

Chemical Activity of the Nitrogenase FeMo Cofactor with a Central Nitrogen Ligand: Density Functional Study

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Abstract: We investigate the chemical consequences of a central ligand in the nitrogenase FeMo cofactor using density functional calculations. Several studies have shown that the central ligand most probably is a nitrogen atom, but the consequences for the chemical reactivity of the cofactor are unknown. We investigate several possible routes for insertion of the central nitrogen ligand and conclude that all routes involve barriers and intermediate states, which are inaccessible at ambient conditions. On this basis we suggest that the central nitrogen ligand is present at all times during the reaction. Furthermore, we investigate how the FeMoco with the central ligand can interact with N_2 and reduce it.

1. Introduction

The enzyme nitrogenase catalyzes one of the most important reactions in biology, the conversion of molecular nitrogen (N_2) to ammonia (NH_3) and molecular hydrogen (H_2) under ambient conditions.^{1–5} The most common Mo-containing form of nitrogenase consists of two metalloproteins, the Fe protein and the MoFe protein. The Fe protein contains a ferredoxin (4Fe-4S) and the MoFe protein contains two unique metal clusters, the P-cluster and the FeMo cofactor (FeMoco).² The latter one is most likely the place where N_2 binds and is reduced. The crystal structure of nitrogenase was first solved by Kim and Rees in 1992. Since then, numerous crystal structures of all parts of the nitrogenase complex with resolutions up to 1.6 Å have been published.⁶ A recent crystal structure of the MoFe protein by Einsle et al. has revealed that the FeMoco contains a light atom as a central ligand.⁷ Even at the high resolution of 1.16 Å, the central atom could not be identified unambiguously, but the possibilities could be limited to nitrogen, oxygen, or carbon. Einsle et al. suggested that the central ligand plausibly is a nitrogen atom, as nitrogen is present as a substrate. In a theoretical study we have shown that the central ligand most

probably is indeed a nitrogen atom,⁸ and other theoretical studies have come to the same conclusion.^{9–11}

These new developments raise the question of how the central ligand changes our understanding of the mechanism of the FeMoco and of the reaction pathway for N_2 reduction. The first question would be whether the central ligand is actually present during turnover. Lee et al. have addressed this issue by performing electron nuclear double resonance (ENDOR) and electron spin echo envelope modulation (ESEEM) experiments.¹² They observe no new ^{15}N signals or changes in ^{14}N hyperfine spectra. From this it follows that if the central ligand is nitrogen, then it is associated with one of the observed ^{14}N signals and it does not exchange during catalysis. These results imply that there is no thermodynamically and kinetically accessible pathway for insertion of N into the cluster or extraction of N from the cluster during catalysis. Of course, this study and our study only cover the situation during turnover and not, e.g., during cluster biosynthesis or assembly. In this paper, we present a detailed theoretical investigation of possible insertion pathways. Although the ENDOR study¹² shows that there is no exchange of N during turnover, it is still of considerable interest to investigate insertion pathways in order to gain an understanding of why insertion is impossible and what the chemistry of such reactions looks like.

Assuming that the central N is present at all times during turnover, the next question is whether it is directly involved in the reduction of N_2 and which parts of the FeMoco take part in binding and reduction.

In the present paper, we address these questions by investigating the reactivity of the FeMoco with the central N ligand by density functional theory. After an account of the calcula-

- (1) Howard, J. B.; Rees, D. C. *Chem. Rev.* **1996**, *96*, 2965.
- (2) Burgess, B. K.; Lowe, D. J. *Chem. Rev.* **1996**, *96*, 2983.
- (3) Smith, B. E. *Adv. Inorg. Chem.* **1999**, *47*, 159.
- (4) Rees, D. C.; Howard, J. B. *Curr. Opin. Chem. Biol.* **2000**, *4*, 559.
- (5) (a) Rees, D. C.; Howard, J. B. *Science* **2003**, *300*, 929. (b) Lee, S. C.; Holm, R. H. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3595.
- (6) (a) Kim, J.; Rees, D. C. *Science* **1992**, *257*, 1677. (b) Kim, J.; Rees, D. C. *Nature* **1992**, *360*, 553. (c) Chan, M. K.; Kim, J.; Rees, D. C. *Science* **1993**, *260*, 792. (d) Georgiadis, M. M.; Komiya, H.; Chakrabarti, P.; Woo, D.; Kornuc, J. J.; Rees, D. C. *Science* **1992**, *257*, 1653. (e) Kim, J.; Woo, D.; Rees, D. C. *Biochemistry* **1993**, *32*, 7104. (f) Bolin, J. T.; Ronco, A. E.; Morgan, T. V.; Mortenson, L. E.; Xuong, N. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1078. (g) Peters, J. W.; Stowell, M. H. B.; Soltis, M.; Finnegan, M. G.; Johnson, M. K.; Rees, D. C. *Biochemistry* **1997**, *36*, 1181. (h) Mayer, S. M.; Lawson, D. M.; Gormal, C. A.; Mark Roe, S.; Smith, B. E. *J. Mol. Biol.* **1999**, *292*, 871. (i) Schindelin, H.; Kisker C.; Schlessman, J. L.; Howard, J. B.; Rees, D. C. *Nature* **1997**, *387*, 370. (j) Strop, P.; Takahara, P. M.; Chiu, H.-J.; Angove, H. C.; Burgess, B. K.; Rees, D. C. *Biochemistry* **2001**, *40*, 651.
- (7) Einsle, O.; Tezcan, F. A.; Andrade, S. L. A.; Schmid, B.; Yoshida, M.; Howard, J. B.; Rees, D. C. *Science* **2002**, *297*, 1696.

- (8) Hinnemann, B.; Nørskov, J. K. *J. Am. Chem. Soc.* **2003**, *125*, 1466.
- (9) Lovell, T.; Liu, T.; Case, D. A.; Noodleman, L. *J. Am. Chem. Soc.* **2003**, *125*, 8377.
- (10) Dance, I. *Chem. Commun.* **2003**, *3*, 324.
- (11) Vrajmasu, V.; Münck, E.; Bominaar, E. *Inorg. Chem.* **2003**, *42*, 5974.
- (12) Lee, H.-I.; Benton, P. M. C.; Laryukhin, M.; Igarashi, R. Y.; Dean, D. R.; Seefeldt, L. C.; Hoffman, B. M. *J. Am. Chem. Soc.* **2003**, *125*, 5604.

tional details, we present an investigation of a possible reaction to insert the central ligand into the cofactor. We find that the studied pathways involve very high barriers, which are inaccessible at room temperature. This agrees with the finding of Lee et al.¹² that such a reaction does not happen during turnover.

Furthermore, we study the reactivity of the FeMoco with the central ligand toward N₂ and its ability to reduce it. Revisiting a previous model¹⁸ for binding and reduction of N₂ and recalculating it for the new cofactor structure, we find that although the 3-fold Fe atoms are coordinated to the central ligand, they can still facilitate N₂ binding and reduction.

There are numerous experimental studies on the reactivity of the FeMoco toward N₂ and other substrates, which are reviewed by Burgess¹³ and Burgess and Lowe.² On the basis of kinetic data, Thorneley and Lowe¹⁴ have developed a kinetic model for ammonia synthesis. In their model, N₂ is irreversibly activated after three to four Fe protein cycles and electron transfers. H₂ is formed by the enzyme with a minimum stoichiometry of N₂:H₂ = 1.¹⁵ Nitrogen is directly reduced with one of the intermediates being hydrazine. H₂ can evolve after a minimum of two enzyme turnovers. It should be remarked that there is an uncertainty of how many transferred electrons actually are transferred to the FeMoco instead of to the surroundings. Electron paramagnetic resonance (EPR) measurements¹⁹ indicate that only two of the first three electrons transferred to the protein reach the cofactor, and it is uncertain what happens to the third electron.

There are many theoretical studies of the structure and the reactivity of the FeMoco from the time before the central ligand in the FeMoco was known.^{16–18} There are theoretical studies on the structure of the FeMoco with the central ligand,^{8–11} which support that the central ligand is nitrogen.

2. Computational Details

We perform all density functional calculations with the program dacapo,²⁰ which uses a plane-wave expansion of the Kohn–Sham wave functions and the generalized gradient approximation (GGA) for the

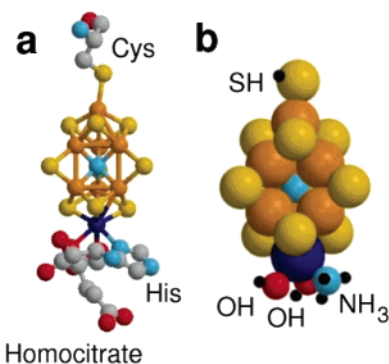


Figure 1. (a) Nitrogenase FeMo cofactor from the crystal structure 1MIN.⁷ (b) Calculated model system. The color scheme is dark blue (molybdenum), orange (iron), yellow (sulfur), red (oxygen), light blue (nitrogen), gray (carbon), and black (hydrogen).

exchange-correlation terms.^{21,22} Due to the plane-wave expansion we have to treat periodic systems. We accomplish this by repeating a supercell containing the cluster in all three dimensions, including enough vacuum around the cluster in order to make the interactions between the images as small as possible and at the same time maintaining a unit cell volume, which is computationally accessible. The criterion for choosing the shape of the unit cell was to minimize the distances between the images while at the same time keeping the unit cell volume as small as possible. Here we use a triclinic supercell with the axes $a = b = 11 \text{ \AA}$ and $c = 15.4 \text{ \AA}$ and the angles $\alpha = 90^\circ$, $\beta = 69^\circ$, and $\gamma = 120^\circ$. We have found that binding energies are converged up to a few kilojoules per mole. We draw conclusions based on energy differences, which are at least 1 order of magnitude higher. In all calculations, we include plane waves with energies up to 25 Ry.

To describe the core parts of the atoms, we use ultrasoft pseudopotentials,²³ except for sulfur, where a soft pseudopotential is used.²⁴ As the exchange-correlation functional, we use the spin-dependent revised Perdew–Burke–Erzerhoff (RPBE) functional, which has been shown to perform well in describing chemisorption.²⁵ In particular, this functional describes N₂ adsorption and activation on iron surfaces, where there are experiments available for comparison, very accurately.²⁶

For the k -point sampling of the Brillouin zone, Γ -point sampling has been used. The Fermi population of the Kohn–Sham orbitals is set to $k_B T = 0.01 \text{ eV}$, and Pulay mixing is applied to the resulting density.²⁷

As starting structures, we used coordinates from the structures 3MIN^{6g} and 1MIN⁷ or coordinates from previously relaxed structures. To reduce the system to a tractable size, we truncate the ligands after the first ligating atom. Thus, we substitute Cys by SH, His by NH₃, and homocitrate by two OH groups. The FeMoco from the 1MIN structure and our model are shown in Figure 1. All color figures are prepared by use of MolScript and Raster3D.^{28,29}

The sums of atomic densities are used as an initial guess for the electron density. The antiferromagnetic ground state^{8,18} with $S = 3/2$ is obtained by starting with nonzero spin densities on the iron atoms. The spin density on the Fe atoms is between 1.5 and 3 Bohr in all cases, independent of the magnitude of the initial (nonzero) spin density.

- (13) Burgess, B. K. In *Molybdenum Enzymes*; Spiro, T. G., Ed.; Wiley-Interscience: New York, 1985; Chapt. 5, pp 161–219.
- (14) Thorneley, R. N. F.; Lowe, D. J. In *Molybdenum Enzymes*; Spiro, T. G., Ed.; Wiley-Interscience: New York, 1985; Chapt. 5, pp 221–285.
- (15) Guth, J. H.; Burris, R. H. *Biochemistry* **1983**, *22*, 5111. (b) Simpson, F. B.; Burris, R. H. *Science* **1984**, *224*, 1095.
- (16) (a) Deng, H.; Hoffmann, R. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1062. (b) Plass, W. *J. Mol. Struct. (THEOCHEM)* **1994**, *315*, 53. (c) Machado, F. B. C.; Davidson, E. R. *Theor. Chim. Acta* **1995**, *92*, 315. (d) Zhong, S.-J.; Liu, C.-W. *Polyhedron* **1997**, *16*, 653. (e) Stavrev, K. K.; Zerner, M. C. *Chem. Eur. J.* **1996**, *2*, 83. (f) Stavrev, K. K.; Zerner, M. C. *Theor. Chem. Acc.* **1997**, *96*, 141. (g) Stavrev, K. K.; Zerner, M. C. *Int. J. Quantum Chem.* **1998**, *70*, 1159. (h) Dance, I. G. *Aus. J. Chem.* **1994**, *47*, 979. (i) Dance, I. *J. Biol. Inorg. Chem.* **1996**, *1*, 581. (j) Dance, I. *Chem. Commun.* **1997**, *2*, 165. (k) Dance, I. *Chem. Commun.* **1998**, *5*, 523. (l) Siegbahn, P. E. M.; Westerberg, J.; Svensson, M.; Crabtree, R. H. *J. Phys. Chem. B* **1998**, *102*, 1615. (m) Szilagy, R. K.; Musaev, D. G.; Morokuma, K. *J. Mol. Struct. (THEOCHEM)* **2000**, *506*, 131. (n) Szilagy, R. K.; Musaev, D. G.; Morokuma, K. *Inorg. Chem.* **2001**, *40*, 766. (o) Durrant, M. C. *Inorg. Chem. Commun.* **2001**, *4*, 60. (p) Durrant, M. C. *Biochem. J.* **2001**, *355*, 569. (q) Durrant, M. C. *Biochemistry* **2002**, *41*, 13934. (r) Durrant, M. C. *Biochemistry* **2002**, *41*, 13946. (s) Lovell, T.; Li, J.; Liu, T.; Case, D. A.; Noodleman, L. *J. Am. Chem. Soc.* **2001**, *123*, 12392. (t) Lovell, T.; Li, J.; Case, D. A.; Noodleman, L. *J. Am. Chem. Soc.* **2002**, *124*, 4546. (u) Lovell, T.; Torres, R. A.; Han, W.-G.; Liu, T.; Case, D. A.; Noodleman, L. *Inorg. Chem.* **2002**, *41*, 5744. (v) Lovell, T.; Li, J.; Case, D. A.; Noodleman, L. *J. Biol. Inorg. Chem.* **2002**, *7*, 735. (w) Barriere, F.; Pickett, C. J.; Talarmin, J. *Polyhedron* **2001**, *20*, 27.
- (17) (a) Rod, T. H.; Hammer, B.; Nørskov, J. K. *Phys. Rev. Lett.* **1999**, *82*, 4054. (b) Rod, T. H.; Logadottir, A.; Nørskov, J. K. *J. Chem. Phys.* **2000**, *112*, 5343.
- (18) Rod, T. H.; Nørskov, J. K. *J. Am. Chem. Soc.* **2000**, *122*, 12751.
- (19) Fisher, K.; Newton, W. E.; Lowe, D. J. *Biochemistry* **2001**, *40*, 3333.
- (20) The program dacapo is freely available at our website, <http://www.fysik.dtu.dk/CAMPOS>.

- (21) (a) Hohenberg, P.; Kohn, W. *Phys. Rev.* **1964**, *136*, B864. (b) Kohn, W.; Sham, L. J. *J. Phys. Rev.* **1965**, *140*, A1133.
- (22) (a) Jones, R. O.; Gunnarson, O. *Rev. Mod. Phys.* **1989**, *61*, 689. (b) Parr, R. G.; Wang, W. *Density Functional Theory of Atoms and Molecules*; Oxford University Press: Oxford, U.K., 1989. (c) Payne, M. C.; Teter, M. P.; Allan, D. C.; Arias, T. A.; Joannopoulos, J. D. *Rev. Mod. Phys.* **1992**, *64*, 1045.
- (23) Vanderbilt, D. *Phys. Rev. B* **1990**, *41*, 7892.
- (24) Troullier, N.; Martins, J. L. *Phys. Rev. B* **1991**, *43*, 1993.
- (25) Hammer, B.; Hansen, L. B.; Nørskov, J. K. *Phys. Rev. B* **1999**, *59*, 7413.
- (26) Mortensen, J. J.; Hansen, L. B.; Hammer, B.; Nørskov, J. K. *J. Catal.* **1999**, *182*, 479.
- (27) (a) Pulay, P. *Mol. Phys.* **1969**, *17*, 197. (b) Kresse, G.; Furthmüller, J. *Comput. Mater. Sci.* **1996**, *6*, 15.
- (28) Kraulis, P. J. *J. Appl. Crystallogr.* **1991**, *24*, 946.
- (29) Merrit, E. A.; Bacon, D. J. *Methods Enzymol.* **1997**, *277*, 505.

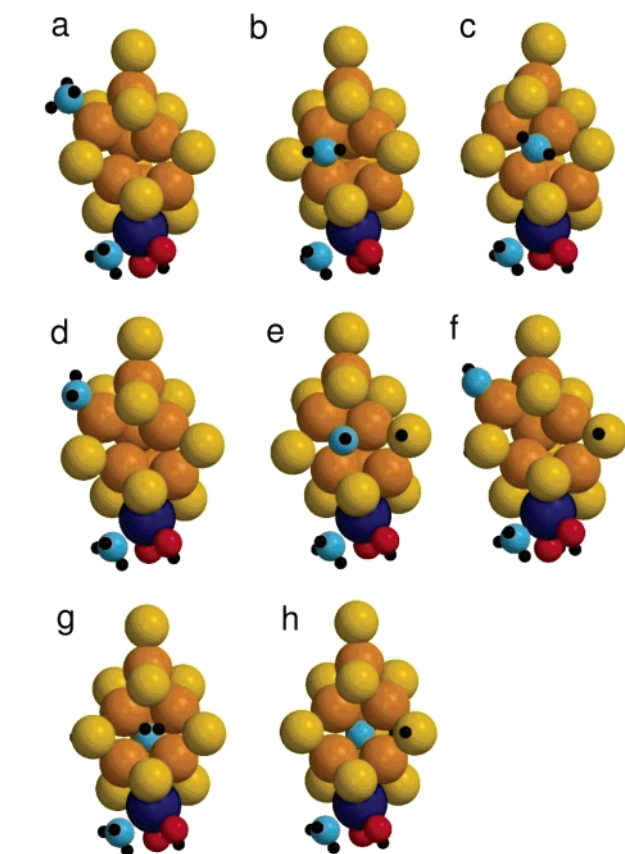
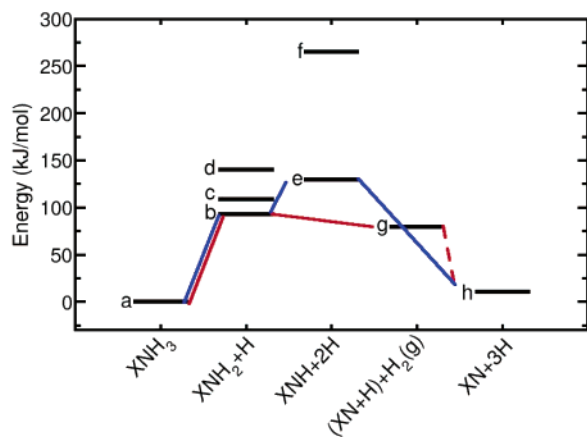


Figure 2. Energy scheme and minimum energy structures for the insertion of the central N ligand. Note that in all structures the H ligand terminating the upper S atom is not visible as it is located behind the sulfur ligand. In structures b–h there is a proton located on the μ_2 S atom to the left, but it is not visible as it points backward. In structure g, the H_2 molecule is so far away from the FeMoco that its interaction with the cluster is negligible, thus $H_2(g)$. It has been included for the barrier calculation (see Figure 3 and text) In structure h, all μ_2 S atoms are protonated.

The spin densities for the Mo atoms and all ligand atoms are small, below 0.2 Bohr.

In all calculations, the total cluster and the unit cell are charge-neutral. We consider this the most natural choice, as a net charge would introduce long-range Coulomb forces. In larger models there is of course the possibility that a small negative charge on the cluster is compensated by a positive charge in the surroundings, as, e.g., discussed for the α -His¹⁹⁵ residue in ref 18, but this is not possible here. We remark that in terms of formal oxidation numbers, the FeMoco with a central N ligand and three protonated μ_2 S ligands (as depicted in Figure 2h) corresponds to the $Mo^{4+}4Fe^{2+}3Fe^{3+}$ assignment proposed by Mössbauer

studies³⁰ and reinforced by previous calculations.^{8–11} One should note that ENDOR studies³¹ propose a different oxidation state assignment, $Mo^{4+}6Fe^{2+}1Fe^{3+}$. Further discussion of these issues takes place in section 3.2.

All atoms are relaxed according to a conjugate-gradient algorithm,³² until the total absolute force is below 0.2 eV/Å. The barrier calculations have been carried out by the nudged elastic band (NEB) method.³³ As initial guess for the reaction path, linear interpolations between the initial and final states were used. The reaction path was modeled with five images between the initial and the final state. For the structures shown in Figure 2 and for the NEB calculations, the Mo atom and the N atom of the NH_3 ligand were kept fixed. This has a negligible effect on the energies but was necessary in order to avoid translation and rotation of the cluster along the NEB path.

It is clear that our model is extremely simple and that effects of the surrounding protein structure, which we neglect, can alter the energies significantly. On the other hand, binding energy differences from one structure to the next should be more accurate, and we base our conclusions on significantly large differences in binding energies.

3. Results

3.1. Insertion of the Central N Atom. In this section, we search for a possible pathway for the insertion or the extraction of the central ligand. If there was such a pathway, it would mean that the central ligand could enter or leave the cycle during turnover. If there is no accessible pathway, the FeMoco could be assembled with the central ligand, which then would be an integral part of the FeMoco and present at all times.

In a previous study,⁸ we have shown that the FeMoco with the central ligand is 56 kJ/mol lower in energy than the FeMoco without a central ligand and NH_3 in the gas phase. We remark that this order of stability is not changed in solution. To judge the stability in solution, one can assume that the FeMoco has the same solution energy with and without a central ligand. The solution enthalpy for NH_3 is 31 kJ/mol,³⁴ which renders the FeMoco with a central ligand stable by 25 kJ/mol relative to the FeMoco without a central ligand.

If one places a single neutral N atom on the face of the FeMoco (without other adsorbates), the N atom moves into the FeMoco spontaneously during the structural relaxation and positions itself as the central ligand. Thus there is no barrier for the insertion of a single N atom. However, adsorbed N_2 cannot be the starting point for N insertion. N_2 would have to dissociate before one N could move into the cluster, which would happen spontaneously. The other N atom, however, has to remain on the cluster, where it is extremely unstable,¹⁸ which would result in a structure of very high energy. Therefore, bound N_2 does not seem to be a suitable starting point for insertion of N as a central ligand.

Another possibility for a starting structure is NH_3 bound at one of the triangular Fe atoms, which is slightly more stable than the FeMoco with N as a central ligand and three H atoms bound to the μ_2 S atoms. Furthermore, one result of the theo-

- (30) Yoo, S. J.; Angove, H. C.; Papaefthymiou, V.; Burgess, B. K.; Münck, E. *J. Am. Chem. Soc.* **2000**, *122*, 4926.
 (31) (a) True, A. E.; Nelson, M. J.; Venters, R. A.; Orme-Johnson, W.; Hoffman, B. M. *J. Am. Chem. Soc.* **1988**, *110*, 1935. (b) Lee, H.-I.; Hales, B. J.; Hoffman, B. M. *J. Am. Chem. Soc.* **1997**, *119*, 11395.
 (32) Press, W. H.; Flannery, B. P.; Teucholsky, S. A.; Vetterling, W. T. *Numerical Recipes*; Cambridge University Press: Cambridge, U.K., 1986.
 (33) (a) Mills, G.; Jónsson, H. *Phys. Rev. Lett.* **1994**, *72*, 1124. (b) Mills, G.; Jónsson, H.; Schenter, G. K. *Surf. Sci.* **1995**, *324*, 305. (c) Jónsson, H.; Mills, G.; Jacobsen, K. W. In *Classical and Quantum Dynamics in Condensed Phase Simulations*; Berne, B. J., Cicotti, G., Coker, D. F., Eds.; World-Scientific: Singapore, 1998.
 (34) Lide, D. R., Ed. *CRC Handbook of Chemistry and Physics*, 83th ed.; CRC Press: Boca Raton, FL, 2002.

retical study¹⁸ was that the NH₃ is bound to the FeMoco at the end of the reduction cycle with a binding energy of ~90 kJ/mol, which makes it hard to remove. Recalculating this energy with our larger model, we obtain 67 kJ/mol. Thus one might ask the question whether, instead of desorbing, the adsorbed NH₃ can in some way get rid of its H ligands and the N be inserted into the cluster? We take FeMoco without a central ligand but with NH₃ bound on one of the triangular iron atoms as a starting point and investigate possible pathways for insertion of the central ligand.

We (and others^{9,10}) have tested whether there could be a molecule, e.g., NH₃, in the cavity and find it impossible because of limited space. Thus, to get into the cavity, NH₃ has to get rid of its hydrogen ligands in some way, which constitutes the main barrier. As the most favorable position for protons to adsorb is on the μ_2 S ligands (and in the cavity,^{16f} but this possibility is not relevant here), the NH₃ could deliver protons to the μ_2 S ligands and move into the cavity. On the basis of these assumptions, we have calculated possible intermediate states, which are depicted in Figure 2. We remark that in our model the FeMoco has an approximate C₃ symmetry apart from the ligands. We have tested that the adsorption energies vary vanishingly little for the different faces of iron atoms. Thus, in our model it is of no significance on which face of the cluster the process takes place. At this point, we remark that Benton et al.^{35,36} and Mayer et al.³⁷ recently have performed mutation studies on the α -Arg⁹⁶ and α -Val⁷⁰ residues, which are located close to the FeMoco.⁷ They observed that upon mutation of α -Val⁷⁰ to α -Ala⁷⁰, short-chain alkynes, propargyl alcohol, and propyne can be reduced effectively, which is not possible with the wild-type enzyme. They outlined a similar gatekeeper role for the residue α -Arg⁹⁶ and therefore provide evidence that substrates bind to a particular face of the FeMoco composed of the Fe atoms 2, 3, 6, and 7 according to the numbering in ref 7. In our model we do not include these surrounding amino acids, which are responsible for the discrimination of one particular face for substrate binding. Thus in our model all faces are equivalent and it is not important on which face the reaction occurs.

We investigated two possible pathways, which in Figure 2 are marked in red and blue. The first step, which is common for the two pathways, is that one proton is delivered to the μ_2 S ligand and NH₂ binds to the cluster. The most favorable binding mode for NH₂ is side-on to the triangular Fe atoms (structure b), and the step from a to b is rather energy-demanding with 93 kJ/mol. One possible way to continue from NH₂ is that H₂ desorbs while N moves into the cluster cavity, and this corresponds to structure g, which is about ~13 kJ/mol lower in energy than b. This pathway is marked in red in Figure 2. The reaction might then continue to h, where two protons are adsorbed on the remaining μ_2 S atoms. Alternatively, NH₃ can of course desorb, which requires 67 kJ/mol.

The red reaction path does involve high energies, but the intermediate states are not completely inaccessible, in particular, because there might be favorable energy shifts due to the environment, which we have not included in our model. Thus, we

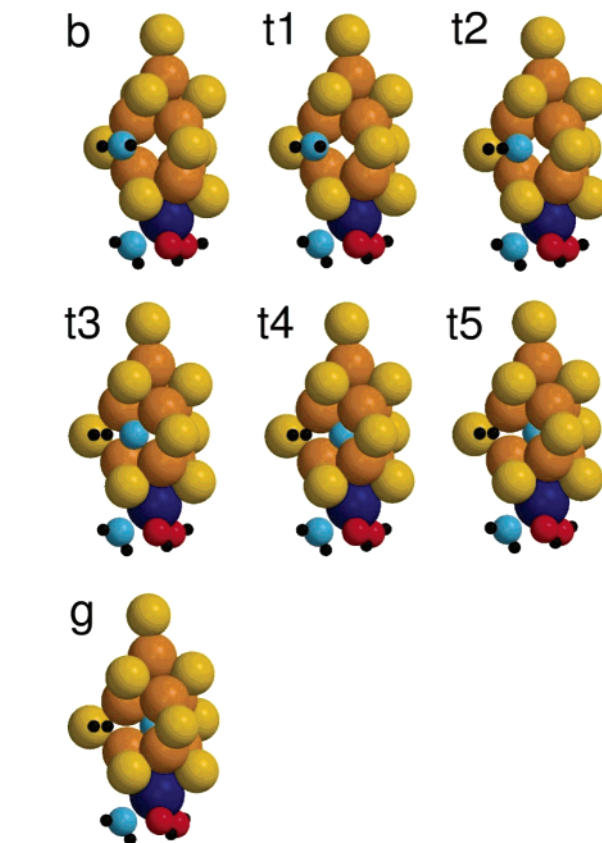
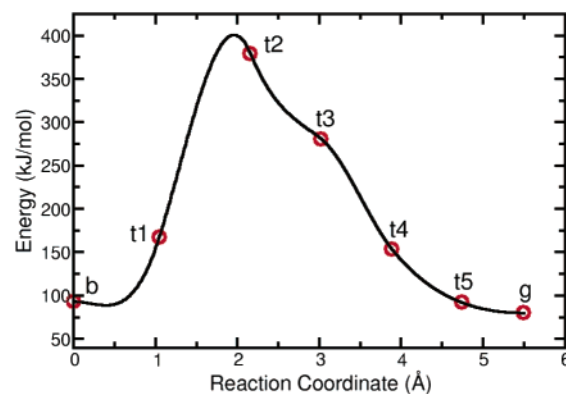


Figure 3. Energies and structures for the optimal reaction path from the nudged elastic band calculation.³⁸ The solid line is a cubic spline fit to the energies and forces of the images. Note that all structures are rotated 60° clockwise relative to Figure 2 for better visibility. The H ligand terminating the upper S atom is not visible, as it points backward.

need to know the height of the energy barriers between the intermediate structures, and we choose to concentrate on the barrier between structures b and g, where H₂ desorbs. The reaction pathway found by using the nudged elastic band (NEB) method and the corresponding structures are shown in Figure 3.³⁸

The barrier for the reaction from structure b to g is thus determined to be 300 kJ/mol. This means that the reaction cannot take place under any circumstances. The barrier is so high because the N atom has to sit above the face at the transition state, where H₂ desorbs, and the N atom is extremely unstable

(35) Benton, P. M. C.; Mayer, S. M.; Shao, J.; Hoffman, B. M.; Dean, D. R.; Seefeldt, L. C. *Biochemistry* **2001**, *40*, 13816.
 (36) Benton, P. M. C.; Laryukhin, M.; Mayer, S. M.; Hoffman, B. M.; Dean, D. R.; Seefeldt, L. C. *Biochemistry* **2003**, *42*, 9102.
 (37) Mayer, S. M.; Niehaus, W. G.; Dean, D. R. *J. Chem. Soc., Dalton Trans.* **2002**, 802.

(38) The remaining forces perpendicular to the reaction path are as follows: t1, 0.93 eV/Å; t2, 0.53 eV/Å; t3, 0.93 eV/Å; t4, 0.98 eV/Å; t5, 0.46 eV/Å. The calculation was stopped because the energy of t2, which is closest to the transition state, changed less than 0.02 eV during the last 15 relaxation steps. Thus, no significant energy gain by further convergence is expected.

on the face compared to the cluster. For this reaction, the environment may not be that helpful either, because the N on the face can be stabilized only by binding to something. Binding would again mean that N could not move into the cluster. Thus we can exclude the red reaction path.

Another possible way to continue from structure b would be that the NH_2 successively delivers another two protons to the $\mu_2\text{S}$ atoms. This path is marked in blue in Figure 2. However, NH is even more unstable on the FeMoco than NH_2 , because the optimal binding mode for NH would be 3-fold, and such a binding site is only available when three of the Fe atoms at the face are contracted, as in structure e, and this contraction is costly in energy. We have also estimated the barrier for the reaction from structure a to b, where NH_3 delivers one proton to a $\mu_2\text{S}$ atom and becomes bound side-on as NH_2 . This barrier is of the same order of magnitude as the other barrier, >200 kJ/mol. From this we can conclude that the blue path is not accessible under ambient conditions. One should note that apart from $\text{NH}_3 \rightarrow \text{N}(3^-) + 3\text{H}^+$, no further reduction or electron transfer has taken place. But in view of the very high barriers, it is unlikely that this would change the picture.

In summary, both the intermediate states and the reaction barriers render it extremely unlikely that N can be inserted into or extracted out of the cavity of the FeMoco. While we cannot exclude that we have missed a more favorable reaction path, it is hard to imagine what such a path would look like, as N would always have to undergo a very unstable and unreduced state on the face of the FeMoco. In a recent paper,¹⁰ Dance suggested a path for N insertion, where as a starting point N_2 binds on the face in an asymmetric position. Then the outer N is hydrogenated by protons coming from the $\mu_2\text{S}$ atoms and dissociated from the inner N, which simultaneously moves into the cluster. As no energies of intermediate states or transition states have been given, we cannot comment on the feasibility of this reaction path. One could think that the barrier for the dissociation is high, as the inner N atom has to undergo an undercoordinated state at the face of the FeMoco. The path suggested by Dance resembles our reaction path in the sense that nitrogen is bound (in our case to H_2 , in Dance's case to NH_3) and it has to dissociate from these ligands and move into the cluster.

In summary, our results are consistent with the study of Lee et al.,¹² as they find that all signals originating from nitrogen are conserved under turnover. Thus, we conclude that our calculations are consistent with a picture where the central nitrogen ligand is present at all times during turnover and that it can be neither inserted nor extracted during turnover.

3.2 Nitrogen Reduction and Ammonia Formation. The coordination of the triangular Fe atoms is quite different when a central ligand is present. With an empty cavity the triangular Fe atoms would be undercoordinated and the FeMoco could distort significantly in order to facilitate binding of N_2 at one of the Fe atoms. With a central N ligand, however, the triangular Fe atoms are bound to the central ligand, and therefore the FeMoco is much less flexible. The central N seems to be truly six-coordinated, as it is located exactly in the middle of the cofactor, and the Fe–N distances to the six surrounding Fe ligands are almost equal.⁸ Thus all Fe atoms are actually four-coordinate and therefore the cluster is more rigid than it would be without a central ligand. This raises the question whether the triangular Fe atoms can be involved in the binding and reduction of N_2 even though the central N ligand is present.

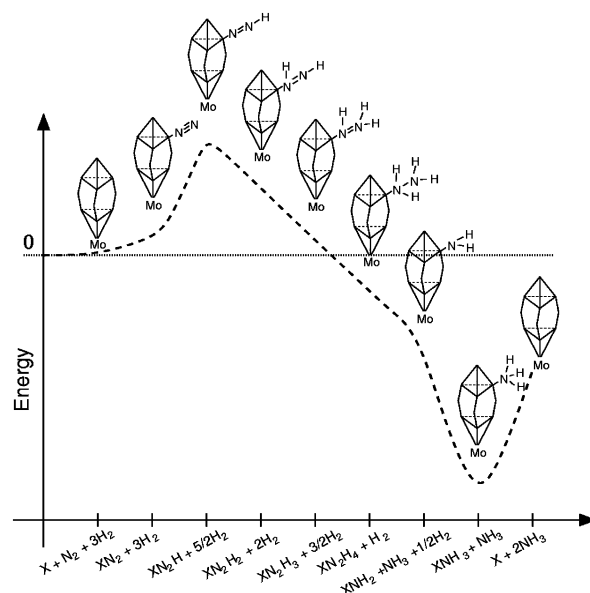


Figure 4. Schematic model for ammonia formation on the FeMoco, based on ref 18. It is emphasized that this schematic results from calculations on the FeMoco without the central ligand. It should serve to illustrate which steps were found to be exothermic and which were endothermic in ref 18.

To investigate this we use ref 18 as a basis, as it suggested a model for N_2 binding and reduction on the triangular Fe atoms. Different ways to bind N_2 to the cluster and to reduce it were studied and it was suggested that the N_2 does not dissociate (see above discussion) but is hydrogenated directly as a molecule. The suggested pathway for ammonia formation is schematically shown in Figure 4. The binding of the N_2 to the cluster is essentially thermoneutral or slightly endothermic, and the most favorable binding position is end-on to one of the triangular iron atoms. The first energy-demanding endothermic step is the addition of the first proton–electron pair to the bound N_2 , which requires an energy of ~ 80 kJ/mol. The following steps are exothermic, with hydrazine being an intermediate. After the addition of five electron–proton pairs, the first molecule of NH_3 desorbs spontaneously. After one subsequent reaction, the second NH_3 molecule desorbs, but this is an endothermic step of ~ 90 kJ/mol. One would expect that in solution the dissociation energy of the second NH_3 molecule is lower, as it will be solvated.

We have calculated the energies for those steps for 0–3 protons present on the $\mu_2\text{S}$ atoms (and 0–3 electrons on the cluster), and the energies and structures are shown in Figure 5. Furthermore, all energies are listed in Table 1. The adsorption of N_2 end-on a triangular Fe atom is slightly more endothermic, 37–65 kJ/mol, depending on the number of protons present on the $\mu_2\text{S}$ ligands. N_2 does not dissociate, as atomic N is very unstable on the cluster.

At this point we note that in this study we restrict ourselves to simultaneous proton and electron transfer. In ref 18, it has been shown that separate proton/electron transfer, where the electron resides on the FeMoco and the proton in the surroundings nearby (e.g., a protonated His residue or water molecule), lowers the energies for N_2 binding and the first reduction. Relative to state a in Figure 5, the energy for N_2 binding has been found to be lowered by approximately 80 kJ/mol and the first reduction step by approximately 130 kJ/mol.¹⁸ Although in ref 18 a simpler model without the central ligand was considered, one can assume that the order of magnitude for the

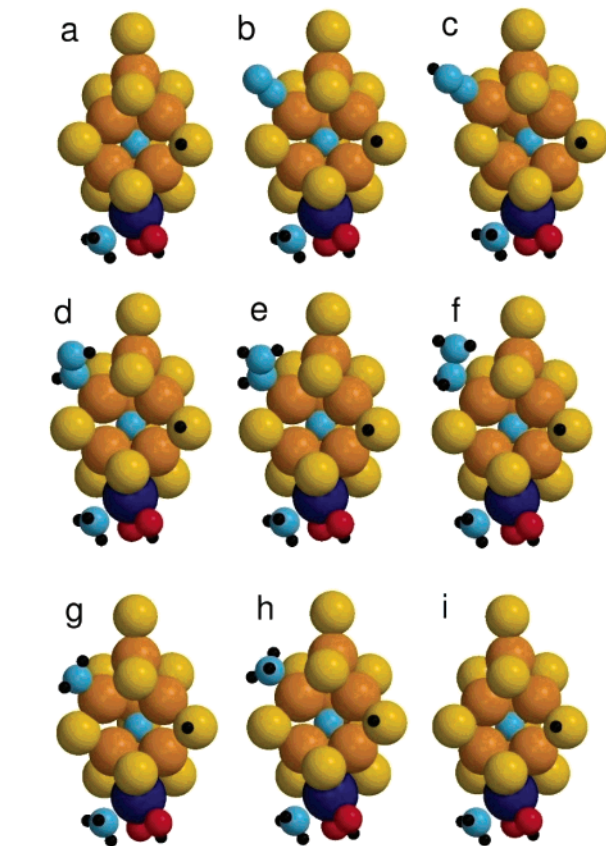
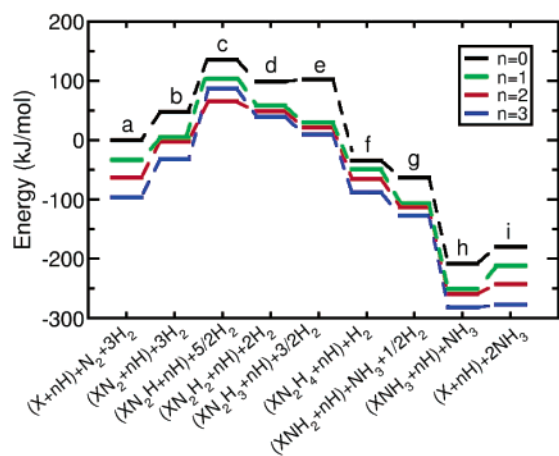


Figure 5. Energy scheme and minimum energy structures for the adsorption and hydrogenation of N_2 with 0–3 protons present on the μ_2S atoms of the FeMoco (and 0–3 electrons on the cluster).³⁹ The minimum energy structures are shown for one proton on the FeMoco; the calculations for two protons have been performed such that the Fe atom involved in the binding of N_2 is not adjacent to a protonated μ_2S ligand. The H ligand terminating the upper S ligand is not visible, as it is pointing backward. The energy zero is the cluster without adsorbates, N_2 , and $1/2H_2$ in the gas phase.

stabilizations should be the same. Therefore we assume that N_2 binding is stabilized by the surroundings and that the first hydrogenation of N_2 is the step with the highest barrier in the mechanism. In the following, we concentrate on this step and look for the reaction path, where the energy for this step is minimized.

It is clear that the most energy-demanding step in the reaction pathway is the first reduction of the bound N_2 and that all following steps are exothermic (with the possible exception of the third reduction for the FeMoco with no protons and electrons

Table 1. All Energies from Figure 5^a

	$n=0$	$n=1$	$n=2$	$n=3$
$(X + nH) + N_2 + 3H_2$	0	-33	-63	-97
$(XN_2 + nH) + 3H_2$	48	5	-2	-32
$(XN_2H + nH) + 5/2H_2$	136	104	65	87
$(XN_2H_2 + nH) + 2H_2$	99	58	49	39
$(XN_2H_3 + nH) + 3/2H_2$	102	30	21	9
$(XN_2H_4 + nH) + H_2$	-35	-49	-65	-88
$(XNH_2 + nH) + NH_3 + 1/2H_2$	-63	-107	-113	-127
$(XNH_3 + nH) + NH_3$	-208	-251	-260	-282
$(X + nH) + 2NH_3$	-180	-212	-243	-277

^a All energies are relative to $X + N_2 + 3H_2$ as in Figure 5 and are given in kilojoules per mole. The number of protons on the μ_2S ligands (and electrons on the cluster) is denoted by n .

adsorbed, which is approximately thermoneutral). Thus the step with the highest barrier in the considered process, which comprises the MoFe–protein cycle, is the first reduction of N_2 , and the energy differences from structure b to c lie between 66 and 119 kJ/mol, depending on the number of protons and electrons on the cluster. These energies are definitely accessible, in particular when the role of a possible proton donor in the vicinity is considered, which has been suggested to reduce the energies of the most endothermic steps.¹⁸ Thus we can conclude that the triangular Fe atoms can still participate in N_2 binding and reduction, even though they are coordinated to a central ligand. Of course, one would also need the barriers of the different steps in the reaction pathway, which we have not calculated. On the other hand, it is not clear to what extent the proton/electron transfers are activated. In a previous study,¹⁸ a nearby electron proton donor was modeled in a simple way and it was found that proton transfer was nonactivated in this setup. In this context we note that the desorption of the first NH_3 molecule upon addition of the fifth proton/electron pair is also nonactivated, as it happens spontaneously during the structural relaxation.

As energy zero, we have chosen the FeMoco without adsorbates and N_2 and $1/2H_2$ in the gas phase. The energy zero for hydrogen is a matter of choice, but $1/2H_2$ as the zero point is related to the standard hydrogen electrode (SHE) and is therefore an especially practical choice. The actual chemical potential of the transferred protons and electrons depends on the redox potential of the $[4Fe-4S]^{1+/2+}$ cluster pair, which has been measured to be -430 mV/SHE for *Azotobacter vinelandii* in the presence of bound MgATP.^{41,2} Therefore, this can be taken as a typical value for the redox potential with which the electrons are delivered. One should note that at pH 7 this does not lead to a shift of the energies, as the correction for pH 7 approximately equals the redox potential of the $[4Fe-4S]^{1+/2+}$ cluster pair.

Having concluded that the energy levels for N_2 binding and reduction are generally accessible under ambient conditions, we now turn to a discussion of the detailed structure of the energy levels. The energies required to proceed at each step depend on the number of protons and electrons transferred to the cluster, but the energy changes are small. Furthermore, it is probable

(39) For the FeMoco with three H atoms on the μ_2S atoms, we had to keep the Fe–N distance fixed, as otherwise N_2 would desorb. We fixed the distance at the value 2.08 Å, which is the Fe–N distance we obtained from other structures.

(40) Atkins, P. W. *Physical Chemistry*; Oxford University Press: Oxford, U.K., 1998.

(41) Ryle, M. J.; Lanzilotta, W. N.; Mortenson, L. E.; Watt, G. D.; Seefeldt, L. C. *J. Biol. Chem.* **1995**, *270*, 13112.

that protons and electrons transferred to the FeMoco are first stored (protons on the $\mu_2\text{S}$ ligands, electrons delocalized on the cluster) and then used for reduction. Thus, it is by no means required that the number of adsorbed protons and electrons on the cluster stays constant during N_2 reduction, and this number is varied not only to supply protons and electrons for the reduction but also to change the energy levels of the different steps slightly. With respect to Figure 5, this means that the whole reaction does not necessarily follow the path of one color, but that one can switch between different colors and obtain the energetically most favorable path.

Let us now assume that the starting structure for the FeMoco is the one where the three $\mu_2\text{S}$ ligands are protonated and there are three surplus electrons on the cluster, as this structure has lowest energy (see Figure 5) and it agrees with experimental results for the resting state.^{8–11,30} This corresponds to the level of the blue curve (Figure 5a with three protons and electrons on the cluster). The next step could be that one more coupled proton and electron transfer triggers H_2 evolution (with H adsorbed on one of the triangular Fe atoms turning into a hydride, transfer of one proton from $\mu_2\text{S}$ to hydride, and evolution of H_2 , as outlined in ref 18). Then the FeMoco accomplishes the binding and first reduction of N_2 with a 2-fold protonation and electron transfer, as the respective energy differences are lowest (the red curve in Figure 5). This means that it is the second electron/proton transfer which accomplishes the first reduction of N_2 to N_2H (state c in Figure 5). The third electron/proton transfer causes the second reduction of N_2H to N_2H_2 (state d in Figure 5). At this point the reaction is over the hill and irreversible, as it has passed the state c which has the highest energy. From state d onward, all further reductions are downhill in energy and can occur after further electron/proton transfers.

Thus, in our model there have to be three electron/proton transfers in order for N_2 to bind and react irreversibly. This mechanism might explain the fact of the Thorneley–Lowe model that 3–4 electron/proton transfers have to be accomplished before N_2 can bind and react. EPR measurements¹⁹ indicate that only two of the first three electrons transferred actually reach the cofactor; therefore there can be a discrepancy of one proton/electron pair. The fate of the third electron could not be resolved yet,¹⁹ but it could possibly be transferred to the surroundings of the FeMoco. Anyhow, we take a possible insecurity of one proton/electron pair into account. Our model would also suggest a possible reason for the obligatory hydrogen evolution: namely, that this is needed in order to take the FeMoco to an oxidation state where it has a more suitable reactivity.

In light of the previous discussion, only the FeMoco states with two and three protonated $\mu_2\text{S}$ ligands (and two or three electrons on the cluster) are relevant, and they correspond to the red and blue curves in Figure 5. But also in a more general context it is interesting that the energies in Figure 5 are dependent on the number of protons and electrons adsorbed on the cluster. The energy dependence is only small, but this still points at a mechanism to influence the energetics of the different steps by adding protons and electrons to the cluster and thereby changing the oxidation state of the Fe ions. Adding a proton to a $\mu_2\text{S}$ atom changes the spin density of the two adjacent triangular Fe atoms. Adding proton–electron pairs to the cluster not only provides supply for the reductions but also modifies

the reactivity of the cluster. In this way, the cluster can be fine-tuned to reduce the energy for certain critical steps during the reaction. This might explain why, as described in the Thorneley–Lowe model, it is relevant how many protons and electrons are supplied before a particular reaction step is accomplished and not just that they are supplied.

The energy required for desorbing the second NH_3 is low. Especially for the 3-fold protonated cluster, the desorption of the second NH_3 atom is essentially thermoneutral. In solution, desorption of the second NH_3 molecule should happen more easily, as it will be solvated as NH_3 or NH_4^+ and thereby gain energy. Thus, in accordance with the Thorneley–Lowe model, we do not expect NH_3 to be bound to the FeMoco. Nonetheless, it is an interesting observation that desorption of the second NH_3 from the FeMoco is easier in the presence of the central ligand than in its absence. This is of course related to the fact that, in the presence of the interstitial N, the FeMoco is much less flexible and thereby cannot bind substrates or products very strongly. This might actually be advantageous if the necessary binding of substrates is accomplished by tuning the redox state of the FeMoco, because then one does not run into the problem of active-site-poisoning by products. Active-site poisoning by intermediates or products is an important problem for industrial ammonia catalysts,⁴² and by limiting the structural flexibility, the FeMoco might have devised a clever way to avoid this problem.

In summary, we have shown that reduction of N_2 on the triangular Fe sites is still possible in the presence of the central N ligand and that the central ligand is probably not directly involved in the reaction. Both the steric situation and the number of proton–electron pairs on the cluster influence the energetics. Of course our model is simple and we do not account for the protein environment, which introduces some energy shifts. The protein environment also introduces changes to the details of the reaction. Although we have found the most stable isomer for each reduction step (N_xH_y), in some cases the energy differences between the isomers are very small. For example, for bound 2-fold reduced N_2 we find the isomer NH–NH to be slightly more stable than N–NH_2 , but the energy differences are too small for a decisive order of stability.⁴³ Also, other details of the reaction may be altered due to the influence of the protein environment. Furthermore, we have not calculated reaction barriers, which ultimately are needed to judge whether the presented reaction pathway is feasible. As most of the reaction steps are electron/proton transfers, it is not sure that all steps are activated. The dissociation of the first NH_3 molecule upon the fifth reduction step is nonactivated, as it occurs spontaneously during the structural relaxation.

In this study, we have limited ourselves to simultaneous proton and electron transfers. One would have to investigate for which steps a separate proton and electron transfer would be favorable. First steps toward this were taken recently,¹⁸ where the electron was transferred to the FeMoco and the proton to a simple model of a nearby proton donor. After N_2 was bound, the proton was transferred to the FeMoco and accomplished

(42) (a) Stoltze, P.; Nørskov, J. K. *Phys. Rev. Lett.* **1985**, *55*, 2502. (b) Stoltze, P.; Nørskov, J. K. *J. Catal.* **1988**, *110*, 1.

(43) The N_2H_2 isomer shown in Figure 5 and Table 1 is *trans*- NH–NH . For *cis*- NH–NH , the energies in Table 1 are 122, 82, 76, and 67 kJ/mol, and for N–NH_2 the energies are 130, 83, 78, and 44 kJ/mol. These energies are for $n = 0, 1, 2$, and 3 H atoms on the $\mu_2\text{S}$ atoms and should be compared to the energies in Table 1, line 5.

the first reduction step. It was found that the binding energy of N_2 is lowered so that binding becomes clearly exothermic. Furthermore, it was observed that the energy required to accomplish the first reduction of bound N_2 was lowered. The first reduction of N_2 is endothermic but requires significantly less energy. Thus, it would be very interesting to extend our reaction pathway to nonsimultaneous proton and electron transfers. One possible candidate for a proton donor is the nearby α -His¹⁹⁵ residue, on which some mutation studies have been carried out.⁴⁴ If the α -His¹⁹⁵ is mutated with a α -Gln¹⁹⁵, the FeMoco can still bind N_2 but it cannot be reduced and the reaction does not proceed. If α -His¹⁹⁵ is mutated with any other amino acid, N_2 cannot be bound. This might suggest that the role of α -His¹⁹⁵ is both to hydrogen-bond N_2 and to accomplish the first proton transfer onto the bound N_2 . Histidine is the only amino acid side chain that has a pK in the vicinity of $pH = 7$ and therefore can both donate and accept a proton at neutral pH .⁴⁵ Another possible candidate for a proton donor is a nearby water molecule, which would be well-positioned for forming a hydrogen bond and transferring a proton. Investigating these

possible proton donors with a larger model will shed light on the detailed mechanism and energetics.

4. Conclusion

In this work we have investigated possible routes for insertion of the central N ligand into the FeMoco. All considered routes are associated with intermediate states and especially barriers of high energy so they are impossible under ambient conditions. This agrees with the findings of Lee et al.,¹² who show by means of ENDOR and ESEEM spectroscopy that nitrogen neither enters nor leaves the cluster during turnover. Furthermore, we have shown that reduction of adsorbed N_2 on a triangular Fe atom is possible in the presence of the central ligand. For this reaction pathway, the central N ligand changes the catalytic properties of the FeMoco only marginally.

Acknowledgment. The Center of Atomic-scale Materials Physics is sponsored by the Danish National Research Foundation. We acknowledge support from the Danish Center of Scientific Computing through Grant HDW-1101-05.

Supporting Information Available: Technical details of the DFT calculations and coordinates of the minimized structures (PDF). This information is available free of charge via the Internet at <http://pubs.acs.org>.

JA037792S

- (44) (a) Scott, D. J.; May, H. D.; Newton, W. E.; Brigle, K. E.; Dean, D. R. *Nature* **1990**, *343*, 188. (b) Kim, C.-H.; Newton, W. E.; Dean, D. R. *Biochemistry* **1995**, *34*, 2798. (c) Dilworth, M. J.; Fisher, K.; Kim, C.-H.; Newton, W. E. *Biochemistry* **1998**, *37*, 17495.
- (45) Fersht, A. *Structure and Mechanism in Protein Science*; W. H. Freeman and Company: New York, 1999.