Thermodynamically Favourable States in the Reaction of Nitrogenase without Dissociation of any Sulfide Ligand

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Abstract: We have used combined quantum mechanical and molecular mechanical (QM/MM) calculations to study the reaction mechanism of nitrogenase, assuming that none of the sulfide ligands dissociates. To avoid the problem that there is no consensus regarding the structure and protonation of the E\(_4\) state, we start from a state where N\(_2\) is bound to the cluster and is protonated to N\(_2\)H\(_2\) after dissociation of H\(_2\). We show that the reaction follows an alternating mechanism with HNNH (possibly protonated to HNNH\(_2\)) and H\(_2\)NNH\(_2\) as intermediates and the two NH\(_3\) products dissociate at the E\(_7\) and E\(_8\) levels. For all intermediates, coordination to Fe\(_6\) is preferred, but for the E\(_4\) and E\(_5\) intermediates, binding to Fe\(_2\) is competitive. For the E\(_6\), E\(_7\) and E\(_8\) intermediates we find that the substrate may abstract a proton from the hydroxy group of the homocitrate ligand of the FeMo cluster, thereby forming HNNH\(_2\), H\(_2\)NNH\(_2\) and NH\(_3\)H\(_2\) intermediates. This may explain why homocitrate is a mandatory component of nitrogenase. All steps in the suggested reaction mechanism are thermodynamically favourable compared to protonation of the nearby His-195 group and in all cases, protonation of the NE2 atom of the latter group is preferred.

Introduction

Nitrogen is an essential element of all lifeforms, being a component of all amino acids and nucleic acids. Although the atmosphere of Earth contains 78% of N\(_2\), nitrogen is still a limiting element for plant growth and a prominent component of all amino acids and nucleic acids. Although the atmosphere of Earth contains 78% of N\(_2\), nitrogen is still a limiting element for plant growth and a prominent component of all amino acids and nucleic acids. While the nitrogenase (EC 1.18.19.6.1), which work at ambient temperature and pressure, only a single group of enzymes can cleave the N–N bond in N\(_2\), the nitrogenases (EC 1.18.19.6.1), which work at ambient temperature and pressure. Crystallographic studies have shown that the most active type of nitrogenase contains a MoFe\(_5\)S\(_3\)(homocitrate) cluster (the FeMo cluster) in the active site, connected to the protein by a histidine and a cysteine residue at the opposite ends of the cluster (Figure 1). There also exist alternative nitrogenases with the Mo ion replaced with either vanadium or iron, which have lower activities towards N\(_2\).[18]

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The nitorgenases catalyse the reaction

\[
\text{N}_2 + 8 \text{e}^- + 8 \text{H}^+ + 16 \text{ATP} \rightarrow 2 \text{NH}_3 + 2 \text{H}_2 + 16 \text{ADP} + 16 \text{Pi}
\]

The mechanism is normally discussed in terms of nine intermediates E\(_4\)–E\(_8\) differing in the number of added electrons and protons, according to the Lowe–Thorneley scheme.[11] Thorough biochemical, kinetic and spectroscopic studies have indicated that the resting E\(_4\) state needs to be reduced to the E\(_5\) state before N\(_2\) may bind.[13,14,15,16] It has also been suggested that H\(_2\) formation through reductive elimination is a prerequisite for the binding of N\(_2\), explaining why H\(_2\) is a compulsory byproduct in the reaction. It is normally assumed that N\(_2\) is directly reduced and protonated to N\(_2\)H\(_2\) upon binding to the enzyme.[13,14]

It has long been debated whether the nitrogenases follow a sequential or alternating reaction mechanism. In the sequential mechanism, the first three protons bind to the same N atom of N\(_2\), which then dissociates as NH\(_3\) from the E\(_5\) intermediate, before the second N atom starts to be protonated. This mechanism was originally suggested by Chatt and has gained support from inorganic model complexes.[19–23] In the alternating mechanism, the protons are instead added alternatively to the two N atoms, so that HNNH and H\(_2\)NNH\(_2\) (hydrazine) are intermediates, binding to Fe2 is competitive. For the E\(_4\) and E\(_5\) intermediates we find that the substrate may abstract a proton from the hydroxy group of the homocitrate ligand of the FeMo cluster, thereby forming HNNH\(_2\), H\(_2\)NNH\(_2\) and NH\(_3\)H\(_2\) intermediates. This may explain why homocitrate is a mandatory component of nitrogenase. All steps in the suggested reaction mechanism are thermodynamically favourable compared to protonation of the nearby His-195 group and in all cases, protonation of the NE2 atom of the latter group is preferred.

The nitrogenases have been thoroughly studied also by computational methods.[13,15–42,27–34] Unfortunately, these studies have given very diverging and disparate suggestions. In fact,
there is not even any consensus about the structure of the key E₄ intermediate. Important reasons for this are that different density-functional theory (DFT) methods give very different predictions of the relative stability of various intermediates, with differences of 600 kJ/mol[43] and that there are very many possibilities for the structures and electronic states of the intermediates.[44,45]

Hoffman and coworkers have suggested a structure of the E₄ intermediate with two hydride ions bridging the Fe2 and Fe6 ions, as well as the Fe3 and Fe7 ions, and with two protons on

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**Figure 1.** Structure of the FeMo cluster (with trans-HNNH bound to Fe6), illustrating also the QM system used in all calculations, as well as the names of the nearby residues (a). (b) shows only the FeMo cluster with atom names indicated.
the S2B and the S5A sulfides, all positioned on the same face of the FeMo cluster\textsuperscript{35,47,48} (the name of the various Fe and sulfide ions are shown in Figure 1b). They have shown that this structure is lower in energy than a few other structures and that it may bind N\textsubscript{2} after reductive elimination of the two hydride ions, leaving the cluster in a doubly reduced state.\textsuperscript{15,41,47,48}

On the other hand, Siegbahn has suggested that the FeMo cluster needs to be reduced by four electrons from the resting state before the true E\textsubscript{0} state is reached, which involves a triply protonated central carbide and a strongly distorted cluster.\textsuperscript{49} Then, this state is reduced by another four electrons to reach the E\textsubscript{0} state, from which H\textsubscript{4} dissociates and N\textsubscript{2} binds, bridging two Fe ions. It is successively protonated in a manner that is a mixture of the alternating and sequential mechanism, involving NNH\textsubscript{2}, H\textsubscript{2}NNH\textsubscript{2}, but also HNNH\textsubscript{2}. The first NH\textsubscript{2} dissociates at the E\textsubscript{1} level.

Dance has presented a mechanism in which E\textsubscript{3} contains two terminal hydride ions on Fe2 and Fe6, and two protons on S2B and S3B. N\textsubscript{2} then binds side-on to Fe6, without any dissociation of H\textsubscript{2} and is alternatively protonated to H\textsubscript{2}NNH\textsubscript{2}, at which level the N–N bond is cleaved, forming two NH\textsubscript{3} fragments on Fe2 and Fe6.\textsuperscript{27,30,51}

On the other hand, Nørskov and coworkers have suggested a mechanism in which the E\textsubscript{4} state is doubly protonated and a sulfide ligand dissociates from the cluster during the reaction mechanism.\textsuperscript{37} This forms a binding site, where N\textsubscript{2} binds in an end-on fashion, bridging two Fe ions and it is then sequentially protonated on the outer N atom. The dissociation of the sulfide ion was inspired by several crystallographic studies of both Mo and V nitrogenase, showing that the S2B group can be replaced and V nitrogenase, showing that the S2B group can be replaced without dissociation of S2B. We show that also such a reaction is possible and thermodynamically favourable, following an alternating mechanism.

Methods

The protein

The calculations were based on the 1.0-Å crystal structure of Mo nitrogenase from Azotobacter vinelandii (PDB code 3U7Q).\textsuperscript{7} The setup of the protein is identical to that of our previous studies.\textsuperscript{43,57-61} The entire heterotetramer was considered in the calculations, because the various subunits are entangled without any natural way to separate them. The quantum mechanical (QM) calculations were concentrated on the FeMo clusters in the C subunit because there is a buried imidazole molecule from the solvent rather close to the active site (≈ 11 Å) in the A subunit. The two P-clusters and the FeMo cluster in subunit A were modelled by MM in the fully reduced and resting states, respectively, using a QM charge model.\textsuperscript{59}

The protonation states of all residues were the same as before.\textsuperscript{59} All Arg, Lys, Asp and Glu residues were assumed to be charged, except Glu-153, 440 and 231D (a letter “D” after the residue number indicates that it belongs to that subunit; if no letter is given, it belongs to subunit C; subunits A and B are identical to the C and D residues). Cys residues coordinating to Fe ions were assumed to be deprotonated. His-274, 451, 297D, 359D and 519D were assumed to be protonated on the ND1 atom, His-31, 196, 285, 383, 90D, 185D, 363D and 457D were presumed to be protonated on both the ND1 and NE2 atoms (and therefore positively charged), whereas the remaining 14 His residues were modelled with a proton on the NE2 atom. The homocitrate ligand was modelled in the singly protonated state with a proton shared between the hydroxy group (which coordinates to Mo) and the O1 carboxylate atom. This protonation state was found to be the most stable one in an extensive QM/MM, molecular dynamics and quantum-refinement study\textsuperscript{59} and this protonation state is also supported by another QM/MM study.\textsuperscript{62}

The protein was solvated in a sphere with a radius of 65 Å around the geometrical centre of the protein. 160 Cl\textsuperscript{-} and 182 Na\textsuperscript{+} ions were added at random positions (but not inside the protein)\textsuperscript{59} to neutralise the protein and give an ionic strength of 0.2 M.\textsuperscript{59} The final system contained 133 915 atoms. The added protons, counter ions and water molecules were optimised by a simulated annealing calculation (up to 370 K), followed by a minimisation, keeping the other atoms fixed at the crystal-structure positions.\textsuperscript{59}

All MM calculations were performed with the Amber software.\textsuperscript{64} For the protein, we used the Amber ff14SB force field\textsuperscript{66} and water molecules were described by the TIP3P model.\textsuperscript{66} For the metal sites, the MM parameters were the same as in our previous investigation.\textsuperscript{59} The metal sites\textsuperscript{45,59} were treated by a non-bonded model\textsuperscript{67} and charges were obtained with the restrained electrostatic potential method, obtained at the TPSS/def2-SV(P) level of theory\textsuperscript{68,69} and sampled with the Merz–Kollman scheme.\textsuperscript{70}

The FeMo cluster was modelled by MoFe\textsubscript{5}S\textsubscript{3}C-(homocitrate)(CH\textsubscript{3}S) (imidazole), where the two last groups are models of Cys-275 and His-442. In addition, all groups that form hydrogen bonds to the FeMo cluster were also included in the
QM model, viz. Arg-96, Gln-191 and His-195 (sidechains), Ser-278 and Arg-359 (both backbone and sidechain, including the Cα and C and O atoms from Arg-277), Gly-356, Gly-357 and Leu-358 (backbone, including the Cα and C and O atoms from Ile-355), as well as two water molecules. Finally, Phe-381 and Val-70 were also included because they are close to the putative N2 binding site and therefore may affect the binding of the substrate. The QM system involved 183–190 atoms in total (depending on the number of added protons and N atoms) and is shown in Figure 1a. The net charge of QM region was $-3$.

**QM calculations**

All QM calculations were performed with the Turbomole software (version 7.5). All structures were studied with both the TPSS[70] and B3LYP[72–74] functionals with def2-SVP(P) basis set. The most stable states were examined also with the larger def2-TZVPD basis set. The calculations were sped up by expanding the Coulomb interactions in an auxiliary basis set, the resolution-of-identity (RI) approximation.[75,76] Empirical dispersion corrections were included with the DFT-D4 approach, as implemented in Turbomole. All minima were fully optimized without any restraints. Transition states (for N→N cleavage and NH$_3$ dissociation) were determined as the highest point on the potential energy surface along the reaction coordinates, which were scanned with a step of 0.1 Å near the transition states.

Experiments have shown that the ground spin state of E$\alpha$ is a doublet,[7,17,21] and we used this state for E$\alpha$ models. For the other E$\beta$ states, we used mainly the doublet or triplet states, but for the most interesting structures, we checked which of the two or three lowest spin states has the most favourable energy at the TPSS and B3LYP/def2-SVP(P) levels of theory.

The electronic structure of all QM calculations was obtained with the broken-symmetry (BS) approach.[79] Each of the seven Fe ions were modelled in the high-spin state, with either a surplus of $\alpha$ (four Fe ions) or $\beta$ (three Fe ions) spin. Such a state can be selected in 35 different ways (\[7,17\]). The various BS states were obtained either by swapping the coordinates of the Fe ions[79] or with the fragment approach by Szilagyi and Winslow.[21] The various BS states are named by listing the number in the Noodleman nomenclature (BS1—10),[79] followed by the numbers of the three Fe ions with minority spin (however, in the tables, only the latter three numbers are given). Most structures were studied in the BS10-147 state, i.e. with $\beta$ spin on Fe1, Fe4 and Fe7, because it was found to be lowest in both this and in our previous study.[21,37] However, sometimes the calculations converged to other states (especially BS7-235). For twelve of the most stable structures, the relative stabilities of all 35 states were examined (with structures fully optimised for each BS state). Moreover, for all structures within 20 kJ/mol of the most stable structure at each E$\alpha$ level, the BS7-235 state was also studied.

As have been discussed before,[43,10] TPSS/def2-SVP(P) calculations give geometries that reproduce the crystal structure of the resting state of nitrogenase excellently with average and maximum deviations of 0.05 and 0.09 Å for the metal–metal distances, and 0.02 and 0.06 Å for metal–ligand distances, and a root-mean-squared-deviation (RMSD) of 0.06 Å for the metals and the first-sphere ligands. This is similar to the results obtained with the TPSSH[10] approach and appreciably better than with the B3LYP/def2-SVP(P) method, which gives average and maximum deviations of 0.08 and 0.12 Å for the metal–metal and 0.04 and 0.11 Å metal–ligand distances, respectively and a RMSD of 0.08 Å. Therefore, we discuss primarily the TPSS/ def2-SVP(P) results.

**QM/MM calculations**

QM/MM calculations were performed with the ComQum software.[82,83] In this approach, the protein and solvent are split into three subsystems: System 1 (the QM region) was relaxed by QM methods. System 2 contained all residues and water molecules with at least one atom within 6 Å of any atom in system 1 and it was optionally relaxed by MM. It included residues 49, 59–74, 92, 95–98, 189–199, 226–231, 234, 235, 253–255, 273–282, 300, 353–355, 358–364, 377–383, 385, 386, 401 422–427, 438, 440–444, 450 and 451 from subunit C and residues 93, 97, 98, 101 and 105 from subunit D, in total 94 residues and 39 water molecules. Finally, system 3 contained the remaining part of the protein and the solvent, and it was kept fixed at the original coordinates (equilibrated crystal structure to avoid the risk that different calculations end up in different local minima). The total system was spherical and non-periodic with 133 915 atoms. Most calculations were performed without relaxing system 2, but for the most interesting structures, calculations with relaxed surroundings were also performed. The effect of the relaxed surroundings are described in the Supporting Information.

In the QM calculations, system 1 was represented by a wavefunction, whereas all the other atoms were represented by an array of partial point charges, one for each atom, taken from the MM setup. Thereby, the polarisation of the QM system by the surroundings is included in a self-consistent manner (electrostatic embedding). When there is a bond between systems 1 and 2 (a junction), the hydrogen link-atom approach was employed: The QM system was capped with hydrogen atoms (hydrogen link atoms, HL), the positions of which are linearly related to the corresponding carbon atoms (carbon link atoms, CL) in the full system.[82,84] All atoms were included in the point-charge model, except the CL atoms.[85]

The total QM/MM energy in ComQum is calculated as[82,83]

$$E_{\text{QM/MM}} = E_{\text{QM1+sub23}}^{\text{HL}} + E_{\text{MM123}}^{\text{CL}} - E_{\text{MM1}}^{\text{CL}} - E_{\text{MM1}}^{\text{HL}}$$

(2)

in which $E_{\text{QM1+sub23}}^{\text{HL}}$ is the QM energy of the QM system truncated by HL atoms and embedded in the set of point charges modelling systems 2 and 3 (but excluding the self-energy of the point charges). $E_{\text{MM1}}^{\text{CL}}$ is the MM energy of the QM system, still truncated by HL atoms, but without any electrostatic interactions. Finally, $E_{\text{MM123}}^{\text{CL}}$ is the classical energy of all atoms in the system with CL atoms and with the
charges of the QM region set to zero (to avoid double-counting of the electrostatic interactions). Thus, ComQum employs a subtractive scheme with van der Waals link-atom corrections. No cut-off is used for any of the interactions in the three energy terms in Equation (3).

The geometry optimisations were continued until the energy change between two iterations was less than 2.6 J/mol (10⁻⁶ a.u.) and the maximum norm of the Cartesian gradients was below 10⁻⁶ a.u.

QM/MM calculations give comparable energies only if they contain exactly the same number of electrons and atoms of each element in both the QM and MM systems. Therefore, we compare only structures within the same Eₙ level. On the other hand, it means that we can study proton transfers within the QM system, for example from the homocitrate ligand or from His-195 to the substrate. For each transition from Eₙ to Eₙ₊₁ an electron and a proton is added to the QM system, and we compare the energies of structures with this proton in different positions.

**Result and Discussion**

In this investigation, we study the later part of the reaction mechanism of nitrogenase, assuming that the S₂B ligand does not dissociate. We describe in separate sections states at different oxidation levels, from E₄ to E₉.

**N₂-bound E₄ structures**

We start with the N₂-bound E₄ state. As in our previous study of the reaction mechanism with a dissociated S₂B ligand, we avoid the problem there is no consensus regarding the protonation of the E₄ state by starting from a state where N₂ has already bound to the cluster and is protonated to N₂H₂. The immediate protonation of the substrate upon binding is normally assumed although it has not been experimentally observed. Mutations and other studies have shown that the substrate most likely bind either the Fe₂ or Fe₆ ions of the FeMo cluster (cf. Table S1 in the Supporting Information). Mo has a Mulliken spin population of 0.3 whereas Fe₂ and Fe₆ have 1.6 and 1.7, respectively. The N₂ bond length is 1.26 Å, which is slightly longer than in isolated trans-HNNH, optimised with the same level of theory, 1.25 Å. The Mulliken spin populations are (in absolute terms) 3.2–2.7 e on the seven Fe ions, except Fe₆, which has only 1.6 e (cf. Figure 2a). The other N atom of the substrate receives a hydrogen bond from the HN group bound to Fe₆ to the alcohol O₇ atom of homocitrate (which coordinates to Mo), with a H···O distance of 1.95 Å (cf. Figure 2a). The other N atom of the substrate receives a hydrogen bond from the HN group bound to Fe₂ to the alcohol O₇ atom of homocitrate (which coordinates to Mo), with a H···O distance of 1.95 Å (cf. Figure 2a). The other N atom of the substrate receives a hydrogen bond from the HN group bound to Fe₆ to the alcohol O₇ atom of homocitrate (which coordinates to Mo), with a H···O distance of 1.95 Å (cf. Figure 2a). The other N atom of the substrate receives a hydrogen bond from the HN group bound to Fe₆ to the alcohol O₇ atom of homocitrate (which coordinates to Mo), with a H···O distance of 1.95 Å (cf. Figure 2a). The other N atom of the substrate receives a hydrogen bond from the HN group bound to Fe₆ to the alcohol O₇ atom of homocitrate (which coordinates to Mo), with a H···O distance of 1.95 Å (cf. Figure 2a).

It can be seen that in nearly all structures, the HIE protonation state was more favourable than HID protonation by 21–132 kJ/mol. The only exception was the Fe₂(63)-cHNNH state with B3LYP. All structures were studied in the BS10-147 state, but sometimes it shifted to the BS7-235 state (the spin on Fe₆ is often small and may change sign; the latter state was also studied for all low-energy structures). However, BS10-147 was always 9–37 kJ/mol lower in energy than BS7-235 when both states were found, except for the Fe₆-cHNNH(3) state, for which BS7-235 was 1 kJ/mol more stable at both the TPSS and B3LYP levels.

The most favourable structure has trans-HNNH end-on bound to Fe₆ (Figure 2a). The Fe₆-N distance is 1.91 Å and the N–N bond length is 1.26 Å, which is slightly longer than in isolated trans-HNNH, optimised with the same level of theory, 1.25 Å. The Mulliken spin populations are (in absolute terms) 3.2–2.7 e on the seven Fe ions, except Fe₆, which has only 1.6 e (cf. Table S1 in the Supporting Information). Mo has a population of ~0.3 e. This structure is stabilised by a hydrogen bond from the HN group bound to Fe₆ to the alcohol O₇ atom of homocitrate (which coordinates to Mo), with a H···O distance of 1.95 Å (cf. Figure 2a). The other N atom of the substrate receives a hydrogen bond from the HE₁ atom of Gln-191 (the HE₁–N distance is 2.17 Å) and the other H atom of the substrate is directed towards S₂B, with a H···S distance of 2.29 Å, but the N–H–S angle is only 124°. S₂B receives another hydrogen bond from the HE₂ atom of His-195 (2.13 Å, with a more ideal geometry). The corresponding structure in the quartet state is 30 kJ/mol less stable with the TPSS functional, but 7 kJ/mol lower in energy compared to the BS7-235 state.
more stable with the B3LYP. A full investigation of all 35 BS states, collected in Table S2, shows that the BS10-147 state is indeed the most stable BS state, 10–67 kJ/mol more stable than the other BS states (BS7-235 second lowest).

A structure with NHH₂ bound end-on to Fe6 is only 4 kJ/mol less stable with TPSS (also with the larger def2-TZVPD basis set and 3 kJ/mol with relaxed surroundings) and it is actually 6 kJ/mol more stable with B3LYP. In this structure, the substrate has abstracted the alcohol proton from homocitrate, giving HNHH₂ (Figure 2b). It has a Fe–N distance of 1.85 Å and a N–N bond length of 1.29 Å (1.21 Å in neutral NHH₂ and 1.23 Å in HNHH⁺ optimised with the same method). It is stabilised by hydrogen bonds from the Fe6-bound NH group to the alcohol O7 atom of homocitrate (H···O7 = 1.72 Å), from the NH group to S2B (H···S2B = 2.19 Å), and from the other atom of NH group to S2B (H···S2B = 2.31 Å; Figure 2b). The S2B atom also receives a hydrogen bond from His-195 (H(E2)···S2B = 2.12 Å) and the O1 atom of homocitrate receives another hydrogen bond from Gln-191 (H(E1)···O1 = 1.93 Å). This structure has also a low spin population on Fe6 (1.8 e). Again, a full investigation of all 35 BS states showed that BS10-147 is the most favourable BS (Table S2), although it is only 4 kJ/mol more stable than BS10-135 (14 kJ/mol more stable than BS7-235).

A structure with trans-HNHH₂ binding to Fe2, directed to the S3A side (Figure 2c), is 20 kJ/mol less stable than Fe6-HNHH₂ structure (22 kJ/mol with the larger basis set, 34 kJ/mol with relaxed surroundings and 5 kJ/mol with B3LYP). The Fe2-N distance is 1.95 Å and the N–N bond length is 1.25 Å. The two H atoms of the substrate point towards S2B (H···S2B = 2.19 Å), but the N–H–S angles are far from straight (83° and 131°, respectively). The Fe spin population on Fe2 (2.1 e) is only slightly less than on the other Fe ions (3.2–2.4 e in absolute terms). A full investigation of all 35 BS states showed that BS10-147 indeed is the most stable BS state. The structure with the substrate directed to the S5A side is 41 kJ/mol less stable. The structure with the substrate directed to the S5A side is 41 kJ/mol less stable.
239 kJ/mol less stable than Fe6-thNNH, structures with cis-
HNNH bound end-on to either Fe2 or Fe6 are 35–182 kJ/mol less stable and structures with NNH$_2$ bound to Fe2 are 66–
255 kJ/mol less stable.

We also considered structures with S2B dissociated from either Fe2 or Fe6 (but still bound to the other ion), because such structures have been suggested to be competitive by other authors.[35,42,48,58] However, with our methods, such structures were always high in energy, by 108–255 kJ/mol for structures with NNH$_2$ bridging Fe2 and Fe6 and by 152–204 kJ/mol for structures with cis-HNNH bridging Fe2 and Fe6.

The present results are somewhat different from those obtained in our previous study of the binding of N$_2$H$_2$ to the FeMo cluster,[46] in that study, we found that the structure with trans-HNNH bound to Fe2 was 10 kJ/mol more stable than the Fe6-thNNH state and 9 kJ/mol more stable than the Fe6-HNNH$_2$ state (19 and 3 kJ/mol with the larger def2-TZVPD basis set). The difference is most likely connected to the larger QM system used in the present study (models of Val-70, Gln-191 and Phe-
381, all situated around the binding site, were not included in the previous study). The present results should be more reliable.

The results also differ from those obtained with a dissociated S2B ligand (and a rotated conformation of Gln
191),[36] for which a structure with NNH$_2$ bridging Fe2 and Fe6 was found to be most favourable. Such structures were at least 108 kJ/mol less stable than Fe6-thNNH in this study and led to half-dissociation of S2B. Clearly, the active site with a bridging S2B group is so crowded that it disfavours structures with N$_2$H$_2$ simultaneously bridging Fe2 and Fe6.

Bjornsson and coworkers studied structures with trans-
HNNH bound to the FeMo cluster in the E$_4$ state.[42] They also found that binding to Fe6 was more favourable than to Fe2, in agreement with our results, but the difference was larger, 69 kJ/mol. In their models, S5A was protonated and one of the protons on HNNH was abstracted from homocitrate.

**E$_5$ structures**

Next, we added an electron and a proton to the FeMo cluster (i.e. to the QM system) to obtain structures at the E$_5$ level. They were studied in the triplet state with BS10-147. The structures are described in Table 2 and the best are shown in Figures 3 and S2. As for the E$_4$ structures, HIE structures were always more stable than the corresponding HID structures, by 16–
173 kJ/mol.

The most favourable state has H$_2$NNH$_2$ (hydrazine) bound to Fe6, where the extra proton is abstracted from the hydroxy group of homocitrate (Figure 3a). The Fe6-N distance is 2.09 Å and the N–N bond length is 1.43 Å, which is the same as for isolated hydrazine, optimised at the same level of theory. The non-coordinating NH$_2$ group is directed to the S3A side of the cluster. The two H atoms of the Fe-bound NH$_2$ group forms hydrogen bonds to O7 of homocitrate (H···O7 = 1.94 Å) and S3B (H···S3B = 2.73 Å). The other two H atoms of hydrazine point towards O1 of homocitrate (H···O1 = 2.66 Å) and S2B (H···S2B = 2.34 Å). The spin density on Fe6 is 2.1 e (Table S3). The singlet state was 16 kJ/mol more stable than the triplet state with TPSS, but 38 kJ/mol less stable with B3LYP. The quintet was 4–
33 kJ/mol less stable than the triplet. A full investigation of all BS states (Table S2) showed that BS7-235 is actually 9 kJ/mol lower in energy than BS10-147. In fact, nine different BS states were found within 11 kJ/mol of the lowest state. There are several structures with similar energies with slight variations in the hydrogen-bond lengths and the relative conformations of the two NH$_2$ groups. For example, a structure with the non-
bonded NH$_2$ group directed towards SSA is only 1 kJ/mol less stable (6 kJ/mol more stable by B3LYP, but 38 kJ/mol less stable with relaxed surroundings).

Other structures are appreciably less stable. The second-
best structure had HNNH$_2$ bound end-on to Fe6 with the NH group and with the NH$_3$ group directed toward SSA (Figure 3b; again with a proton abstracted from homocitrate). It is 54–
68 kJ/mol less stable than the Fe6-H$_2$NNH$_2$ structure at the

**Figure 3.** The best E$_5$ structures: (a) Fe6-H$_2$NNH$_2$(3) and (b) Fe6-HNNH$_2$(5) both with the HIE state of His-195.
various levels of theory. A similar structure with the NH$_3$ group directed towards S3A is 10–18 kJ/mol less stable. A structure with HNNH$_2$ bound end-on to Fe6 with the NH group (i.e. without the proton transfer from homocitrate) is 66–84 kJ/mol less stable than the Fe6-HNNH$_2$ structure. A structure with HNNH$_2$ bound to Fe2 with the NH$_2$ group directed towards S3A is 54–94 kJ/mol less stable than the best structure. The structure with the NH$_2$ group pointing in the opposite direction is 50 kJ/mol less stable. Structures with NNH$_3$ bound to Fe2 are 184–196 kJ/mol less stable than the best Fe6-HNNH$_3$ structure.

We also studied a number of structures with HNNH$_2$ or NNH$_3$ bridging Fe2 and Fe6 on either side of S2B, but all of them were high in energy (182–471 kJ/mol less favourable than Fe6-HNNH$_3$). Moreover, we studied structures with a proton on His-195, instead of on the substrate, giving the HIP state. These structures were at least 233–430 kJ/mol higher than Fe6-H$_2$NNH-HIE, with Fe6-HNNH$_2$ lowest, 6 kJ/mol lower than Fe6-HNNH. This may illustrate a possible path for the transfer of protons to the substrate and it is apparently strongly downhill.

For the best Fe6-HNNH$_3$ structure, we tested to cleave the N-N bond. However, this reaction turned out to prohibitive with an activation barrier of 119 kJ/mol and a reaction energy of 78 kJ/mol to a product with NH bound to Fe6 and NH$_3$ dissociated from the cluster, but forming hydrogen bonds to O1 of homocitrate, S2B and S3B. This indicates that nitrogenase does not follow a sequential reaction mechanism. On the other hand, the N-N bond can be cleaved in Fe2-NNH$_3$, with a barrier of 49 kJ/mol, but the reactant and the Fe2-N product are 196 and 149 kJ/mol less stable than the Fe6-H$_2$NNH$_2$ structure, showing that they are not expected to form during the reaction mechanism.

| Structure | His | BS | TP | B3 | TZ | Rlx | N-N | Fe-N |
|-----------|-----|----|----|----|----|-----|-----|-----|
| Fe2-HNNH$_3$ (3) | HID | 147 | 344 | 344 | 1.45 | 1.86 |
| Fe2-HNNH$_3$ (5) | HIE | 147 | 344 | 334 | 1.45 | 1.86 |

Table 2. Energies (in kJ/mol), N-N and Fe-N distances (in Å) of the various structures of the E$_5$ states. All states were studied in the S=1 state unless otherwise stated. The entries are the same as in Table 1.

For the best Fe6-HNNH$_3$ structure, we tested to cleave the N-N bond. However, this reaction turned out to prohibitive with an activation barrier of 119 kJ/mol and a reaction energy of 78 kJ/mol to a product with NH bound to Fe6 and NH$_3$ dissociated from the cluster, but forming hydrogen bonds to O1 of homocitrate, S2B and S3B. This indicates that nitrogenase does not follow a sequential reaction mechanism. On the other hand, the N-N bond can be cleaved in Fe2-NNH$_3$, with a barrier of 49 kJ/mol, but the reactant and the Fe2-N product are 196 and 149 kJ/mol less stable than the Fe6-H$_2$NNH$_2$ structure, showing that they are not expected to form during the reaction mechanism.
**E₆ structures**

Adding a proton and an electron to the previous structures gives intermediates at the E₆ level. These were studied primarily in the doublet BS10-147 state. The results are collected in Table 3 and the best structures are shown in Figures 4 and S3. As for the E₄ and E₅ structures, HIE protonation was found also to be 1–138 kJ/mol more favourable than HID, except for Fe₂/₆(3)-H₂NNH₂ with TPSS (2 kJ/mol), where His-195 accepts a hydrogen bond from H₂NNH₂ in the HID state.

Table 3. Energies (in kJ/mol), N–N and Fe–N distances (in Å) of the various structures of the E₆ states. All states were studied in the S = 1/2 state unless otherwise stated. The entries are the same as in Table 1.

| Structure                  | His | BS | TP | B3 | TZ | Rx | N–N  | Fe–N  |
|----------------------------|-----|----|----|----|----|----|------|-------|
| Fe₂-H₂NNH₂(3)              | HID | 147| 163| 163| 14.3| 2.10|
| Fe₂-H₂NNH₂(5)              | HIE | 147| 17 | 33 | 29 | 31 | 28   | 1.43  | 2.11  |
| Fe₆-H₂NNH₂(3)              | HID | 147| 166| 161| 162| 1.32| 2.11 |
| Fe₆-H₂NNH₂(5)              | HIE | 147| 60 | 55 | 54 | 1.43| 2.01 |
| Fe₆-H₂NNH₂(3)              | HIE | 147| 83 | 84 | 1.45| 2.10|
| Fe₆-H₂NNH₂(5)              | HIE | 147| 0  | 0  | 0  | 0  | 0    | 1.45  | 2.11  |
|                           | 235 | 51 | 9  | 1.45| 2.05|
| Fe₆-H₂NNH₂(3)              | S = 3/2 | 147| 43 | 38 | 1.45| 2.27|
| Fe₆-H₂NNH₂(5)              | S = 3/2 | 147| 16 | 16 | 1.45| 2.12|
| Fe₂/₆-H₆-NNH₂(3)           | HID | 147| 93 | 87 | 1.45| 2.15|
| Fe₂/₆-H₆-NNH₂(5)           | HIE | 147| 2  | 9  | 1.45| 2.15|
| Fe₂/₆-H₆-NNH₂(3)           | HIE | 147| 17 | 18 | 1.45| 2.09|
| Fe₂/₆-H₆-NNH₂(5)           | HIE | 147| 226| 76 | 1.47| 2.12,2.02|
| Fe₂-H₂NNH₂(3)              | HID | 235| 323| 197| 1.45| 2.36,2.06|
| Fe₂-H₂NNH₂(5)              | HIE | 147| 109| 82 | 1.45| 2.07|
| Fe₂-H₂NNH₂(3)              | HIE | 147| 282| 246| 1.44| 2.13|
| Fe₂-H₂NNH₂(5)              | HIE | 147| 150| 108| 1.44| 2.09|
| Fe₆-H₂NNH₂(3)              | HID | 147| 243| 197| 1.44| 2.02|
| Fe₆-H₂NNH₂(5)              | HIE | 147| 105| 108| 1.44| 2.02|
| Fe₆-H₂NNH₂(3)              | HIE | 147| 200| 204| 1.44| 2.11|
| Fe₆-H₂NNH₂(5)              | HIE | 147| 119| 122| 1.44| 2.11|
| Fe₂/₆-H₆-NH₃(3) + NH₂      | HIE | 147| 232| 179| 1.44| 2.08,1.88|
| Fe₂/₆-H₆-NH₃(5) + NH₂      | HIE | 147| 194| 194| 1.44| 2.11|
| Fe₆-H₂NNH₂(3)              | HIE | 147| 309| 286| 1.44| 2.11|
| Fe₆-H₂NNH₂(5)              | HIE | 147| 265| 285| 1.44| 2.11|
| Fe₆-H₂NNH₂(3)              | HIE | 147| 110| 116| 1.44| 2.11|
| Fe₆-H₂NNH₂(5)              | HIE | 147| 78 | 31 | 1.44| 2.11|
| Fe₂-H₂NNH₂(3)              | HIP | 147| 324| 345| 1.37| 1.96|
| Fe₂-H₂NNH₂(5)              | HIP | 147| 478| 547| 1.45| 1.95,2.01|
| Fe₂-H₂NNH₂(3)              | HIP | 147| 445| 501| 1.46| 1.97,1.99|
| Fe₂-H₂NNH₂(5)              | HIP | 147| 285| 249| 1.42| 1.89|
| Fe₆-H₂NNH₂(3)              | HIP | 147| 156| 167| 1.44| 2.09|
| Fe₆-H₂NNH₂(5)              | HIP | 147| 164| 169| 1.45| 2.15|
| Fe₂-NH₂                   | HIP | 147| 409| 409| 1.45| 2.05|
| Fe₂-NH₂                   | HIP | 147| 502| 537| 1.47| 1.91,1.91|
| Fe₆-H₂NNH₂(3)              | HIP | 235| 469| 533| 1.46| 1.91,1.92|
| Fe₆-H₂NNH₂(5)              | HIP | 147| 254| 267| 1.45| 1.99|

[a] A structure with a H–N–N–H torsion of 98–99°.
The best structure at the TPSS level has $\text{H}_2\text{NNH}_2$ bound end-on to Fe6, with the non-coordinating NH$_3$ group pointing towards S3A (thus, the new proton in the E$_2$ state is added to homocitrate and not the substrate; Figure 4a). It has a Fe6-N distance of 2.10 Å and a N-N bond length of 1.45 Å, which is slightly longer than for the E$_3$ structures and the same molecule optimised in vacuum, 1.43 Å. The Fe ions have spin populations of 2.2–3.2 e, but 2.0 e on Fe2 and 1.4 e on Fe6 (Table S4). The spin on Mo is minor and slightly positive, 0.1 e. It is stabilised by a hydrogen bond from the Fe6-bound NH$_3$ group pointing to the hydroxy O7 atom of homocitrate (H···O7 = 2.25 Å) and by a hydrogen bond from the other NH$_3$ group to S2B (H···S2B = 2.31 Å). The other H atom of the latter NH$_3$ group points in the direction of S1B, but the distance is long, 3.02 Å. It is also 2.94 Å from O1 of homocitrate. The fourth H atom of the substrate is 2.73 Å from S3B, but the geometry is far from ideal. It is also close to a methyl group of Val-70 (1.83 Å H–H distance).

The quartet state was 16 kJ/mol less stable at the TPSS level, but 53 kJ/mol more stable with B3LYP. An investigation of all BS states (Table S2) showed that BS10-147 is most stable, 6 kJ/mol better than BS10-135. With B3LYP, a structure in which the two NH$_3$ groups are twisted with respect to each other (H–N–N–H torsion of 99°), as in the structure for the free hydrazine, is 38 kJ/mol more stable than the structure in Figure 4a, but at the TPSS level, the other structure is 43 kJ/mol more stable.

The corresponding structure with the non-coordinating NH$_3$ group pointing towards S5A (Figure 4b) is only 2–19 kJ/mol less stable with TPSS but 33 kJ/mol more stable B3LYP. It is stabilised by a hydrogen bond to the O1 atom of homocitrate (H···O1 = 2.04 Å), whereas the other H atoms interact with S2B, S3B and S1B as for the other conformation (H–S distances of 2.35, 3.13 and 2.79 Å). With B3LYP, this structure is further stabilised by 62 kJ/mol when studied in the BS7-235 state, whereas with TPSS the BS10-147 state is 48 kJ/mol more stable.

The corresponding structure with hydrazine bound end-on to Fe2 (Figure 4c) is 17–33 kJ/mol less stable. The two H atoms of the NH$_3$ group bound to Fe2 form hydrogen bonds to SG of Cys275 (H···SG = 2.43 Å) and S2B (H···S2B = 2.84 Å). The other two H atoms interact with ND1 of His-195 and S1A (H···ND1 = 2.53 and H···S1A = 3.15 Å), but with poor geometries. A full investigation of all BS states (Table S2) showed that this structure is most stable in the BS6-157 state, which is 16 kJ/mol more stable than the BS10-147 state. End-on bound HNNH$_2$ structures are 105–282 kJ/mol less stable, whereas side-on (Fe2/6) structures are 117–309 kJ/mol less stable and those with HNNH$_2$ dissociate to NH and NH$_3$.

We have also studied the same structures as for E$_3$, but with HIP and an extra electron. They were all high in energy, 156–547 kJ/mol less stable than the best Fe6-H$_2$NNH$_2$ structure, showing that proton transfer from His-195 is strongly favourable. In several of this type of structures, the substrate automatically abstracts a proton from His-195, forming HID states instead (which are less stable than the HIE structures, as has already been discussed).

$E_2$ structures

After adding yet another proton and electron, we reach the $E_2$ level intermediates. They were studied in the triplet BS10-147 state. The structures are listed in Table 4 and the most stable structures are shown in Figures S5 and S4. As usual, the HID structures were less stable than the corresponding HIE structures by 3–176 kJ/mol.

Only a few structures were obtained with H$_2$NNH$_2$ bound to the FeMo cluster. They had a N–N distance of 1.42–1.43 Å. The most stable one had H$_2$NNH$_2$ bound end-on to Fe6, with the NH$_3$ directed towards the S5A side (Figure 5a), but binding to Fe2 was only 4 kJ/mol less favourable with TPSS (30 kJ/mol by B3LYP). An investigation of all BS states (Table S2) showed that this complex is most stable in the BS2-234 state, which is actually 36 kJ/mol more stable than the BS10-147 state. The N–N bond can readily be cleaved in this structure with an activation barrier of only 32 kJ/mol and an exothermic reaction energy of −154 kJ/mol. The product has NH$_3$ bound end-on to Fe6 and NH$_3$ dissociated, but hydrogen bonded to the cluster (Figure 5b). The two H atoms of NH$_3$ form hydrogen bonds to O1 of homocitrate (H···O1 = 2.47 Å) and S2B (H···S2B = 2.53 Å). The dissociated NH$_3$ molecule forms hydrogen bonds to NH$_3$ (H···N = 2.06 Å) and O2 of homocitrate (H···O2 = 2.23 Å), whereas the third H atom does not form any favourable interaction, but instead is quite close to a methyl group of Val-70 (2.03 Å H–H distance). A structure with H$_2$NNH$_2$ dissociated from the Fe ions, but still hydrogen-bonded to the cluster is 83 kJ/mol more stable than the bound Fe6-H$_2$NNH$_2$ structure (Figure 5c). When it is dissociated, it is appreciably harder to cleave the N–N bond – the calculated barrier is 91 kJ/mol.

Structures with a cleaved N–N bond and both NH$_3$ and NH$_3$ coordinated to the cluster are up to 84 kJ/mol more stable than the bound Fe6-H$_2$NNH$_2$ structure, but 21 kJ/mol less stable than the structure with NH$_3$ dissociated. The best one has NH$_3$ bridging Fe2 and Fe6 on the side facing S5A, whereas NH$_3$ binds to Fe6 (Figure 5d). The two Fe–NH$_3$ distances are 1.93 and 1.95 Å, whereas the Fe6–NH$_3$ distance is 2.27 Å. S2B has moved considerably, but it still binds to Fe2 and Fe6, and it receives a hydrogen bond from His-195 (H···S2B = 2.36 Å). NH$_3$ forms a hydrogen bond to O1 of homocitrate (H···O1 = 2.00 Å), whereas the second H atom points towards S2B (H···S2B = 2.65 Å). The third H atom does not form any favourable interactions. The two H atoms of NH$_3$ point towards S3A and S2B (H···S3A = 3.21 and H···S2B = 2.63 Å). The spin populations on Fe2 and Fe6 are relatively low, 2.1 and 2.2 e, respectively, but that on Fe7 is even lower, 1.5 e (Table S5). NH$_3$ may dissociate from this structure, but the activation barrier is rather high, 78 kJ/mol.

There are several other structures with comparable energies (cf. Table 4), for example with NH$_3$ bridging Fe2 and Fe6 on either side of S2B and with NH$_3$ either on Fe2 or Fe6. The relative energies sometimes differ rather much between TPSS and B3LYP.

As for the other $E_3$ states, we tested also structures with HIP protonated, but all these were at least 183 kJ/mol less stable.
E3–E8 structures with only one N atom

We studied also structures with only a single N atom, i.e. after N–N bond cleavage and dissociation of a NH2 product. These were studied at four levels of oxidation and protonation (E3–E8), even if the results in the previous subsections indicate that only the E3 and E6 states are involved in the reaction mechanism. The results are collected in Table 5 and the best structures are shown in Figures 6, 7 and S5.

The best E3 structure has N bound end-on to Fe6 with a Fe–N distance of 1.60 Å (Figure 6a). The N atom receives a hydrogen bond from HE1 of Gln-191 (2.55 Å, but this hydrogen also forms a hydrogen bond to O1 of homocitrate with a H–O1 distance of 2.40 Å). We tested also the singlet and quartet states for this structure. The latter was 38 kJ/mol less stable at the TPSS level (33 kJ/mol with B3LYP). However, the singlet was 39 kJ/mol more stable than TPSS, but 11 kJ/mol less stable with B3LYP. A structure with NH bound to Fe6 (with the proton abstracted from homocitrate) is only 2 kJ/mol less stable (38 kJ/mol by B3LYP), but it is 14 kJ/mol more stable with the larger basis set and 9 kJ/mol more stable if the surroundings are relaxed.

The corresponding structure with N bound end-on to Fe2 (Figure 6b) is also 2 kJ/mol less stable (35 kJ/mol with B3LYP), but 1 kJ/mol more stable with the larger basis set, 41 kJ/mol more stable if the surroundings are relaxed. It has an even shorter Fe2–N bond length of 1.54 Å. The N atom does not receive any polar hydrogen bond, but it is 2.00 Å from a HB of Ser-278. The corresponding structures with N bridging Fe2 and Fe6 are 30 kJ/mol (on the S3A side) and 57 kJ/mol (S5A side) less stable. In both cases, S2B moves to a position where it interacts with more Fe ions than Fe2 and Fe6. Moreover, N receives the hydrogen bond from His-195 (instead of S2B; 2.44 and 2.50 Å, respectively). The corresponding HID structures are 78–126 kJ/mol less stable. Interestingly, for the three most stable structures B57-235 was found to be 5–37 kJ/mol more stable.

Table 4. Energies (in kJ/mol) of NH–Fe distances (in Å) of the various structures of the Ei states. All states were studied in the S=1 state unless otherwise stated. The entries are the same as in Table 1. Fe–N distances of NH3 precede those of NH2.

| Structure | His | BS | TP | B3 | TZ | Rx | N-N | Fe-N |
|-----------|-----|----|----|----|----|----|------|------|
| Fe2-H,NNH(3) | HID | 147 | 277 | 297 | 1.42 | 2.14 |
|           | HIE | 147 | 137 | 162 | 1.43 | 2.05 |
| Fe2-H,NNH(5) | HID | 147 | 248 | 256 | 1.43 | 2.17 |
|           | HIE | 147 | 143 | 159 | 1.43 | 2.14 |
| Fe6-H,NNH(3) | HID | 147 | 193 | 216 | 1.43 | 2.28 |
|           | HIE | 147 | 217 | 237 | 1.43 | 2.26 |
| Fe6-H,NNH(5) | HID | 147 | 133 | -12 | 28 | 132 | 1.43 | 2.38 |
|           | HIE | 147 | 97 | 1.42 | 2.43 |

We studied also structures with only a single N atom, i.e. after N–N bond cleavage and dissociation of a NH2 product. These were studied at four levels of oxidation and protonation (E3–E8), even if the results in the previous subsections indicate that only the E3 and E6 states are involved in the reaction mechanism. The results are collected in Table 5 and the best structures are shown in Figures 6, 7 and S5.

The best E3 structure has N bound end-on to Fe6 with a Fe–N distance of 1.60 Å (Figure 6a). The N atom receives a hydrogen bond from HE1 of Gln-191 (2.55 Å, but this hydrogen also forms a hydrogen bond to O1 of homocitrate with a H–O1 distance of 2.40 Å). We tested also the singlet and quartet states for this structure. The latter was 38 kJ/mol less stable at the TPSS level (33 kJ/mol with B3LYP). However, the singlet was 39 kJ/mol more stable than TPSS, but 11 kJ/mol less stable with B3LYP. A structure with NH bound to Fe6 (with the proton abstracted from homocitrate) is only 2 kJ/mol less stable (38 kJ/mol by B3LYP), but it is 14 kJ/mol more stable with the larger basis set and 9 kJ/mol more stable if the surroundings are relaxed.

The corresponding structure with N bound end-on to Fe2 (Figure 6b) is also 2 kJ/mol less stable (35 kJ/mol with B3LYP), but 1 kJ/mol more stable with the larger basis set, 41 kJ/mol more stable if the surroundings are relaxed. It has an even shorter Fe2–N bond length of 1.54 Å. The N atom does not receive any polar hydrogen bond, but it is 2.00 Å from a HB of Ser-278. The corresponding structures with N bridging Fe2 and Fe6 are 30 kJ/mol (on the S3A side) and 57 kJ/mol (S5A side) less stable. In both cases, S2B moves to a position where it interacts with more Fe ions than Fe2 and Fe6. Moreover, N receives the hydrogen bond from His-195 (instead of S2B; 2.44 and 2.50 Å, respectively). The corresponding HID structures are 78–126 kJ/mol less stable. Interestingly, for the three most stable structures B57-235 was found to be 5–37 kJ/mol more stable.
stable than the BS10-147 state. However, the Fe6-N structure was still the best structure.

The most stable E6 structure has NH2 bound end-on to Fe6 with the extra proton on the substrate abstracted from homocitrate (Figure 6c). It has a Fe6–N bond length of 1.85 Å and the H atoms of NH2 point toward O1 and O7 of homocitrate (H···O1 = 2.01 Å, H···O7 = 2.17 Å) and S2B (H···S2B = 2.59 Å). The spin population on Fe6 (2.0 e) is slightly lower than that on Fe2 (2.1 e; Table S6). This structure is 126 kJ/mol more stable (63–90 kJ/mol with B3LYP, the larger basis set or relaxed surroundings) than a structure with NH binding end-on to Fe6 (Fe6–N bond length of 1.73 Å). The corresponding HID structures are 51–221 kJ/mol higher in energy. Structures with HIP are 305–373 kJ/mol higher in energy, showing that proton transfer from His-195 is favourable.

The most stable E7 structure has NH3 bound end-on to Fe6 (with a proton abstracted from homocitrate). The Fe6–N bond length is 2.06 Å (Figure 6d). One of the three H atoms forms hydrogen bonds to both O1 and O7 of homocitrate (H···O1 = 2.01 Å and H···O7 = 2.03 Å). The other two are rather close to S2B (H···S2B = 2.76 and 2.86 Å), but also to S1B and S3B (H···S1B = 2.96 Å and H···S3B = 3.09 Å). The spin population of Fe6 is 2.1 e (Table S6). NH3 cannot dissociate from this structure (the energy keeps rising by more than 120 kJ/mol when the Fe6–N bond is elongated). A full investigation of all BS states (Table S2) showed that BS6-156 is actually lowest, but the BS10-147 state is only 8 kJ/mol higher in energy and there are five BS states within 10 kJ/mol of BS6-156.

This Fe6-NH3 structure is 99–113 kJ/mol more stable than a structure with NH2 bound to Fe6 (and a proton on homocitrate). The structure with NH2 bound to Fe2 is 95–119 kJ/mol less stable. Structures with NH2 bridging Fe2 and Fe6 are 191–350 kJ/mol higher in energy than the best structure. Structures with HID are ~90 kJ/mol less stable than the corresponding HIE structures. Structures with HIP are 281–418 kJ/mol less stable, again indicating that proton transfer from His-195 is strongly favourable.

Finally, we studied also E8 states. Again, the most stable structure has NH3 bound end-on to Fe6 (with a proton abstracted from homocitrate). The Fe6–N bond length is 2.06 Å (Figure 6d). One of the three H atoms forms hydrogen bonds to both O1 and O7 of homocitrate (H···O1 = 2.01 Å and H···O7 = 2.03 Å). The other two are rather close to S2B (H···S2B = 2.76 and 2.86 Å), but also to S1B and S3B (H···S1B = 2.96 Å and H···S3B = 3.09 Å). The spin population of Fe6 is 2.1 e (Table S6). NH3 cannot dissociate from this structure (the energy keeps rising by more than 120 kJ/mol when the Fe6–N bond is elongated). A full investigation of all BS states (Table S2) showed that BS6-156 is actually lowest, but the BS10-147 state is only 8 kJ/mol higher in energy and there are five BS states within 10 kJ/mol of BS6-156.

This Fe6-NH3 structure is 99–113 kJ/mol more stable than a structure with NH2 bound to Fe6 (and a proton on homocitrate). The structure with NH2 bound to Fe2 is 95–119 kJ/mol less stable. Structures with NH2 bridging Fe2 and Fe6 are 191–350 kJ/mol higher in energy than the best structure. Structures with HID are ~90 kJ/mol less stable than the corresponding HIE structures. Structures with HIP are 281–418 kJ/mol less stable, again indicating that proton transfer from His-195 is strongly favourable.
favourable interactions. The Fe spin populations are 2.2–3.2 e, but 2.0 e on Fe6 (Table S6). The structure was studied in the doublet state and the corresponding quartet state is 23 kJ/mol less stable with TPSS, but 35 kJ/mol more stable with B3LYP. A

| Table 5. Energies (in kJ/mol), N–N and Fe–N distances (in Å) of the various structures of the E5-E8 states with a single N atom. All states were studied in the S = 1 (E5 or E7) or S = ½ (E6 or E8) state unless otherwise stated. The entries are the same as in Table 1. |
|-------------------------------------------------|-------|---|---|---|---|---|---|
| State   | Structure   | His | BS | TP | B3 | TZ | Rlx |
| E5      | Fe2-N       | 147 | 124 | 157 | 1.53 |
|         |             |     | 147 | 2  | 35  | –1 | –41 | 1.54 |
|         |             |     |     | 235 | –5  | 87 | 1.52 |
|         | Fe6-N       | 147 | 126 | 126 | 1.60 |
|         |             |     |     | 147 | 0  | 9  | 0  | 0  | 1.60 |
|         |             |     |     | 235 | –37 | –13 | 1.52 |
|         | S = 0       | 147 | –39 | 11  | 1.52 |
|         | S = 2       | 147 | 38  | 33  | 1.60 |
|         | Fe2/6-N(3)  | 147 | 155 | 309 | 1.69,1.79 |
|         |             |     |     | 235 | 134 | 256 | 1.71,1.72 |
|         | Fe2/6-N(5)  | 43  | 111 | 302 | 1.69,1.81 |
|         |             |     |     | 147 | 57  | 23  | 1.71,1.81 |
| Fe6-N   |             | 147 | 117 | 16  | 1.77 |
|         |             |     |     | 147 | 2  | 38  | –14 | –9  | 1.72 |
|         |             |     |     | 235 | –17 | 28  | 1.62 |
| E6      | Fe2-N       | 147 | 226 | 199 | 1.85 |
|         |             |     |     | 147 | 132 | 118 | 139 | 69  | 1.83 |
|         | Fe6-N       | 147 | 204 | 196 | 1.89 |
|         |             |     |     | 147 | 126 | 90  | 131 | 69  | 1.79 |
|         | S = 3/2     | 147 | 154 | 133 | 1.77 |
|         | Fe2/6-NH(3) | 147 | 239 | 328 | 1.80,1.87 |
|         |             |     |     | 147 | 129 | 107 | 149 | 79  | 1.84,1.86 |
|         | Fe2/6-NH(5) | 147 | 124 | 167 | 1.86,1.87 |
|         |             |     |     | 147 | 138 | 188 | 175 | 116 | 1.85,1.89 |
| Fe6-NH2 |             | 147 | 86  | 113 | 1.86 |
|         |             |     |     | 147 | 0  | 0  | 0  | 0  | 1.85 |
|         |             |     |     | 235 | 26  | –48 | 1.84 |
| E7      | Fe2-N       | 147 | 305 | 273 | 1.53 |
|         |             |     |     | 147 | 373 | 288 | 1.72,1.85 |
|         | Fe6-N       | 147 | 319 | 296 | 1.78 |
|         | Fe2-NH2     | 147 | 207 | 201 | 1.89 |
|         |             |     |     | 147 | 117 | 107 | 119 | 95  | 1.87 |
|         | Fe6-NH3     | 147 | 200 | 195 | 1.86 |
|         |             |     |     | 147 | 112 | 109 | 113 | 99  | 1.85 |
|         | S = 0       | 147 | 118 | 150 | 1.87 |
|         | S = 2       | 147 | 149 | 101 | 1.85 |
|         | Fe2/6-NH(3) | 147 | 242 | 213 | 1.97,1.96 |
|         |             |     |     | 147 | 224 | 240 | 1.98,2.03 |
|         | Fe2/6-NH(5) | 147 | 281 | 350 | 1.94,1.95 |
|         |             |     |     | 147 | 191 | 263 | 1.92,1.94 |
| Fe6-NH4 |             | 147 | 89  | 85  | 2.06 |
|         |             |     |     | 147 | 0  | 0  | 0  | 0  | 2.06 |
|         |             |     |     | 156 | –8  | 2.09 |
|         | S = 0       | 147 | –13 | 47  | 2.08 |
|         | S = 2       | 147 | 33  | –37 | 2.06 |
|         | Fe6-NH4     | 147 | 418 | 394 | 1.82 |
|         | Fe6-NH4     | 147 | 283 | 281 | 1.82 |
| Fe2/6-NH4 |             | 147 | 126 | 128 | 2.06 |
|         |             |     |     | 147 | 11  | 8  | 14  | –28 | 2.06 |
|         |             |     |     | 235 | 35  | –2  | 2.13 |
| Fe6-NH4 |             | 147 | 88  | 81  | 2.12 |
|         |             |     |     | 147 | 0  | 0  | 0  | 0  | 2.13 |
|         |             |     |     | 235 | 17  | 12  | 2.07 |
|         | S = 3/2     | 147 | 23  | –35 | 2.20 |
|         | Fe2/6-NH(3) | 147 | 326 | 336 | 2.09,2.51 |
|         |             |     |     | 147 | 324 | 301 | 2.11,2.34 |
| Fe6-NH4 |             | 147 | 273 | 273 | 2.10,3.14 |
|         | NH4 dissociated | 147 | 88  | 74  | 3.04 |
|         |             |     |     | 147 | –2  | 6  | 1  | –32 | 3.03 |
|         |             |     |     | 235 | 17  | –20 | 3.09 |
| Fe6-NH4 |             | 147 | 292 | 228 | 1.88 |
|         | Fe6-NH4     | 147 | 152 | 146 | 2.06 |
full investigation of all possible BS states (Table S2) showed that the BS10-147 state is best, but there are four other BS states within 10 kJ/mol. NH$_3$ can dissociate from this structure with a barrier of 42 kJ/mol. The dissociation energy of NH$_3$ (compared to the quartet BS7-235 $E_0$ state and NH$_3$ in a water-like continuum solvent with a dielectric constant of 80) is 16 kJ/mol (−20 kJ/mol with B3LYP), which can easily be overcome by the gain in translational and rotational entropy of the released NH$_3$ ligand, ~60 kJ/mol$^{[92,93]}$.

A structure, in which NH$_3$ has abstracted the proton from O7 of homocitrate, forming NH$_4^+$, is actually 2 kJ/mol more stable than the Fe6-NH$_3$ structure. NH$_4^+$ has dissociated from Fe6 and the four H atoms form hydrogen bonds to O1 of...
homocitrate (H--O1 = 1.43 Å), S2B (H--S2B = 2.80 Å), S1B (H--S1B = 2.19 Å) and S3B (H--S3B = 2.56 Å; Figure 7b).

A structure with NH₃ bound to Fe2 is 8–11 kJ/mol less stable than the Fe6-NH₃ structure (but 28 kJ/mol more stable with relaxed surroundings). It has a slightly shorter Fe--N bond (2.06 Å; cf. Figure 7c). Two of the H atoms of NH₂ approximately towards SG of Cys-275 and S2B (H···SG = 2.89 Å). A full investigation of all BS states showed that BS10-147 indeed is the most stable state, but only 2 kJ/mol more stable than the BS6-157 state. We also tried to find structures with NH₄ bridging Fe2 and Fe6, but they were at least 326 kJ/mol less stable. Structures with HIP were 152–292 kJ/mol less stable, showing that proton transfer from His-195 is strongly favourable.

Conclusions

In this investigation, we have studied possible reaction intermediates of nitrogenase, assuming that the S2B remains bound to the FeMo cluster. To avoid the problem that the structure of the E₄ intermediate is not known and that different DFT functionals give very different relative stabilities of various protonation states, we started our study after H₂ has dissociated and N₂ has bound to the cluster and has become doubly protonated to N₃H₂⁺ so that no protons remain bound to the cluster. Based on the accumulated experimental evidence, as well a systematic study of the binding of N₃H₂ to the FeMo cluster, and in agreement with most previous computational studies, we have assumed that N₃H₂ binds either to Fe2 or Fe6.

Our study has led to the following conclusions:

- For the E₄ state, Fe6-THNNH, Fe2-THNNH and Fe6-HNNH₂ structures are all competitive (within 5 kJ/mol with at least one of the four levels of theory included in Table 1).
- For the E₅ state, Fe6-H₂NNH₂ is lowest in energy. Fe6-NNH₂ is 54–68 kJ/mol higher and the N--N bond in cannot be cleaved.
- For the E₆ state, Fe6-H₂NNH₂ structure is lowest in energy, 28–43 kJ/mol lower than Fe6-H₂NNH₂. Cleavage of N--N in the latter has a barrier of 95 kJ/mol.
- The N--N bond in the H₂NNH₂ E₇ complexes can easily be cleaved, the reaction is exothermic and NH₃ moves spontaneously into the second coordination sphere of the cluster, whereas NH₃ binds with similar affinities to both Fe2 and Fe6. However, the most stable structure is obtained if NH₃ abstracts the hydroxy proton from homocitrate, forming NH₃ bound to Fe6, which cannot dissociate at this level of reduction.
- In the E₈ state, NH₃ binds preferably to Fe6 (binding to Fe2 is 8–11 kJ/mol higher in energy). It can readily dissociate from the FeMo cluster.

Based on these results, we suggest the reaction mechanism in Figure 8. In this mechanism, the substrate binds to Fe6. In the E₄ state, it is protonated to H₂NNH₂ whereas in E₅ the proton is added to homocitrate, so that the ligand remains H₂NNH₂. In the E₅ state, NH₃ is lowest in energy. Fe₆-NNH₂ binds either to Fe2 or Fe6.

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Our study has led to the following conclusions:

- For the E₅ state, Fe6-H₂NNH₂, Fe2-H₂NNH₂ and Fe6-HNNH₂ structures are all competitive (within 5 kJ/mol with at least one of the four levels of theory included in Table 1).
has investigated different proton paths from the solvent to the FeMo cluster. He suggested that protons are transferred from the surface via a chain of water molecules to a water molecule close to homocitrate and the S3B atom. He has also studied how the proton may be transferred within the FeMo cluster, starting on the S3B atom and ultimately ending up close to the substrate-binding site. His-195 may also provide protons to the substrate, as has often been assumed. In fact, our calculations show that such a transfer (via the HIP state of His-195) is always downhill. However, this gives the HID state of His-195, which is nearly always unfavourable. Moreover, Dance has shown that rotation of the sidechain of His-195 is unlikely in the protein. Therefore, His-195 can probably not provide more than one proton to the substrate (at the most).

However, it should be remembered that QM studies of nitrogenase are extremely complicated. Our survey of the most stable structures at the various $E_1-E_4$ levels have indicated that several structures often have quite similar energies and that there are many conformations of the bound intermediates, depending on the direction of the non-coordinated N-group, the $H-N-N-N$ dihedral angle and the formation of hydrogen bonds and other polar interactions. Moreover, sometimes other BS states than BS10-147 are most stable and a full BS investigation of every structure is currently too demanding. This makes it harder to settle the most stable structures and it cannot be excluded that we might have overlooked some low-energy structures.

For all states, we have optimised structures with both TPSS and B3LYP. In general, the two methods give relative energies that agree reasonably. However, in some cases, differences > 50 kJ/mol are observed, without any significant differences in the geometry. This is often observed for the various spins states (B3LYP typically prefers higher spin states than TPSS, as expected). However occasionally, such differences also occur for structures with differences only in the hydrogen-bond pattern or coordination mode, making interpretation of the results harder.

For the most interesting states, we have also recalculated energies with the larger def2-TZVPD basis set. As in our previous studies, this has typically only a minor effect on the energies, < 20 kJ/mol. However, calculations with relaxed surroundings occasionally have larger effects, up to 26 kJ/mol, which may indicate that the structure need to relax more than is allowed in the rather large QM system or problems with local minima in the surroundings. However, a more detailed study of the relaxation of the surroundings (in the Supporting Information) indicates that the large energy differences are connected to major movements of water molecules and other groups far from the substrate-binding site, suggesting that it reflects more occurrences of multiple local minima, rather than important relaxation of the surroundings.

For many of the $E_n$ states, we have observed that a transfer of the hydroxyl proton of homocitrate to the $N_j$-intermediate is favourable. This indicates homocitrate may constitute a proton buffer, which can be used to stabilise certain intermediates of the reaction, especially $H_2NNH_2$ and $NH_2$. This provides an attractive explanation why homocitrate is mandatory for the function of nitrogenase. Björnsson and coworkers have also suggested that the hydroxy proton of homocitrate is employed to form trans-HNNH from $N_j$ in the $E_n$ intermediate, although the reaction was uphill.

For all $E_n$ levels, binding of the intermediates to Fe6 seems to be preferred and therefore we have suggested such binding in the mechanism in Figure 8. This site has more hydrogen-bond possibilities (besides sulfide ions), involving His-195, Glu-191 and the homocitrate ligand. It can also employ the suggested proton buffer of homocitrate and it is also closer to the end of the suggested proton-transfer path, involving a chain of water molecules, ending close to homocitrate and S3B. However, for the $E_r$, $E_s$, and $N_j$-intermediates to Fe2 are competitive. Moreover, it has been suggested that a likely $N_j$-binding channel at Fe2.

We have previously studied the reaction mechanism of nitrogenase, assuming that S2B dissociates from the FeMo cluster, opening up for an obvious binding site of the substrate. This gave rise to a mainly alternating reaction mechanism, in which the substrate and the intermediates bound in a bridging mode (with one or both N atoms) between Fe2 and Fe6. In the present study, bridging intermediates were always found to be much less stable than end-on intermediates, except for $E_s$ structures with both $NH_2$ and $NH_2$. The reason for this is that the FeMo cluster is too crowded if both the substrate and S2B bridges Fe2 and Fe6. In fact, it is often observed that S2B moves to other positions or reacts with the substrate or other sulfide ions in such high-energy structures with a bridging substrate. Thus, it seems clear that bridging substrate structures are unlikely when S2B remains bound.

In conclusion, this study shows that the second part of the nitrogenase reaction (after binding of $N_j$) is possible also if the S2B ligand has not dissociated. Such a reaction follows an alternating mechanism with the substrate and intermediates binding to Fe6. It has also pointed out an important role for the homocitrate ligand as a proton buffer. In future studies, we study the binding of $N_j$ and dissociation of $H_2$ to the $E_n$ state of the cluster.

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Conflict of Interest

The authors declare no conflict of interest.
Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: alternating mechanism • homocitrate • nitrogenase • nitrogen fixation • QM/MM

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