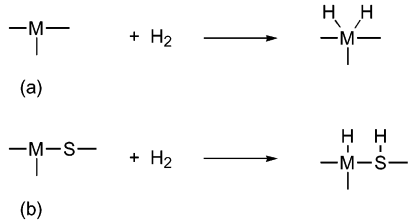
A final consideration is the stability of the Mo hydride/deuteride species itself. As shown in Figure 2, the hydride **16**, which is formally Mo^{IV} and has a protonated carboxylate group, is thermodynamically unstable with respect to elimination of H_2 and reclosure of the homocitrate ring to give **17**. Thus, there would probably be a limited time scale for scrambling to occur before elimination of H_2 returned the enzyme to an earlier state in the Lowe−Thorneley scheme.

DISCUSSION

Mechanism of the HD Formation Reaction. Production of HD from D_2 by nitrogenase requires capture of D_2 from solution followed by cleavage of the D−D bond. As mentioned in the Introduction, there are two longstanding opinions as to how this reaction might occur. The first of these dates from 1960 and a proposal by Hoch (*30*) that the reaction involves a diazene level intermediate. Subsequent detailed experimental work on the reaction (*13*) lent strong biochemical support to this view. However, it was also shown in the 1960s that N_2 can displace two hydride ligands, as H_2, from a metal to give a dinitrogen complex (*31*). This observation was incorporated into the Lowe−Thorneley scheme such that N_2 can only bind to the FeMoco via displacement of two hydride ligands as H_2. In the Lowe−Thorneley scheme, HD is produced via reversible displacement of N_2 by D_2.

[2] Of all the structures considered in this and the preceding paper, the relative energies of **14** and **15** are most sensitive to the constraints placed on the Mo coordination sphere. This appears to be a reflection of the very small size of the hydride ligand, together with the substantial trans interaction between the hydride and SH ligand on Mo.

Scheme 2: Schematic Mechanisms for (a) Oxidative Addition and (b) σ-bond Metathesis



As expected, the calculations on free diazene and isodiazene presented in this work indicate that their reaction with D_2 to give 2HD plus N_2 is energetically highly favorable. Even when bound to the FeMoco, these reactions are still thermodynamically allowed. However, the very high calculated energy barriers for the reactions of bound N_2H_2 species, of +69 kcal mol^{-1} or more, indicate that the direct reaction of a diazene level intermediate with D_2 is not a kinetically viable route to HD in the nitrogenase catalytic cycle. Furthermore, there are no close chemical precedents for this type of reaction. Perhaps the most relevant chemistry described to date concerns a *trans*-diazene complex, [μ-N_2H_2-{Ru(PCy_3)("S_4")}$_2$][Cy = cyclohexyl, "S_4" = $(CH_2SC_6H_4S^-)_2$], described by Sellmann (*32*). This complex reacts with D_2 to give HD plus the equivalent N_2D_2 complex. However, this reaction clearly does not proceed via direct interaction of D_2 with the diazene ligand, since this is in an enforced trans geometry. Rather, the reaction was found to involve heterolytic cleavage of D_2 to give a metal deuteride.

Given the problems attending the production of HD by nitrogenase via direct reaction of a diazene level intermediate, the alternative possibility of HD production via metal hydrides requires further consideration. The factors controlling this type of chemistry are now quite well understood. Since the first isolation of 'nonclassical' dihydrogen complexes in 1984 (*33*), a great deal of experimental and theoretical work has been directed toward elucidating the interconversion of dihydrogen and hydride ligands, and the theoretical aspects of this topic have recently been reviewed (*29*). Two pathways leading to cleavage of the H−H bond have been described, namely, oxidative addition and σ-bond metathesis (Scheme 2).

Oxidative addition involves the formation of two M−H bonds, with concomitant H−H bond cleavage and oxidation of the metal by two units. The Lowe−Thorneley scheme for HD formation implies the formation of such a dihydride (*2*, *14*). By combining the production of hydrides from H_2 with reversible mutual displacement of H_2 and N_2, Lowe and Thorneley produced a mechanism which accounts for the experimental data relating to H_2 inhibition, HD formation, and the limiting H_2/N_2 stoichiometry. However, this model does not readily explain the α-Asn^{195} mutant data, discussed in the Introduction, which implicate a reduced N_2 intermediate in HD formation (*5*). A further problem relates to the complete lack of activation of H_2 toward oxidative addition on both the Fe and Mo sites of the FeMoco, as indicated by the DFT results presented above and also, in the case of the Fe sites, by the DFT studies of Rod and Nørskov (*22*). This is in line with expectations from other studies, which show that second- and third-row metals, in relatively low oxidation states and with strongly electron-releasing co-ligands such

as phosphines, are generally required for oxidative addition of H_2 (*29*). For example, DFT calculations on a series of complexes prepared by oxidative addition of H_2 to $[MoX_2(PMe_3)_4]$ (X = F, Cl, Br, or I) gave Mo−H bond energies of at most 64 kcal mol^{-1}; experimentally, these complexes are quite unstable, and the iodide complex, for which the calculated Mo−H bond energy was 55 kcal mol^{-1}, could not be isolated at all (*34*). The Mo−H bond energy of 45 kcal mol^{-1} determined for **12** in the present work is less than this value. Hence, it is difficult to imagine that any site on the FeMoco could sustain cleavage of D_2 via oxidative addition during turnover.

The second established pathway for H−H bond cleavage, σ-bond metathesis, has also been considered as the basis for various HD formation pathways in nitrogenase. Published DFT calculations suggest that metathesis of H_2 on the FeMoco to give an iron hydride and a protonated sulfide is energetically feasible (*22*), while elegant synthetic chemistry has provided experimental precedents for a model in which H_2 is initially cleaved to an iron hydride and a protonated sulfur at a diazene level intermediate (*32*). Furthermore, it is highly likely that H−H bond cleavage occurs via heterolytic σ-bond metathesis to give a metal hydride and a protonated cysteine in hydrogenases (*8*−*12*). However, the comparison with hydrogenases also highlights a critical difference with nitrogenase. In hydrogenase, HD formation occurs via D^+/H^+ exchange; hence, HD formation is accompanied by stoichiometric release of D^+ into solution. In contrast, tritium labeling experiments showed that the rate of HD formation by nitrogenase is ∼50 times greater than the rate of incorporation of T^+ into solution (*13*). If σ-bond metathesis of the type shown in Scheme 2 was to occur in nitrogenase, then the most likely acceptor sites for the D^+ would be the three central S atoms of the FeMoco (*22*). However, two of these are connected to hydrogen bonded chains which would readily allow exchange of D^+ with bulk water (*35*). Hence, it is difficult to reconcile a σ-bond metathesis process involving the metal and sulfide sites of the FeMoco with the available experimental data on nitrogenase.

There is, however, a special type of σ-bond metathesis which could also occur on the FeMoco. This is the case of hydrogen atom exchange, which is now well established by both experiment and theory (*29*). In nitrogenase, exchange of a hydride with D_2 could provide a mechanism for HD formation with minimal leakage of deuterium into solution, since metal hydrides generally exchange more slowly with water than do protonated sulfides (*8*, *10*, *12*). Furthermore, hydride/dihydrogen exchange is by definition thermoneutral and frequently has a low activation energy. To operate in nitrogenase, such a mechanism would require the formation of a hydride contingent upon the presence of both N_2 and D_2 during catalysis. This criterion can be met by consideration of a well-established feature of dinitrogen reduction in model chemical complexes, namely, that initial protonation of N_2 competes with protonation at the metal. This was first recognized in the equilibrium between the complexes $[MX(N_2H)(dppe)_2]$ and $[MXH(N_2)(dppe)_2]$ (M = Mo or W; X = F, Cl or Br; dppe = $Ph_2PCH_2CH_2PPh_2$) (*26*), and a recent infrared and theoretical study of these complexes confirmed and refined this picture (*27*). The equilibrium between $[WX(N_2H)(dppe)_2]^{0/+}$ and $[WXH(N_2)(dppe)_2]^{0/+}$ (X = F or Br) has also been observed electrochemically in solution (*36*). Kinetic studies on the protonation of $[Mo(N_2)_2(dppe)_2]$ highlighted competition for protonation at the metal and the N_2 ligand (*23*, *24*); it was found that protonation at the metal tends to labilize the N_2 ligand, as well as reducing its basicity.

In the present context, it can be envisaged that protonation of N_2 bound to the Mo atom of the FeMoco competes with protonation of the FeMoco core itself. The DFT calculations presented above indicate that addition of H to the Mo atom is preferred over addition to the Fe atoms and, furthermore, that protonation of the MoN_2 species at Mo results in dissociation of N_2 from the FeMoco. In the absence of H_2 or D_2, the diazenido(1−) complex **10** (Figure 1) is energetically preferred over the Mo hydride **13** (Figure 2) by 5 kcal mol^{-1}. This, however, depends on the diazenido(1−) ligand's ability to bridge to the neighboring Fe atom. If D_2 (or H_2) is allowed to occupy the Fe site before protonation of N_2, as in complex **9**, the resulting linear diazenido(1−) species **11** is then formed, and this is essentially isoenergetic with the corresponding Mo hydride **14** plus free N_2.

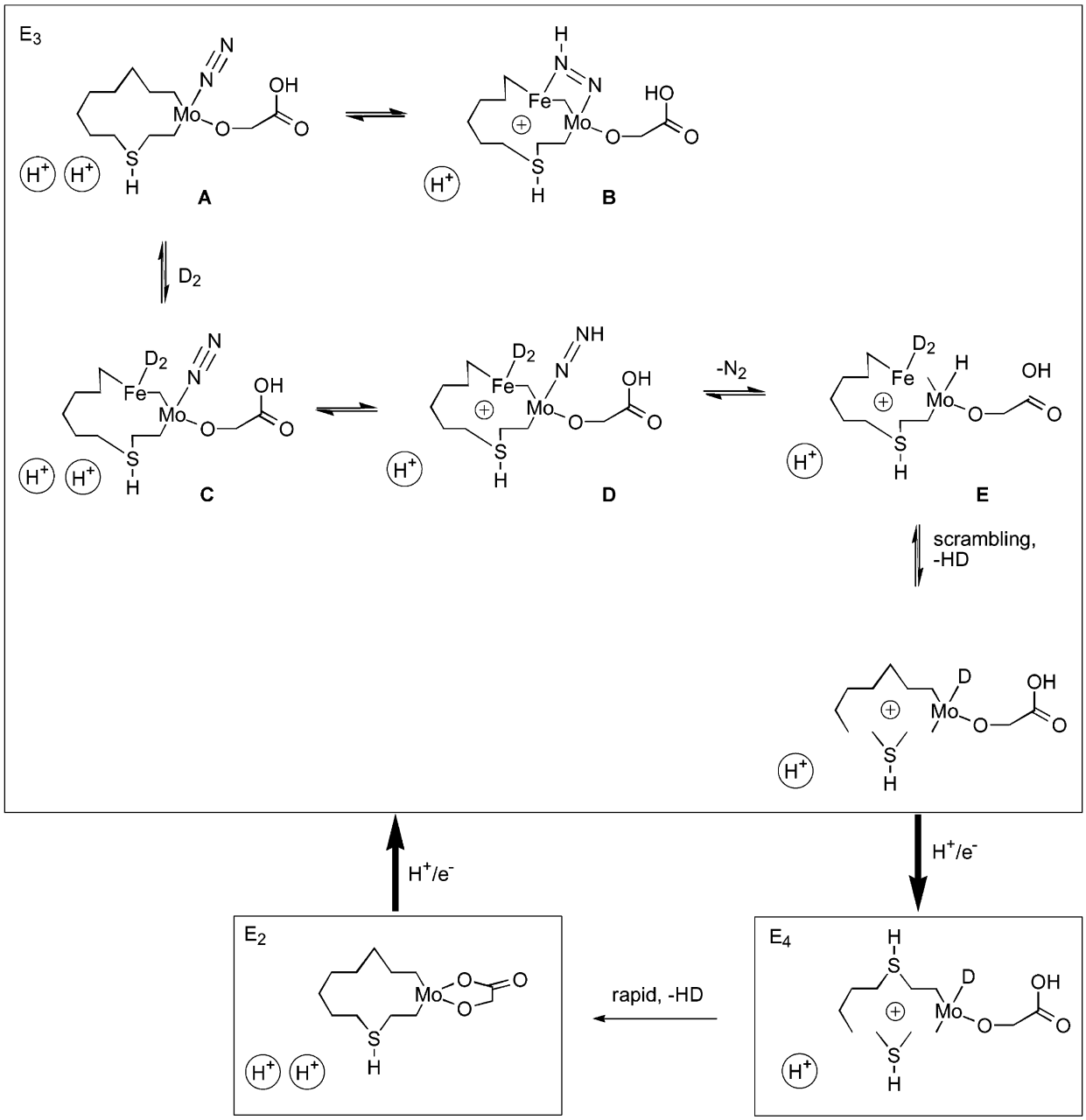
These considerations provide a model for HD formation as shown in Scheme 3. In the absence of D_2, N_2 binds to Mo at state E_3 to give structure **A** and is initially reduced to a bridging diazenido(1−) species, **B**, as described in the preceding paper. In the presence of D_2, the neighboring Fe site in the initial N_2 complex **A** can become blocked by D_2, giving **C**. This results in kinetic destabilization of the diazenido(1−) intermediate **D**, allowing competitive formation of the Mo hydride and loss of N_2 as in **E**. The overall interconversion between **A** and **E** depends on the concentration of both D_2 and N_2. The D_2 ligand in **E** is then very well placed to undergo scrambling with the Mo hydride via hydrogen atom exchange. Loss of the first molecule of HD leaves a molybdenum deuteride; addition of another H^+/e^- couple to give a state equivalent to E_4 results in the formation of a second molecule of HD via rapid hydrolysis, and the enzyme is returned to state E_2. This gives an overall stoichiometry for HD formation from each molecule of D_2 which undergoes scrambling as shown in eq 1

$$D_2 + 2H^+ + 2e^- \rightarrow 2HD \qquad (1)$$

The assignment of the critical step in the HD formation reaction to state E_3 is consistent with the kinetic analysis of Lowe and Thorneley, who showed that H_2 inhibition must occur before state E_4 (*16*). The available experimental data for the nitrogenase HD formation reaction constitute an exacting test of any atomic model for this process. The present model accommodates these data as described below.

1. Requirement for N_2. Rigorous experimental analysis showed that the nitrogenase HD formation reaction requires both D_2 and N_2 (*6*). In the present model, the N_2 requirement allows two inferences to be drawn about the mechanism, as follows. First, state E_3 in the catalytic cycle must undergo homocitrate ring opening in the presence of N_2, but not when H^+ is the only available substrate. This is supported by the analysis of homocitrate ring opening in the preceding paper; in the absence of N_2, ring opening is endothermic, but concomitant binding of N_2 at Mo gives a process which is exothermic overall. This also allows a rationalization of the fact that the Mo atom is coordinatively saturated in the resting

Scheme 3: Proposed Mechanism for the HD Formation Reaction[a]



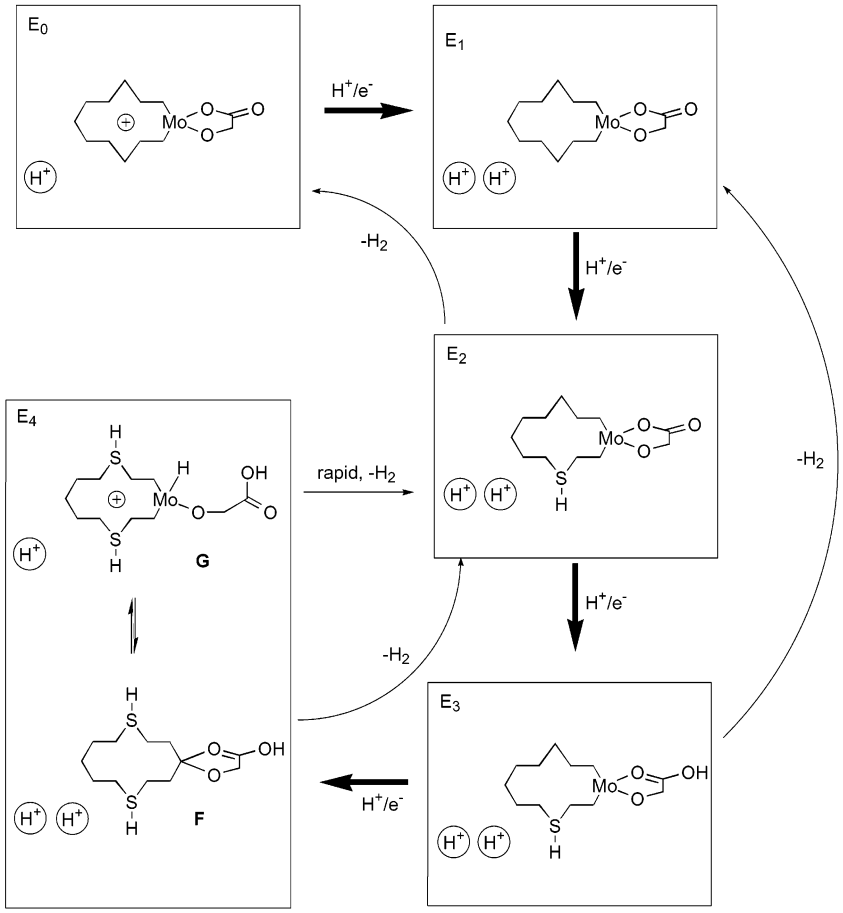
[a] Each of the bold arrows represents the transfer of a single e^- from the Fe protein to the MoFe protein. Circled H^+ represent nonlocated protons associated with the protein or homocitrate.

state of the enzyme; in the absence of N_2, the Mo site is protected by homocitrate at the E_3 level, and so cannot be protonated to give a catalytically counterproductive hydride. The second inference is that a hydride formed at the E_3 level is sufficiently long-lived to undergo scrambling with D_2, but a hydride at the more reduced E_4 level is hydrolyzed too rapidly to give HD. If this were not so, then the Mo hydride formed at the E_4 level in the absence of N_2 (see below and Scheme 4) would be capable of N_2-independent HD formation. The hypothesis of different hydride lifetimes at states E_3 and E_4 can be justified by the Lowe−Thorneley rate constants for evolution of H_2 from these two states, 8 and 400 s^{-1}, respectively (*2*).

2. The Electrons for HD Formation Are Diverted from N_2 Reduction. It has been established that the electrons used in HD formation are diverted exclusively from N_2 reduction rather than from OHE (*7*). In the present model, this follows naturally from the competitive protonation between N_2 and the FeMoco core, as discussed above. OHE is considered to be an entirely separate process, unaffected by the presence of either N_2 or D_2 (see below).

3. Turnover under HD Gives No D_2. When nitrogenase was turned over under a mixture of HD and N_2, no D_2 was detected in the products (*37*). This observation suggests that only a single scrambling event can occur on the catalytic time scale. This is reasonable given that there is always necessarily one molecule of D_2 or, in this case, HD, at the active site when the Mo hydride is formed, but the local FeMoco environment is relatively isolated from bulk solution (see preceding paper); hence, diffusion of D_2 or HD from the protein exterior to the FeMoco is relatively slow. Note that a high pHD might allow for multiple scrambling events,

Scheme 4: Proposed Mechanism for H_2 Evolution in the Absence of Other Substrates[a]



[a] Curved arrows represent loss of H_2 via the k_{+7}, k_{+8}, and k_{+9} pathways in the Lowe−Thorneley scheme (*14*). Other symbols have the same meanings as those in Scheme 2.

resulting in some D_2; however, the pressure of HD used in these experiments was only 0.5 Atm.

4. Minimal Exchange with Solvent. It was found that the rate of incorporation of T^+ into solution is ~50 times slower than the rate of HD formation (*13*). This is consistent with the present model, in that work on hydrogenases has shown that exchange of hydride with solvent is relatively slow compared to exchange involving protonated nonmetal atoms (*8*, *10*, *12*).

5. Competitive Inhibition of N_2 Binding and Reduction. The observation that H_2 inhibits reduction of N_2, but not of H^+, N_2O, N_3^-, C_2H_2, CN^-, MeNC, or N_2H_4 (*4*), is readily understood in that only N_2 achieves critical stabilization of a reduced intermediate by bridging between the Mo and Fe atoms of the FeMoco. Of all known nitrogenase substrates, N_2 is the hardest to reduce; moreover, the first H^+/e^- addition is the most difficult step of N_2 reduction. Hence, although other substrates might bind at Mo via homocitrate ring opening, their initial reduction will be significantly easier than that of N_2, and consequently they will not suffer from competitive formation of hydride on the FeMoco core cluster. Alternative substrates may or may not be bound at Mo; for example, C_2H_2 binds to states equivalent to E_1 and E_2 in the Lowe−Thorneley cycle (*38*), for which Mo is unavailable according to the present model. In this context, it is interesting to note that N_2 inhibits the reduction of C_2H_2, both in whole nitrogenase (*39*) and in solutions of extracted FeMoco (*40*). Following the analysis presented in the preceding paper, this can be interpreted in terms of competitive weak binding of N_2 and C_2H_2 at the central Fe sites of the FeMoco in states E_1 and E_2.

It has been shown that HD production is suppressed as the pN_2 is increased (*41*). This is expected in the present model, given that species **13** in Figure 2 shows dissociation of N_2; thus, a high pN_2 will shift the equilibrium between **D** and **E** in Scheme 3 in favor of **D**. At first sight, it might be argued that a high pN_2 would itself destabilize intermediate **D** by blocking the Fe site with a second N_2 ligand. However, this destabilization is not a problem since high pN_2 also shifts the equilibrium between the MoNNH and MoH + N_2 states back toward the former.

Recent experiments have indicated that in addition to suppressing N_2 reduction, H_2 also competes directly with N_2 for binding sites on the FeMoco (*5*). In the present model, competition between H_2 and N_2 for binding sites can be viewed as a result of the cage effect discussed in the preceding paper. Thus, the FeMoco-containing pocket can hold a strictly limited number of small molecules. In the early stages of catalysis up to state E_3, H_2, and N_2 bind weakly and nonspecifically at the FeMoco Fe sites, and so compete for occupancy of the pocket. The α-Asn^{195} mutant behaves normally in this regard; however, the lack of a hydrogen bond to S2B of the FeMoco in this case (*35*) means that the FeMoco cannot sustain reduction to the level required for the first protonation of N_2, hence no HD can be produced.

6. Limiting HD/H_2 Stoichiometries. Experiments designed to measure the limiting HD/H_2 stoichiometry using pD_2's of up to 400 kPa (4 Atm) gave a limiting value for this ratio of 2 at infinite pD_2 (*41*). For the model shown in Scheme 3, the limiting HD/H_2 ratio can be calculated as follows. As noted above, scrambling should be limited to the single D_2 molecule found at the active site upon formation of the molybdenum hydride, giving the following stoichiometry:

$$\mathrm{MoH} + \mathrm{D_2} \rightarrow 2/3\mathrm{MoD} + 1/3\mathrm{MoH} + 2/3\mathrm{HD} + 1/3\mathrm{D_2} \quad (2)$$

Addition of a further H^+/e^- couple results in hydrolysis of the hydride/deuteride; hence

$$2/3\mathrm{MoD} + 1/3\mathrm{MoH} + 2/3\mathrm{HD} + \mathrm{H^+} + \mathrm{e^-} \rightarrow 4/3\mathrm{HD} + 1/3\mathrm{H_2} \quad (3)$$

Overall, the products of eq 3 require two H^+/e^- couples, since one is used to make the initial Mo hydride. Under limiting conditions for N_2 reduction, six out of every eight electrons are utilized for N_2 reduction, while two are allocated to OHE. Since all electrons used in HD production are diverted from N_2 reduction, the limiting equations for HD production therefore consist of the HD component, eq 4, plus the OHE component, eq 5:

$$2\mathrm{D_2} + 6\mathrm{H^+} + 6\mathrm{e^-} \rightarrow 4\mathrm{HD} + \mathrm{H_2} \quad (4)$$

$$2\mathrm{H^+} + 2\mathrm{e^-} \rightarrow \mathrm{H_2} \quad (5)$$

Combining eqs 4 and 5 gives an overall limiting HD/H_2 ratio of 2, in agreement with experiment. Interestingly, however, use of still higher pD_2, up to 50 Atm, gave a different limiting HD/H_2 ratio, of 6.0−6.8 (*42*). There are two possible explanations for this. First, very high pressures should result in an increase in the number of molecules of D_2 which can access the FeMoco pocket, enabling multiple scrambling events to occur. As the mean number of scrambling encounters increases, the HD/H_2 ratio should approach a limiting value of 6. However, since all of the electrons used for HD formation are diverted from N_2 reduction, the maximum possible HD/H_2 ratio should be 6, regardless of the mechanism of HD formation. Hence, the reported HD/H_2 ratios of up to 6.8 are especially intriguing. Since all chemical reactions are equilibria, a sufficiently high pD_2 will suppress obligatory H_2 evolution. If OHE is not strictly required for binding and initial reduction of N_2, which are the processes associated with HD formation, then HD/H_2 ratios greater than 6 become possible. Taken at face value, then, these experimental data provide evidence that OHE is not an essential feature of nitrogenase mechanism.

Production of H_2 by Nitrogenase. In the absence of any other reducible substrate, nitrogenase catalyses the production of H_2 from protons. A scheme for proton reduction can be deduced by extension of the analysis of the HD formation reaction given above, Scheme 4. The first part this scheme is identical to that for N_2 reduction given in the preceding paper. In the absence of N_2, however, the homocitrate ring remains closed at state E_3. Addition of a further H^+/e^- couple to the FeMoco core to give a state corresponding to E_4 allows homocitrate ring opening and concomitant protonation at Mo, with subsequent rapid loss of H_2 returning the system to state E_2, similar to the scheme for HD formation given above. Experimentally, it has been shown that reduction of a Mo carboxylate complex results in dechelation of the carboxylate ligand and protonation of the metal (*43*). In addition, each of the states E_2−E_4 can lose H_2, as indicated in the Lowe−Thorneley scheme (*2*, *15*), by migration of an H atom from S to Fe on the FeMoco core and subsequent hydrolysis (*35*). Conceptually, Scheme 4 exactly parallels the mechanism of reduction of H^+ to H_2 by $[Fe_4S_4(SPh)_4]^{3-}$, proposed by Grönberg et al. (*44*). In both cases, protonation of the cluster core at sulfur is followed by dissociation of a protonated leaving group [homocitrate in Scheme 4, thiophenol in reference (*44*)]. Further protonation then occurs at the exposed metal site, and the resulting hydride is lost as H_2, allowing religation of the leaving group. The assignment of the reduced iron hydride cluster $[Fe_4S_4H_3(SPh)_3]$, rather than the initially produced $[Fe_4S_4H_3(SPh)_3]^+$, as the primary source of H_2 also lends further support to the hypothesis of faster release of HD from state E_4 compared to E_3 in Scheme 3, as discussed above.

An interesting test of this model is provided by work on $NifV^-$ mutant nitrogenase, whose FeMoco contains citrate rather than homocitrate (*45*, *46*). In both of these studies, the rate of H_2 evolution by $NifV^-$ nitrogenase under argon was found to be ~92% of that of the wild type. Similarly, the rate of H_2 evolution of the wild-type enzyme in the presence of a low concentration of CO was virtually identical to the rate in the absence of CO. However, the $NifV^-$ mutant evolved H_2 at significantly lower rates under low CO concentrations, resulting from CO-induced uncoupling of ATP hydrolysis from electron transfer. It has been shown that CO perturbs the profile of H_2 evolution versus pH for wild-type nitrogenase (*47*), an effect which was explained in terms of a shift in pK_a of a group associated with the FeMoco from ~9 to ~8.5. We have previously suggested that differences in the reactivities of homocitrate- and citrate-containing cofactors are best understood in terms of opening of the (homo)citrate chelate ring, which allows a specific hydrogen bond to Av1 α-His^{442} (Kp1 α-His^{440}) in the case of homocitrate but not of citrate, thereby enhancing the basicity of the Mo atom (*48*). In state E_4 of Scheme 4, there is an equilibrium between protonation at Mo and an unidentified amino acid residue close to the FeMoco. The position of the equilibrium will be determined by the pK_a of the amino acid residue, which should be relatively invariant, and the pK_a of the Mo hydride, which will be influenced by the disposition of the FeMoco. Binding of CO at a trigonal Fe site will reduce the basicity of the FeMoco, as will loss of the hydrogen bond between homocitrate and α-His^{442}. Combining both effects then reduces the pK_a of the Mo hydride below that of the protonatable amino acid residue, switching the position of the equilibrium in state E_4 back from **G** to **F**. Species **F** cannot easily be reduced, since it has no sites available to receive further protons, resulting in the uncoupling of ATP hydrolysis from electron-transfer noted above. Linear regression analysis gave a maximum inhibition of H_2 production by CO in these experiments of 73% (*45*); the residual H_2 production can be accounted for if OHE continues at its normal rate.

Another set of experiments which can be interpreted using the present model concerns the effects of site-directed mutagenesis on Av1 nitrogenase residue α-His^{195} (*49*). The

wild-type enzyme exhibits a significantly lower rate of H_2 evolution under an atmosphere of N_2 compared to that under Ar, due to nitrogen fixation. The α-Gln^{195} mutant showed a very similar inhibition of H_2 evolution under N_2, even though in this case the rate of N_2 fixation is minimal (*50*), while the α-Asn^{195} and other mutants showed a low rate of H_2 evolution under both Ar and N_2 atmospheres (*49*). It was previously suggested (*35*) that these data can be interpreted in terms of a successive attenuation of the FeMoco's basicity in going from the wild type through the Gln mutant to the Asn mutant, meaning that the Gln mutant can access a condition in which N_2 is bound but only inefficiently reduced, whereas the Asn mutant cannot reduce N_2 at all. This interpretation can now be made more specific, as follows. The Gln mutant would operate normally in the absence of N_2, giving rapid H_2 evolution via a state similar to **G** in Scheme 4, except that S2B of the FeMoco would receive only a hydrogen bond from α-Gln^{195}, rather than the formal transfer of a proton as occurs in the wild-type enzyme. In the presence of N_2, this mutant would be diverted into a nonproductive state with the Mo site occupied by N_2 rather than H. The other mutants, lacking even a hydrogen bond to S2B, would not be able to reach the level of reduction required for homocitrate ring opening, resulting in a lower rate of H_2 production even in the absence of N_2.

Obligatory Hydrogen Evolution. The model presented above accommodates competitive inhibition of N_2 binding and reduction by H_2, the HD formation reaction, and the characteristics of H^+ reduction in the absence of other substrates. The one remaining feature of nitrogenase hydrogen chemistry which requires discussion is obligatory hydrogen evolution. Is this a necessary feature of reduction of N_2 by nitrogenase, or merely a consequence of unavoidable leakage of H_2 from reduced states of the enzyme (*2*)? Regardless of the answer to this question, it can be reasonably assumed on chemical grounds that OHE involves metal hydrides. This raises a general problem in seeking a constructive role for OHE in N_2 fixation; addition of H atoms to the nonmetallic elements of the FeMoco (sulfide or homocitrate) reduces the total oxidation state of the metals, enhancing their ability to bind and protonate N_2, whereas addition of H to the metals has the opposite effect. This is consistent with experiments on model complexes (*23*, *24*) and is illustrated by the model for HD formation presented above, where protonation at the metal competes with protonation at N_2.

It could be argued that formation of metal hydrides allows some crucial change in the FeMoco structure, which is required for N_2 binding. Subsequent reductive elimination of H_2 would then allow protonation of N_2 to proceed. The question is, what could the structural change be? One possibility is that formation of a Mo hydride allows opening of the homocitrate chelate ring (*43*). This fits in very well with the model for H^+ reduction in the absence of N_2 shown in Scheme 4 (cf. structure **G**), but the results shown in Figure 2 structure **13** suggest that the resulting hydride blocks binding of N_2 at Mo almost as effectively as does homocitrate.

The limiting H_2/N_2 stoichiometry for Mo nitrogenase is 1, but vanadium nitrogenase has a limiting H_2/N_2 stoichiometry of 3.5 (*3*). There are three possibilities which could explain this difference. First, both limiting stoichiometries might genuinely reflect the enzymes' mechanisms. In this case, V nitrogenase would operate by a mechanism requiring evolution of 3.5 molecules of H_2 per N_2 reduced, which seems singularly unlikely. Second, both nitrogenases might require release of one H_2 per N_2, and the balance of 2.5 H_2 for V nitrogenase would result from unavoidable leakage of reducing potential. Third, all the H_2 produced by both enzymes might result from unavoidable leakage. This last possibility is in line with Occam's razor and also fits in best with the results from both the present and other (*22*) theoretical studies, which failed to uncover any potential constructive role for OHE. Finally, it was argued in a previous study (*35*) that under the experimental conditions used to measure the limiting H_2/N_2 ratio (*51*), wasteful release of H_2 from states $E_2 - E_4$ in the Lowe−Thorneley scheme would not be fully suppressed. The rate of leakage would be essentially constant, dependent only upon the rate of association/dissociation of the nitrogenase component proteins. If correct, this would explain both the phenomenon of OHE and the different limiting stoichiometries for the different enzymes, as well as the HD/H_2 ratios greater than 6 which were found at very high pD_2, as discussed above.

ACKNOWLEDGMENT

I thank C. J. Pickett, D. J. Lowe and B. E. Smith (John Innes Centre), R. L. Richards (University of Sussex), and R. A. Henderson (University of Newcastle) for helpful discussions.

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BI025624R