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The mechanism of nitrogenase: formation and release of the second NH₃ and completion of the cycle†

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The enzyme nitrogenase catalyses the reaction $\text{N}_2 + 8\text{e}^- + 8\text{H}^+ \rightarrow 2\text{NH}_3 + \text{H}_2$. Two prior papers in this series report a computed mechanism for the first and second phases of this catalysis. In the first phase H₂ is formed, the H₂/N₂ exchange occurs, and N₂ is captured in a concerted step forming the bound HNHH intermediate. The second phase breaks the N–N bond and converts this intermediate to bound NH plus the first NH₃, which dissociates. This third paper describes the final phase, which forms and then releases the second NH₃, and recovers the resting state. The mechanism is supported by density functional calculations with a 483+ atom quantum model of the active site, FeMo-co, and relevant surrounding amino acids and water. Calculated reaction trajectories and potential energy profiles generate five mechanistic pathways through this NH₃ formation phase of the reaction. These pathways are evaluated with incorporation of entropic components and possible kinetic contributions by H atom tunneling, leading to the identification of the most favourable pathway for generation of NH₃ and its subsequent dissociation. The steps regenerating the resting state and completing the mechanism cycle are described. All steps in this third and final phase of the mechanism are thermodynamically and kinetically feasible. Atom S2B of FeMo-co, whose retention during enzyme turnover is experimentally controversial, remains intact as a bridge between Fe2 and Fe6 and is an essential H transfer agent in the proposed mechanism. The architecture of the active site and its surrounds that promote the chemical choreography of nitrogenase in its performance space are outlined, and key features and principles of the proposed complete mechanism are summarised.

Received 19th March 2025

Accepted 30th May 2025

DOI: 10.1039/d5dt00658a

rsc.li/dalton

1. Introduction

Nitrogenase, the enzyme that has evolved as the natural source of nitrogen for the earth's biosphere,¹ catalyses the conversion of inert N₂ to NH₃ under ambient conditions which contrast markedly with the extreme pressure and temperature conditions required for the Haber–Bosch industrial manufacture of ammonia.² How does the enzyme achieve a mild catalysis that chemists are unable to replicate?^{3,4} Extensive experimental investigation involving reaction kinetics,^{5,6} mutations of amino acids surrounding the active site,^{7–22} vibrational and spin resonance spectroscopy,^{23–31} spectro-electrochemistry,³²

use of abiological electron sources,^{33,34} many crystal structures,^{35,36} cryo-electron-microscopy,^{4,37–39} and density functional simulations,^{40–51} still leave many aspects of the chemical mechanism unresolved. There is a fundamental experimental impediment to the isolation and direct investigation of intermediates because unavoidable protons are a substrate of the enzyme and mixtures of transient intermediates occur.^{36,52} Furthermore, the overall chemical reaction eqn (1) implies a mechanistic cycle with at least 27 steps: eight introductions of a proton, eight additions of an electron, N₂ binding, breaking the N–N bond, formation of six N–H bonds, two dissociations of NH₃ and formation of one H–H bond.



The protein domain in which this catalysis occurs is shown in Fig. 1. The active site is the iron-molybdenum cofactor, FeMo-co, a CFe₇MoS₉ cluster with homocitrate (HCA) and His442 coordination to Mo, and Cys275 coordination at Fe1: throughout this paper amino acids are numbered according to crystal structure PDB 3U7Q of Mo-nitrogenase species *Azotobacter vinelandii* (Av). Mutagen-reactivity experiments

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†Electronic supplementary information (ESI) available: Description of the protein model and the structural constraints used, including movement of Val70 and the contiguous chain, movement of Arg96, a description of the density functional procedures and their validation, a description of procedures for determination of transition states and reaction trajectories, and an additional potential energy profile. Electronic states and atomic coordinates for reactants, transition states and products are listed. See DOI: <https://doi.org/10.1039/d5dt00658a>

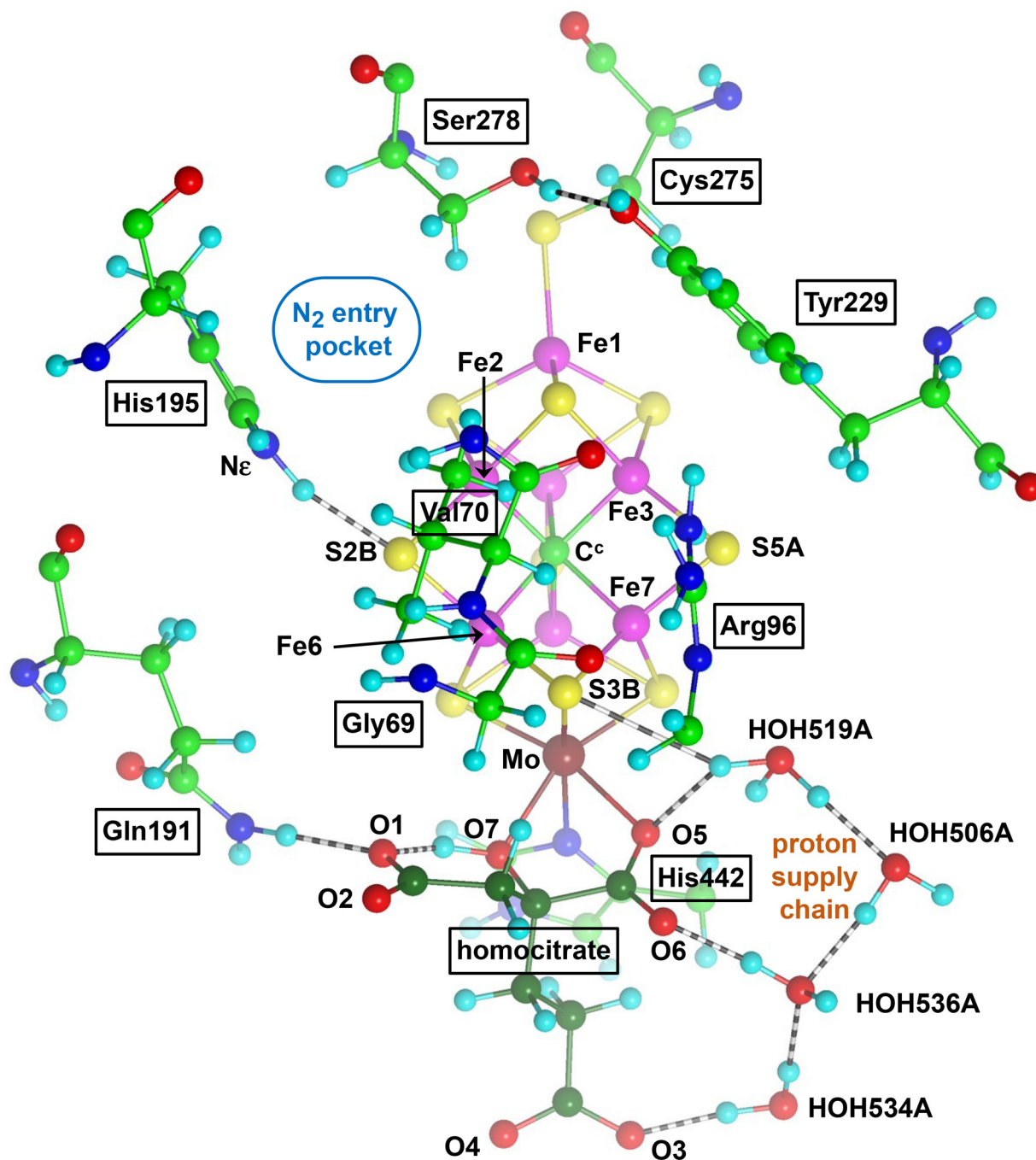


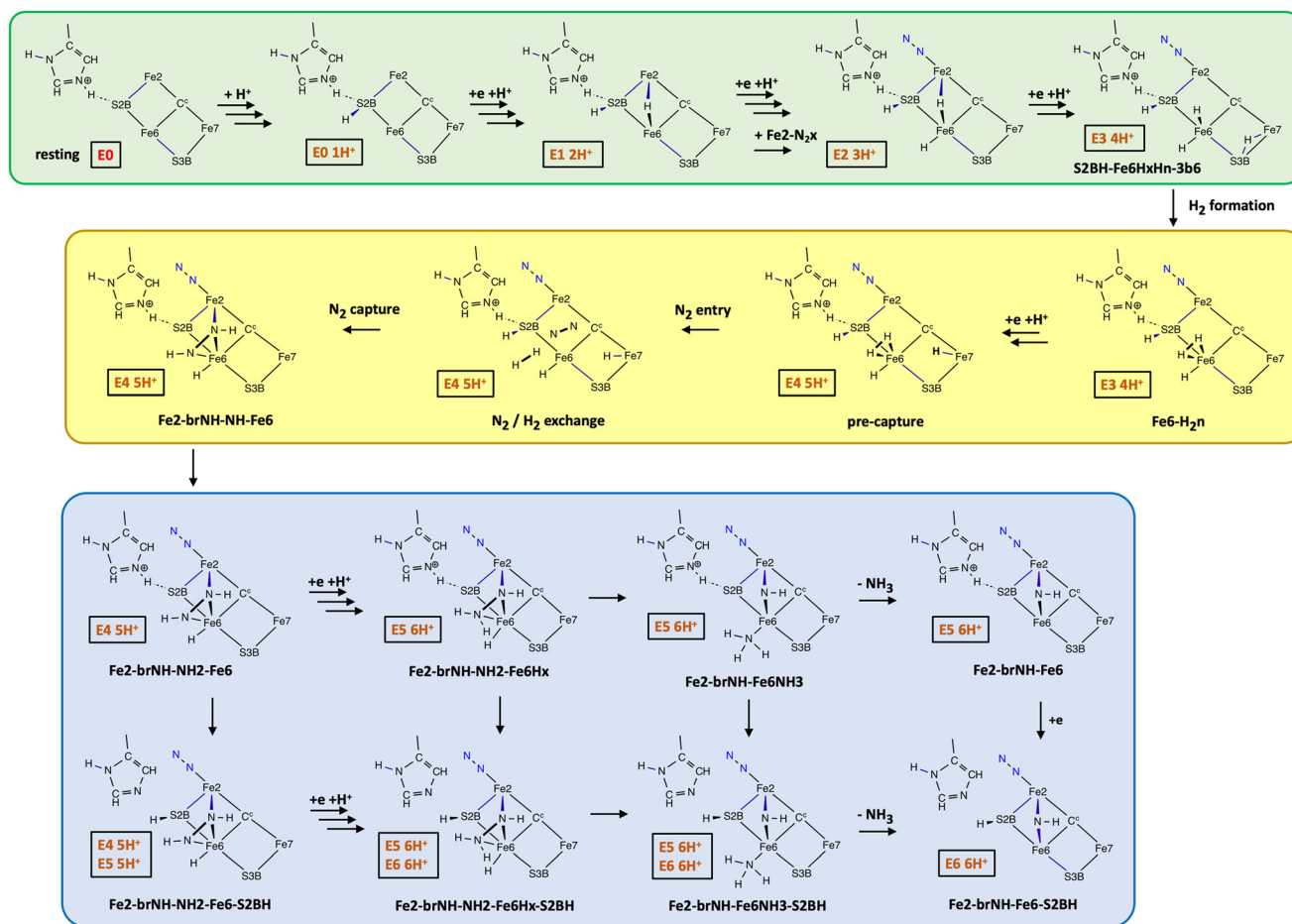
Fig. 1 Structure of the active site of Mo-nitrogenase, with FeMo-co at the centre. Significant surrounding amino acids and water molecules are included, with labels for Av1 protein, crystal PDB 3U7Q. Homocitrate C atoms are dark green, and hydrogen bonds are striped. The reaction zone is the Fe2–Fe3–Fe6–Fe7 face, under the side chain of Val70 and bounded by the side chain of Arg96. The proposed N₂ entry pocket is between His195 and Ser278, approaching Fe2. The four penultimate water molecules of the proton supply chain⁵⁸ are shown, with hydrogen bonds to O3, O6 and O5 of homocitrate, and with the final proton directed towards S3B.

show that the active domain of FeMo-co is the front face, Fe2–S2B–Fe6–S3B–Fe7 enclosed by Val70 and Arg96.^{8,10–12,17,19,53–55,56,57} There is a significant hydrogen bond between Ne of His195 and S2B. Protons are supplied along a conserved water chain, and the four penultimate water molecules of the proton supply chain are shown on Fig. 1,

with hydrogen bonds to O3, O6 and O5 of homocitrate, and the final proton directed towards S3B. Protons are translocated along the complete chain (not shown) by a Grotthuss mechanism.⁵⁸ Electrons reach FeMo-co from a separate P-cluster.^{59–61}

I have developed a complete chemical mechanism by which Mo-nitrogenase effects catalysis of the reaction in eqn (1). The





Scheme 1 Stages of the proposed mechanism preceding the stages described in the current paper. The green enclosure describes the introduction and migration of H atoms to the more stable locations on FeMo-co around the reaction domain. The yellow enclosure includes the formation of H_2 , the H_2/N_2 exchange step, and then the concerted capture of N_2 to form the intermediate with bound HNNH, **Fe2-brNH-NH-Fe6**. Then the lavender enclosure shows the pathways during which another H is added, the N–N bond breaks, NH_3 is formed, and then dissociates. The boxes show the number of added electrons and protons at each intermediate.

previously reported components of this mechanism are outlined in Scheme 1. The labelling system for intermediates includes the incorporated moieties N_2x , H, H_2 , HNNH, HNNH $_2$, NH_2 , NH_3 , and the locations where they are bound, being S2B, Fe2, Fe6, S3B. Suffixes br = bridging, x = *exo* coordination position, n = *endo* coordination position are included in the labels: Fig. 2 defines coordination positions, and the labelling of the six configuration positions for H bonded to S3B. At each intermediate in Scheme 1 the numbers of electrons and protons added beyond the resting state are shown in the red status boxes, as En mH^+ .

The green enclosure in Scheme 1 shows the resting state and the sequence of preparatory additions of protons and electrons, generating H atoms first at S2B, then at the *exo* and *endo* coordination positions of Fe6, and then to the *endo* position of Fe7, forming intermediate **S2BH-Fe6HxHn-3b6**. Calculations indicate that proton addition to S3B from the proton supply chain is triggered by electron addition to FeMo-co.⁶³ The reaction energies and barriers for configurational interchange at S3BH (Fig. 2) and for migration of each H atom

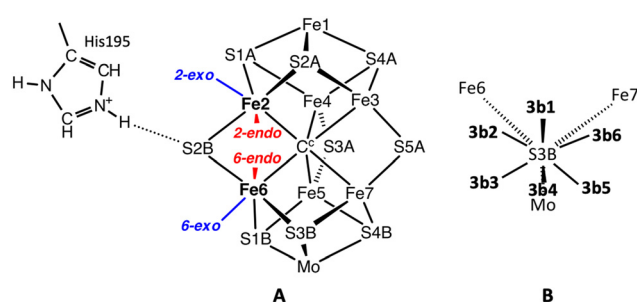


Fig. 2 (A) Locations of the *exo* and *endo* coordination positions at Fe2 and Fe6. Ligand bridging Fe and Fe6 supplants their *endo* coordination positions. (B) Labels used for the configurations of H bonded to S3B.⁶²

from S3B to other locations on FeMo-co are relatively small and have been reported.^{42,62} The N_2 that binds at the *exo*-coordination position of Fe2 (labelled N_2x) during this sequence is not reducible because it is not surrounded by H atom donors. The thermodynamics of Fe2- N_2x binding from the N_2 entry pocket (Fig. 1) are calculated to be variable, and



the occurrence of N₂x in intermediates during the course of the mechanism has been described and rationalised.⁶⁴

The yellow enclosure in Scheme 1 describes crucial steps.⁶⁵ First H₂ is formed at the *endo* position of Fe6, then a fifth H is added at *endo*-Fe7 (intermediate **pre-capture**). At this point the experimentally characterised H₂/N₂ exchange reaction can occur, with diffusion of reducible N₂ from the N₂ entry pocket, past non-reducible N₂x,⁶⁶ and into the reaction space to reach intermediate **N₂/H₂ exchange**. Dissociation of H₂ is followed by capture of N₂ in concert with double hydrogenation of N₂, to form **Fe2-brNH-NH-Fe6** containing HNNH bound and bridging between Fe2 and Fe6. This N₂ capture step, described in detail,⁶⁵ is proposed to be the way in which the enzyme bypasses the inherently difficult first hydrogenation of inert N₂. This concerted double hydrogenation and binding of N₂ at two Fe atoms differs radically from the conventional assumption of sequential Fe–N₂ binding and hydrogenation.

The lavender enclosure in Scheme 1 shows pathways through which the capture intermediate, **Fe2-brNH-NH-Fe6**, proceeds to form NH₃ in concert with severance of the N–N bond. The product of this sequence, **Fe2-brNH-Fe6-S2BH**, has released this first NH₃. Previously reported calculations⁶⁷ indicate that the most likely pathway in the lavender phase is through **Fe2-brNH-NH₂-Fe6Hx-S2BH** and **Fe2-brNH-Fe6NH₃-S2BH**. Intermediates shown in the green and yellow enclosures of Scheme 1 have a status where the number of added protons is one larger than the number of added electrons. Although it has been generally assumed that proton addition is coupled with electron addition, there is no experimental evidence for equal numbers of added protons and electrons at each stage.⁴ There are two parts to the rationale for the additional proton status in this sequence of intermediates. First the mechanism is compliant with the accumulated experimental evidence^{5,6,68,69} that the N₂/H₂ exchange equilibrium occurs after addition of four electrons. Secondly, in addition to the proton on HisNe, five H are required at the **pre-capture** intermediate: two leave as H₂, two form HNNH, and *exo*-Fe6H is required for the N–N breaking step. At completion of the sequence in the lavender enclosure (**Fe2-brNH-Fe6-S2BH**) H is required on S2B as a donor for the next steps to be described below, and it cannot be obtained from S3BH because the bound HNNH entity blocks H migration. It is sourced from the proton reservoir His195NεH⁺ which originated in the resting state. At completion of the lavender phase of the mechanism intermediate **Fe2-brNH-Fe6-S2BH** has status E6 6H⁺.

In this paper I report computational results for addition of the seventh and eighth electrons and protons, with the steps that form the second NH₃, then its dissociation, and finally recovery of the resting state and completion of the mechanism cycle.

2. Methodology

2.1 Protein model

The computed protein model is a 483+ atom extract from crystal PDB 3U7Q, including all relevant amino acids. This is my stan-

dard model for simulations of nitrogenase reactions and reactivity.^{43,45,64–67,70} This model includes nine of the ten active-site residues that are conserved across all analyzed extant nitrogenases:⁷¹ the exception, Gly424, is outside the reaction zone. Details, and the rationale for inclusion of amino acids and truncation of uninvolved side chains, are provided in the ESI.† Investigations of the protonation state of homocitrate using crystal structures at various pH (summarised in ref. 72), together with vibrational circular dichroism spectroscopy,²⁸ QM/MM calculations,^{72,73} and quantum refinement,⁷³ indicate that the coordinated alcoholate O7 atom is protonated and hydrogen bonded to O1, and this is included in the computed model. Some constraints on the protein structure are required during optimization calculations because the modelled protein is incomplete and the influences of the complete protein outside the computational model are absent. The strategy for constraints and the 28 distance constraints are described in the ESI.†

As previously explained⁴⁵ trial calculations on the modes of diffusion and coordination of N₂/H₂ led to the conclusion that the front chain near Val70 should move slightly away from FeMo-co. This is consistent with experimental data on mutants of amino acid 70 with smaller and larger side chains.^{8,54,57} In the present investigation of NH₃ formed in the reaction zone, and diffusing away between the side chains of Val70 and Arg96, it became apparent that a small additional separation of these sidechains should occur, by a libratory movement of the sidechain of Arg96, without changing the resting state hydrogen bond from NH₂ of Arg96 to S5A. This is shown in Fig. S1 of the ESI.† The Cα(Val70)⋯C^c and Cα(Val70)⋯CZ(Arg96) distances were fixed, at 8.2 and 5.18 Å respectively.

The charge on the [CFe₇MoS₉] core of FeMo-co is –1, in agreement with experimental and computational studies.^{43,72,74,75}

2.2 Density functional procedures

My density functional (DF) calculations use the DMol methodology of Delley,^{76–81} with accurate DNP (double numerical plus polarisation) basis sets.⁷⁹ The gradient-corrected functional PBE⁸² is used because validation tests demonstrate that when used with the numerical basis sets of DMol it is more accurate than other commonly used functionals.⁸³ See the ESI† for validation information. The conductor-like screening model (COSMO)^{84–86} is used with a dielectric constant of 5. The dispersion components of the non-bonding intermolecular interactions are treated effectively with the numerical basis sets used here: see the ESI† for additional information. Constraints on interatomic distances use the Lagrange Multiplier Algorithm. Control of electronic states is *via* input specifications of spin populations for Fe atoms (usually Fe1, Fe3, Fe4, Fe5 and Fe7) subsequently optimised during the scf procedure.

2.3 Electronic states

The electronic structure of the FeMo-co cluster is complex, with many electronic states, which are usually described with the signs and magnitudes of the spin densities on the seven



Fe atoms. Investigation of postulated reaction intermediates and reaction trajectories in the enzyme mechanism requires understanding and control of these electronic states. I have expounded a general principle, that maximisation of the weak bonding influences of opposite spin signs on each *axial* Fe pair (*i.e.* Fe2–Fe6, Fe3–Fe7, Fe4–Fe5) is a significant stabilising influence, proportional to the magnitudes of the spin densities.⁸⁷ Ligation of the Fe2 and/or Fe6 atoms, as in the intermediates described here, usually diminishes the magnitude of spin density on the ligated Fe atom, sometimes almost to zero, and so the stabilisation due to opposite spins on Fe2 and Fe6 is similarly diminished. Therefore, opposite spin signs and larger spin densities on the Fe3–Fe7 axial pair or the Fe4–Fe5 axial pair maximise stability, and this prediction is consistent with the findings and procedures of other authors.^{40,41,46–49,72,88–90} Therefore the two most favourable electronic states have the spin sign combinations –Fe3 +Fe4 –Fe5 +Fe7, or +Fe3 –Fe4 +Fe5 –Fe7. Because electronic states are labelled with the numbers of the Fe atoms with negative spin density, these states are labelled ‘35’ and ‘47’ respectively.

2.4 Mapping potential energy surfaces

This investigation comprehensively mapped the potential energy topology in the reaction space, to locate energy minima and then to locate the energy saddle points between them. Explorations deployed the relevant stereochemical principles, as elaborated in the Results. The shape of the potential energy surface was monitored throughout, and the magnitude of the displacement in each energy minimisation step was adjusted to maintain regular small energy changes.

Transition states (TS) and intrinsic reaction coordinates were determined by the procedure described previously^{43,58,91,92} and explained in detail in the ESI.† Variations of Fe spin densities occur during reactions, and continuity of the electronic state throughout each reaction trajectory was checked.

3. Results

The results are organised as follows. First I outline the scope of the results, defining and classifying the reaction intermediates and their labels, and the reaction steps. In section 3.2 I present and discuss some normal and abnormal electronic states. Then, because the minimal skeletal diagrams used in the schemes are minimal and uninformative about geometry, section 3.3 contains pictures of representative intermediates. A key component of this investigation is reaction trajectory analysis, and aspects of this are elaborated in section 3.4. After these preliminaries the calculated reaction potential energies and potential energy barriers for all possible steps are presented in section 3.5. From these it is possible to construct sequences of reaction steps, presented as reaction pathways in section 3.6. Because some of the H atom transfer steps could occur with H atom quantum tunneling, section 3.7 describes

additional details of reaction trajectories in attempt to assess the occurrence of tunneling in this mechanism.

3.1 Scope

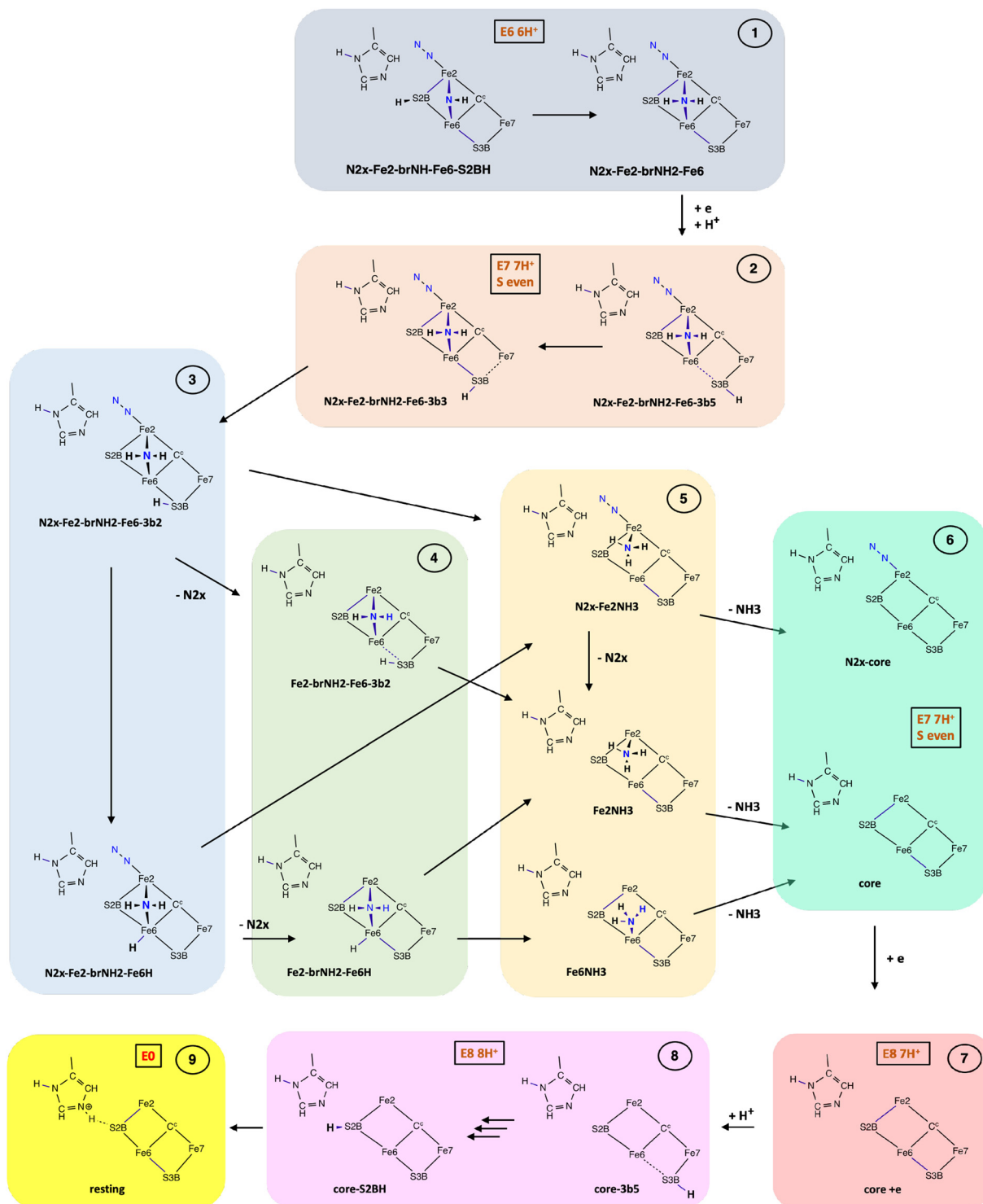
The scope of the results reported here is shown in Scheme 2, which displays all intermediates and transformations investigated, and defines the labels for intermediates. Labels now explicitly include the non-reducible N2x when present, because dissociations/associations of this ligand are part of the mechanism explored. The first step, continuing from the terminus of Scheme 1, is H transfer from S2B to generate the Fe2–NH2–Fe6 bridge (grey enclosure 1). Then the seventh H atom is introduced as 3b5, followed by reconfiguration around S3B to 3b3 (salmon enclosure 2) and then to 3b2 at the top of the blue enclosure 3. There are three pathways from this pivotal intermediate **N2x-Fe2-brNH2-Fe6-3b2**: (i) 3b2 to 6Hx in the blue enclosure 3, (ii) dissociation of 2N2x yielding the corresponding 3b2 and 6Hx in the green enclosure 4, or (iii) formation of NH₃ at the *endo*-Fe2 position (**N2x-Fe2NH₃**) in the buff enclosure 5. The buff enclosure contains the intermediates with bound NH₃, and the intermediates after dissociation of NH₃ are in the turquoise enclosure 6. Completion of the mechanism is shown in the pink 7, lilac 8 and yellow 9 enclosures. Scheme 2 also shows the numbers of electrons and protons added since the beginning of the mechanism cycle. In the top grey enclosure 1 the status is 6 electrons and 6 protons. After addition of an H atom (salmon enclosure 2) all intermediates through to the turquoise enclosure 6 are E7 7H⁺, with even spin S. Finally an electron and proton are added to recover the His195NεH⁺ resting state.

3.2 Electronic states and Fe spin densities

Understanding and control of the electronic states of the intermediates and of structures during the reaction trajectories is essential. Section 2.3 describes the rationale for selection of energetically favourable electronic states, and explanation of the two states labelled ‘35’ and ‘47’. Control of the electronic state is by input specification of Fe spin densities which are optimised during the scf calculation. In this investigation the ‘35’ and ‘47’ electronic states were tested, with specified orbital occupancies yielding several total spins *S*. In general states with smaller values of *S* are calculated to be more stable. Electronic states for reported structures with spin *S* are labelled as *S*, 35 or *S*, 47. Electronic states were monitored throughout by examination of the set of seven Fe calculated spin densities.

Electronic states and Fe spin densities are listed (with atomic coordinates) for all intermediates and transition states in the ESI.† The variable coordination chemistry at Fe2 and Fe6 throughout the reaction sequences results in considerable variation in the spin densities at these and other Fe atoms, illustrated with the selected results in Table 1. The first section, **N2x-Fe2NH₃**, contains typical results for the *S* = 1, 356 and *S* = 1, 476 states (here Fe atoms with spin densities more negative than –1 are added to the state label). Note that the Fe6 spin density is not small, because Fe6 is not ligated, that





Scheme 2 Intermediates and reaction steps investigated in this report, and the labels used.

the signs only on Fe3, Fe4, Fe5 and Fe7 are reversed, and that the two states are almost equi-energetic. The second section, $\text{N2x-Fe2-brNH2-Fe6H}$, shows how a reduction in total spin S from 1 to 0 is associated with a decrease in the spin density at Fe1, and decrease also in this example at Fe3, without significant energy change. Four electronic states for Fe2NH3 are

shown in Table 1. Two (356, 235) are nominally $S = 1$ '35' and equi-energetic, but have spin densities differing appreciably at Fe1, Fe2 and Fe6. This example of electronic state isomerism is occasionally seen in other related systems. The slightly less stable $S = 0$, 47 and $S = 1$, 47 states of Fe2NH3 also have different spin densities at Fe1, Fe2 and Fe6. Note that in the



Table 1 Fe spin densities for selected intermediates. Relative energies in kcal mol^{−1}. Notable features are marked in bold

N2x-Fe2NH ₃				
	S = 1, 356		S = 1, 476	
Fe1	3.16		3.15	
Fe2	0.01		−0.04	
Fe3	−2.70		2.68	
Fe4	2.70		−2.76	
Fe5	−2.25		2.36	
Fe6	−1.70		−1.66	
Fe7	2.29		−2.12	
Relative energy	+2		0	
N2x-Fe2-brNH ₂ -Fe6H				
	S = 1, 47		S = 0, 47	
Fe1	3.03		1.50	
Fe2	−0.49		−0.15	
Fe3	2.70		1.97	
Fe4	−2.53		−2.49	
Fe5	2.20		2.16	
Fe6	−0.85		−0.78	
Fe7	−2.30		−2.21	
Relative energy	0		+2	
Fe2NH ₃				
	356 S = 1	235 S = 1	S = 0, 47	S = 1, 47
Fe1	3.21	1.80	2.89	1.78
Fe2	0.21	−1.16	−1.11	−0.01
Fe3	−2.52	−2.48	2.70	2.46
Fe4	2.60	2.57	−2.57	−2.41
Fe5	−2.47	−2.37	2.35	2.25
Fe6	−2.06	1.59	−2.29	0.11
Fe7	2.45	1.95	−2.30	−2.15
Relative energy	+1	0	+7	+4
Fe6NH ₃				
	S = 0, 47		S = 1, 47	
Fe1	2.87		2.93	
Fe2	−2.62		−2.33	
Fe3	2.74		2.78	
Fe4	−2.50		−2.39	
Fe5	2.22		2.36	
Fe6	−0.88		0.85	
Fe7	−2.36		−2.28	
Relative energy	0		+2	

$S = 1, 47$ state the spin density at Fe6 is negligible even though this atom is not ligated: this is abnormal in the general pattern of spin densities for the electronic states of FeMo-co. In the '47' states of **Fe6NH₃**, the difference between $S = 0$ and $S = 1$ is primarily a flip in the spin sign at Fe6. The results for **N2x-Fe2-brNH₂-Fe6H** and **Fe2NH₃** in Table 1 are unusual, but they demonstrate the importance of monitoring electronic states *via* Fe spin densities during calculations of reaction trajectories.

3.3 Pictures

Fig. 3 illustrates geometrical properties of the reaction site in representative intermediates, with significant dimensions marked (all for the electronic state $S = 1, 35$). **N2x-Fe2-brNH₂-**

Fe6-3b5 and **N2x-Fe2-brNH₂-Fe6-3b3** possess standard characteristics, extension of S3B–Fe6 and S3B–Fe7 respectively, with extension of Fe2–C^c due to *exo*-Fe2–N2x. When the H atom on S3B is conformed as 3b2, there are isomers with long and short S3B–Fe6 distances. The short 3b2 isomer (Fig. 3C) is effectively an S3B–H–Fe6 bridge. When 2N2x is dissociated (Fig. 3E) the Fe2–C^c distance decreases from 2.40 to 1.95 Å, and the S3B–H–Fe6 bridge elongates slightly. The Fe2–N and Fe6–N bonds of the NH₂ bridge are effectively invariant, 1.98 ± 0.03 Å. Fig. 3F and G illustrate how N2x influences coordination geometry at Fe2, and indirectly at Fe6.

There are two postulated intermediates with NH₃ bound at the *endo* position of Fe2. In **N2x-Fe2NH₃**, where Fe2 is near octahedrally coordinated there is a distinctive elongation of Fe2–S1A *trans* to the Fe2–NH₃, and the Fe2–S2A bond is also lengthened. When N2x is absent, **Fe2NH₃**, Fe2 possesses more regular square pyramidal coordination (Fig. 3I), and similar square pyramidal coordination at Fe6 occurs in **Fe6NH₃**.

3.4 Reaction trajectory analyses

Potential energy topologies between the energy minima presented in Scheme 2 were explored to find the lowest energy pathways between them. The pragmatic procedure for calculation of transition states is described in detail in the ESI.† Some of the transformations involve multiple geometrical variables, which need to be recognised and understood during determination of the transition state and reaction trajectory. The following outlines of reaction trajectories refer to the atom labels in Fig. 4.

The formation of NH₃ is by transfer of H, from either 3b2 or 6Hx to N^{br} of the bridging NH₂, involving multiple changes: extension then breaking of the S3B–H bond or of the Fe–H bond, formation of the H–N^{br} bond, and extension then breaking of one of the two N^{br}–Fe bonds. In the 3b2 to Fe2NH₃ reaction step (**N2x-Fe2-brNH₂-Fe6-3b2** → **N2x-Fe2NH₃** and **Fe2-brNH₂-Fe6-3b2** → **Fe2NH₃**), at the transition state the H...N^{br} and S3B...H distances are both *ca.* 1.55 Å, S3B is closing towards Fe6, the transferring H is 1.9–2.0 Å from Fe6, and the originally equal Fe–N^{br} distances are only slightly differentiated by 0.1–0.2 Å, towards Fe2–N^{br}. All of these dimensions are monitored and adjusted during the trajectory analysis. The influence of N2x is small. The formation of NH₃ by transfer of H at 3b2 (*i.e.* from **N2x-Fe2-brNH₂-Fe6-3b2** or from **Fe2-brNH₂-Fe6-3b2**) generated *only* Fe2–NH₃. The possibility that H transfer from 3b2 to the NH₂ bridge would first form NH₃ bonded to Fe6 was thoroughly tested, and no pathway from 3b2 to generate NH₃ at Fe6 could be found.

In the alternative formation of NH₃ by transfer of 6Hx (instead of 3b2) to N^{br} the stereochemistry at N^{br} is an additional factor. In the reactant the angles 6Hx–N^{br}–Ha and 6Hx–N^{br}–Hb are unequal (*ca.* 80, 126°) and their equalisation during the reaction step needs to be incorporated in the trajectory analysis. The formation of Fe–NH₃ by transfer of 6Hx to bridging NH₂ is also affected by electronic state. When the electronic state is 35 and $S = 0$ or 1, the product of H transfer is NH₃ bound at the *endo* position of Fe2, but in electronic



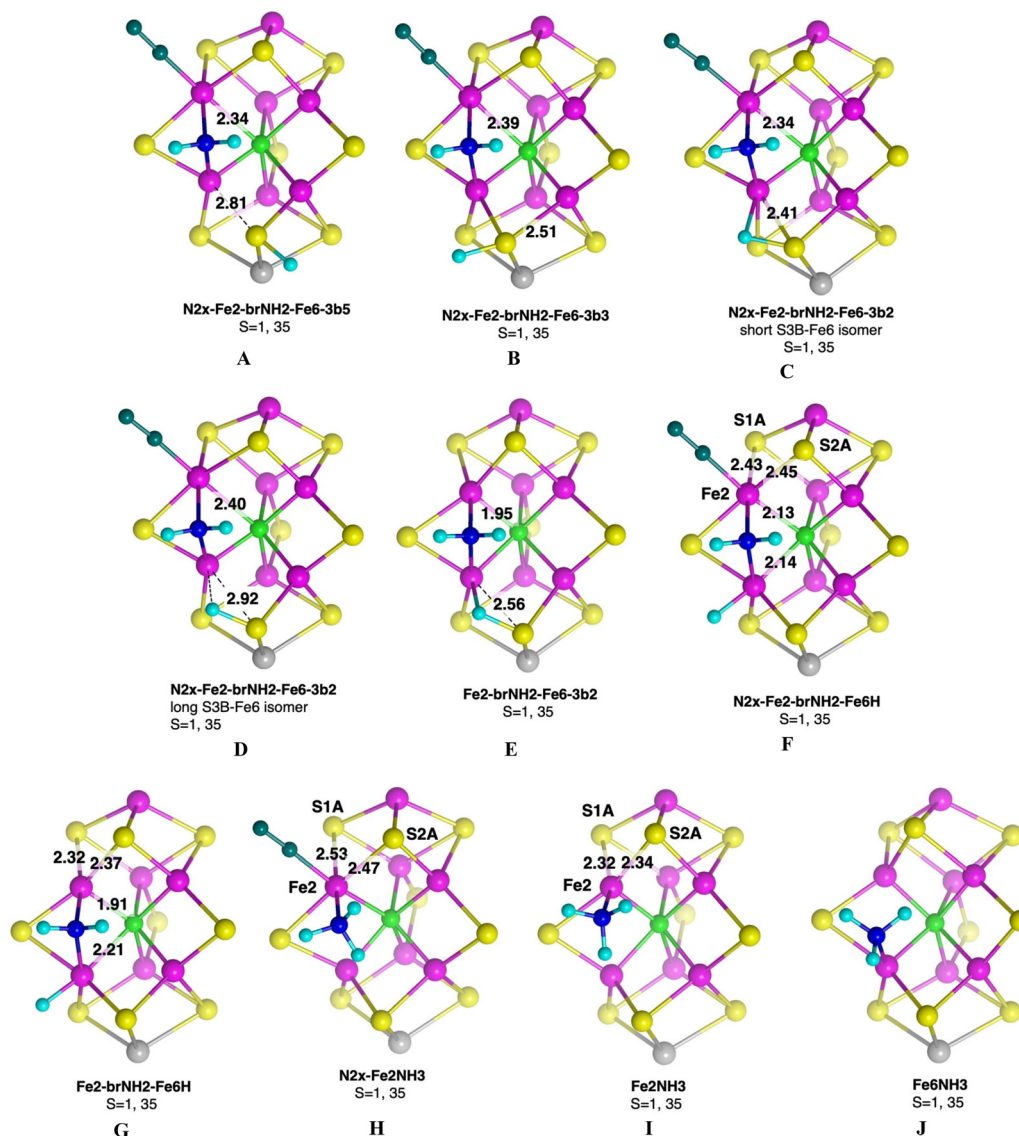


Fig. 3 The core structures of representative intermediates, with significant dimensions (Å) marked. Exo-Fe2–N2x is dark turquoise.

state 47, $S = 0$ or 1, the product NH_3 is bound instead at *endo* Fe6.

NH_3 formed at the *endo* position of Fe2 or Fe6 dissociates from them along a pathway that leads first between the side-chains of Val70 and Arg96, before reaching the hydrophilic mouth of the NH_3 egress pathway.⁹³ The space between Val70 and Arg96 is relatively tight, and I have postulated a minor expansion achieved by a libratory movement of the sidechain of Arg96. This is shown in Fig. 5. Small reconfigurations in the $\text{CH}_2\text{CH}_2\text{CH}_2$ arm of Arg96 effect this movement, which involves no change in the hydrogen bond from the terminal NH_2 of Arg96 to S5A. The 70C α –96CZ separation, 3.66 Å in the resting crystal PDB-3U7Q, was constrained to 5.18 Å in the modelling.

Dissociating NH_3 can wiggle through this space. Fig. 6 shows examples, one (A) at the transition state ($\text{Fe2}\cdots\text{N}$ 2.79 Å), one (B) with NH_3 dissociated from Fe2, and two (C and D) with

NH_3 dissociated from Fe6. These four are representative of the variety of orientations possible for dissociating NH_3 , which are quantified by the three $\text{Fe}\cdots\text{N}\cdots\text{H}$ angles recorded on Fig. 6. The dotted lines identify the closest contacts, which in almost all instances are longer than the destabilisation regime of the $\text{N}\cdots\text{H}$ dispersion energy potential.⁹⁴ Dissociating NH_3 can also orient to form a bent hydrogen bond with S3B (Fig. 6A) but this interaction does not appear to be advantageous.

3.5 Reaction energies

For each of the transformations in Scheme 2 the potential energy profile was calculated, by first determining the transition state (TS), and then following (by small step energy minimisations) the trajectories from TS to reactant and TS to product. The comprehensive results are presented in Scheme 3, with symbols comprising the reaction energy (red), the reaction barrier (black), and the electronic state. The



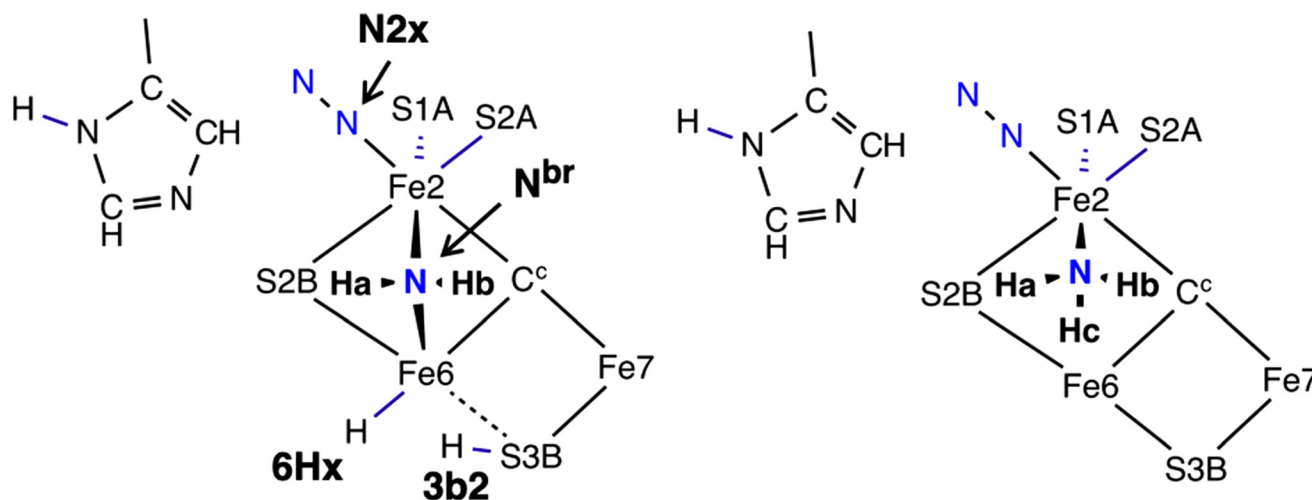


Fig. 4 Atom labels used in descriptions of reaction trajectories.

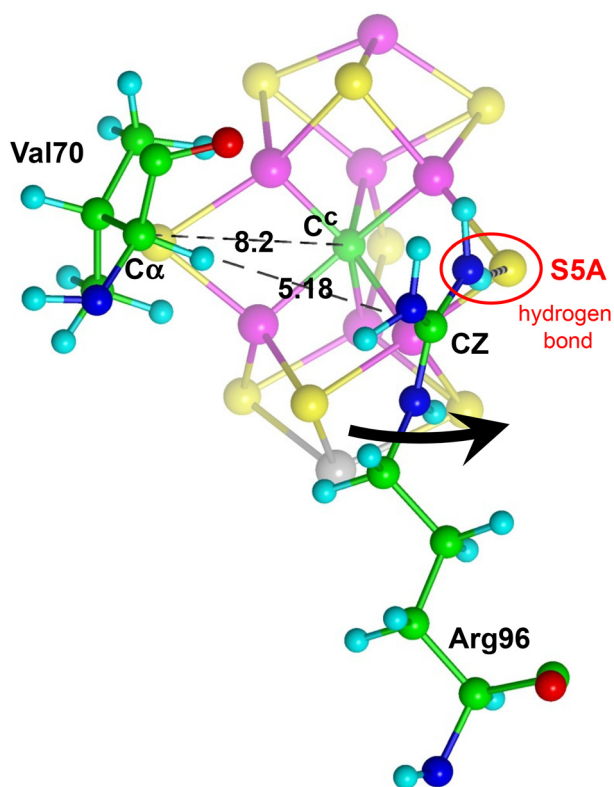


Fig. 5 Expansion of the space between the sidechains of Val70 and Arg96 by a libratory movement of the sidechain of Arg96, black arrow. The hydrogen bond from terminal NH_2 of Arg96 to S5A is unaffected.

uncertainty level is 1 kcal mol^{-1} . Results where larger calculational uncertainty arose, mainly due to anomalous electronic structure, are not included. Panel A shows the preparatory steps from $\text{N2x-Fe2-brNH-Fe6-S2BH}$ (the final intermediate in the previous phase, Scheme 1) to $\text{N2x-Fe2-brNH}_2\text{-Fe6-3b2}$ (the

grey and salmon enclosures of Scheme 2). Characteristically the transfer of H from S2B to N forming $\text{N2x-Fe2-brNH}_2\text{-Fe6}$ is strongly exergonic. Then a new H introduced at 3b5 surmounts a barrier of *ca.* 10 kcal mol^{-1} to reach $\text{N2x-Fe2-brNH}_2\text{-Fe6-3b2}$. Panel B describes the three possible reactions of $\text{N2x-Fe2-brNH}_2\text{-Fe6-3b2}$. The energy profiles for these three steps are quite different: dissociation of N2x is endergonic, as usual;⁶⁴ formation of NH_3 at Fe2 is very strongly exergonic, with moderate barriers; conversion to $\text{Fe2-brNH}_2\text{-Fe6H}$ is exergonic with very small barriers. Panel C shows the two reactions of $\text{N2x-Fe2-brNH}_2\text{-Fe6H}$: slightly endergonic dissociation of N2x with small barriers, and exergonic formation of NH_3 at Fe2 with large barriers. Panel D contains the steps in which NH_3 is formed in the absence of N2x, *i.e.* the green 4 to buff 5 transformations in Scheme 2. All pathways are strongly exergonic, and the barriers are variable, being unusually small for the $\text{Fe2-brNH}_2\text{-Fe6-3b2} \rightarrow \text{Fe2NH}_3$ step. No pathway could be found for the formation of NH_3 at Fe6 from $\text{Fe2-brNH}_2\text{-Fe6-3b2}$. Panel E describes the energy profiles for dissociation of NH_3 from Fe2 and Fe6. In the absence of N2x this dissociation is exergonic with relatively small barriers, but with N2x present dissociation of NH_3 to form N2x-core is appreciably endergonic. Panel F shows recovery of the resting state, after addition of the final electron + proton pair.

Four different NH_3 formation steps have been calculated. All are strongly exergonic. The barriers are 13 or 19 kcal mol^{-1} for H transfer from 3b2 when N2x is present, 22 or 23 kcal mol^{-1} from 6Hx when N2x is present, 5 or 7 kcal mol^{-1} for H transfer from 3b2 when N2x is absent, and 21, 22, or 25 kcal mol^{-1} for transfer from 6Hx when N2x is absent. The smaller barriers when H is transferred from 3b2 reflect the positional variability of 3b2 (see Fig. 3) and the slight stereochemical awkwardness of the 6Hx to N^{br} transfer (section 3.4).

Non-reducible N2x occurs in three structure types, all with a ligand at the *endo* position of Fe2, and all with a positive



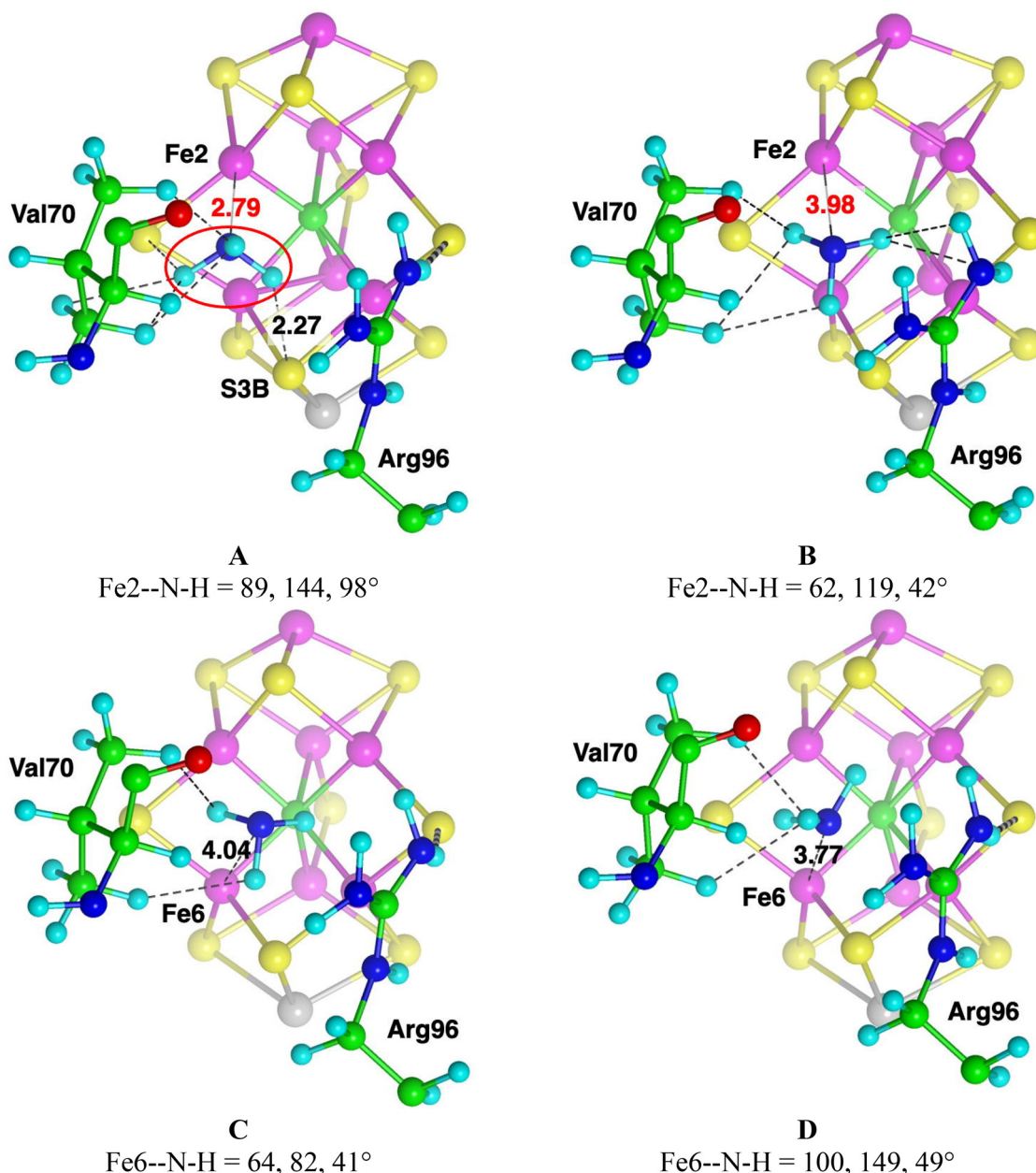


Fig. 6 Representations of typical positions and orientations of NH_3 on its dissociation pathway. Orientations are quantified with the three $\text{Fe}\cdots\text{N}-\text{H}$ angles marked. Dotted lines locate the closest contacts with the sidechains of Val70 and Arg96.

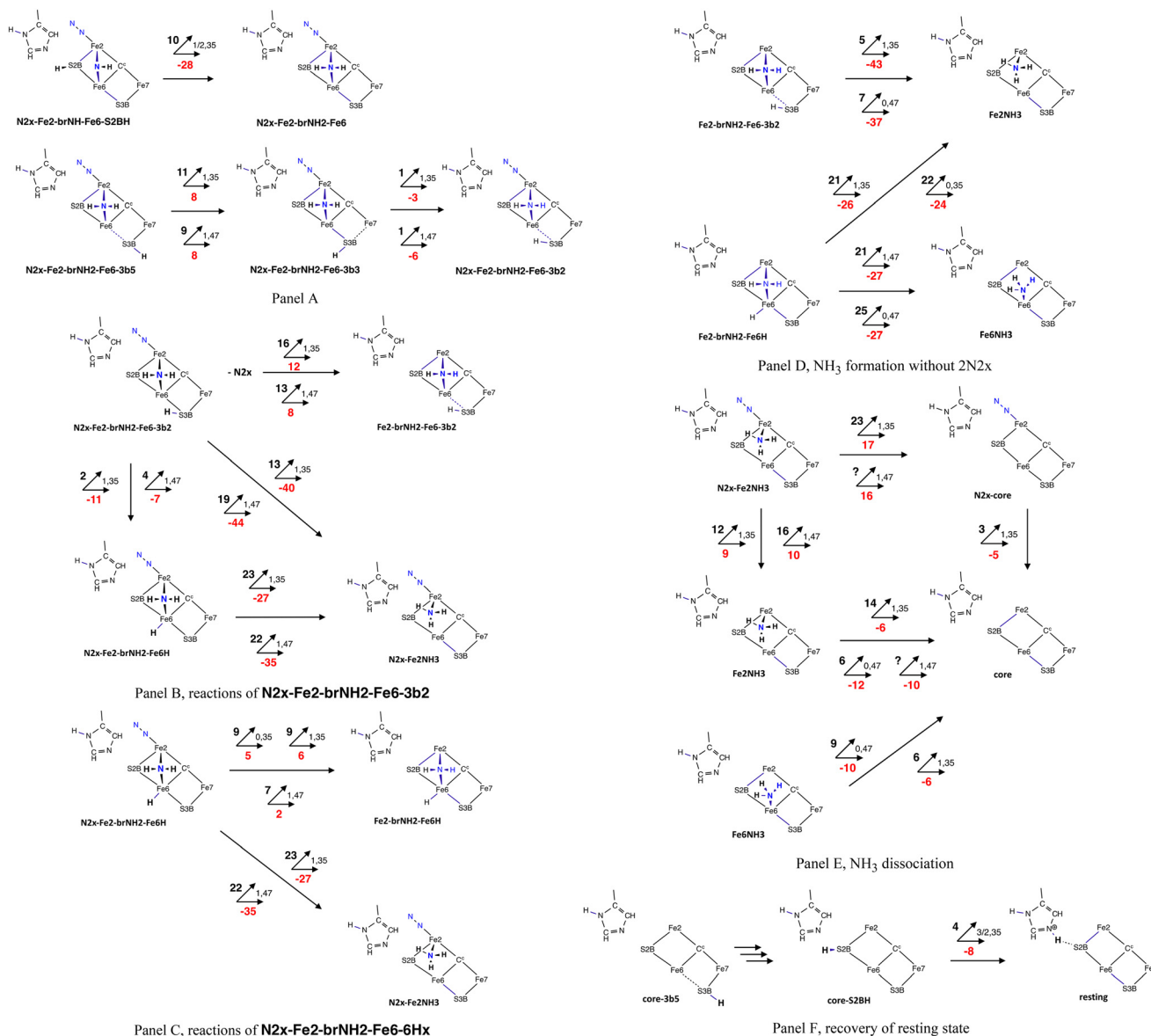
potential energy for dissociation of N_2x . This is consistent with the generalisations previously described.⁶⁴ In the intermediate with NH_2 bridge and 6Hx ($\text{N}_2\text{x-Fe2-brNH}_2\text{-Fe6H}$) the N_2x dissociation barrier (7 to 9 kcal mol^{-1}) and endergonicity (2 to 6 kcal mol^{-1}) are smaller than the barrier (range 12 to 16 kcal mol^{-1}) and endergonicity (range 8 to 12 kcal mol^{-1}) for N_2x dissociation from $\text{N}_2\text{x-Fe2-brNH}_2\text{-Fe6-3b2}$ and $\text{N}_2\text{x-Fe2NH}_3$.

Further discussion of these results is deferred until they are incorporated into possible reaction sequences, in the next section.

3.6 Reaction pathways

I have constructed the possible reaction pathways from $\text{N}_2\text{x-Fe2-brNH}_2\text{-Fe6-3b2}$ to **core**, and the potential energy profiles for these are charted in Chart 1. Note that the variations in energy due to different electronic and spin states are relatively small, and there is consistency in each sequence of ridges and valleys. Paths P1, P2 and P3 continue from the three different reactions of the precursor $\text{N}_2\text{x-Fe2-brNH}_2\text{-Fe6-3b2}$ (see Panel B of Scheme 3). Paths P1 and P2 involve formation of NH_3 from 3b2, preceding or following dissociation of N_2x . Paths P3 and P4 shift 3b2 to 6Hx which is then used to generate NH_3 , with





Scheme 3 Calculated reaction potential energies (red) and reaction barriers (black), in kcal mol⁻¹. Electronic states are marked, as S, 3/2 or 5/2. Panel A describes the preparatory steps forming $N2x-Fe2-brNH_2-Fe6-3b2$ from $N2x-Fe2-brNH_2-Fe6-S2BH$. Panel B describes the three reactions of $N2x-Fe2-brNH_2-Fe6-3b2$ and panel C describes the two reactions of $N2x-Fe2-brNH_2-Fe6-6Hx$. Panel D contains the steps that form NH_3 after prior dissociation of $N2x$. Panel E describes the NH_3 dissociation steps from intermediates with and without $N2x$, and also the two $N2x$ dissociation steps with and without bound NH_3 . Panel F shows recovery of the resting state.

dissociation of $N2x$ after (P3) or before (P4) the formation of NH_3 . Path P5 is the variant of P4 in which NH_3 is formed at Fe6 rather than Fe2. This variation is dependent on the electronic state (illustrated by the black/red difference in P4/P5 at the NH_3 formation step).

Referring to Chart 1, in path P1 the first intermediate, $N2x-Fe2NH_3$, is a deep energy well, followed by endergonic NH_3 dissociation with a barrier of ca. 22 kcal mol⁻¹, disfavoring this path. Path P2 first dissociates $N2x$ (barrier ca. 14 kcal mol⁻¹) and then forms NH_3 with a barrier (ca. 6 kcal mol⁻¹) considerably smaller than the NH_3 formation barrier in path P1. Again there is a deep energy well at $N2x-Fe2NH_3$, from which, in state

$S = 0, 47$, the final exergonic (−12 kcal mol⁻¹) dissociation of NH_3 encounters a barrier of only 6 kcal mol⁻¹. The overall reaction energy is ca −40 kcal mol⁻¹. For path P3 the first substantial barrier, ca. 22 kcal mol⁻¹, is the formation of NH_3 . This intermediate, $N2x-Fe2NH_3$, is again in an energy well of ca −40 kcal mol⁻¹, close to the energy of the final product **core**. This low energy of $N2x-Fe2NH_3$, and the two subsequent energy barriers for dissociation of NH_3 and $N2x$, appear to disfavor completion of this reaction pathway.

Paths P4 and P5 do not include the very stable intermediate $N2x-Fe2NH_3$, and possess a different overall energy profile. The first two steps in both paths, to $N2x-Fe2-brNH_2-Fe6H$ and

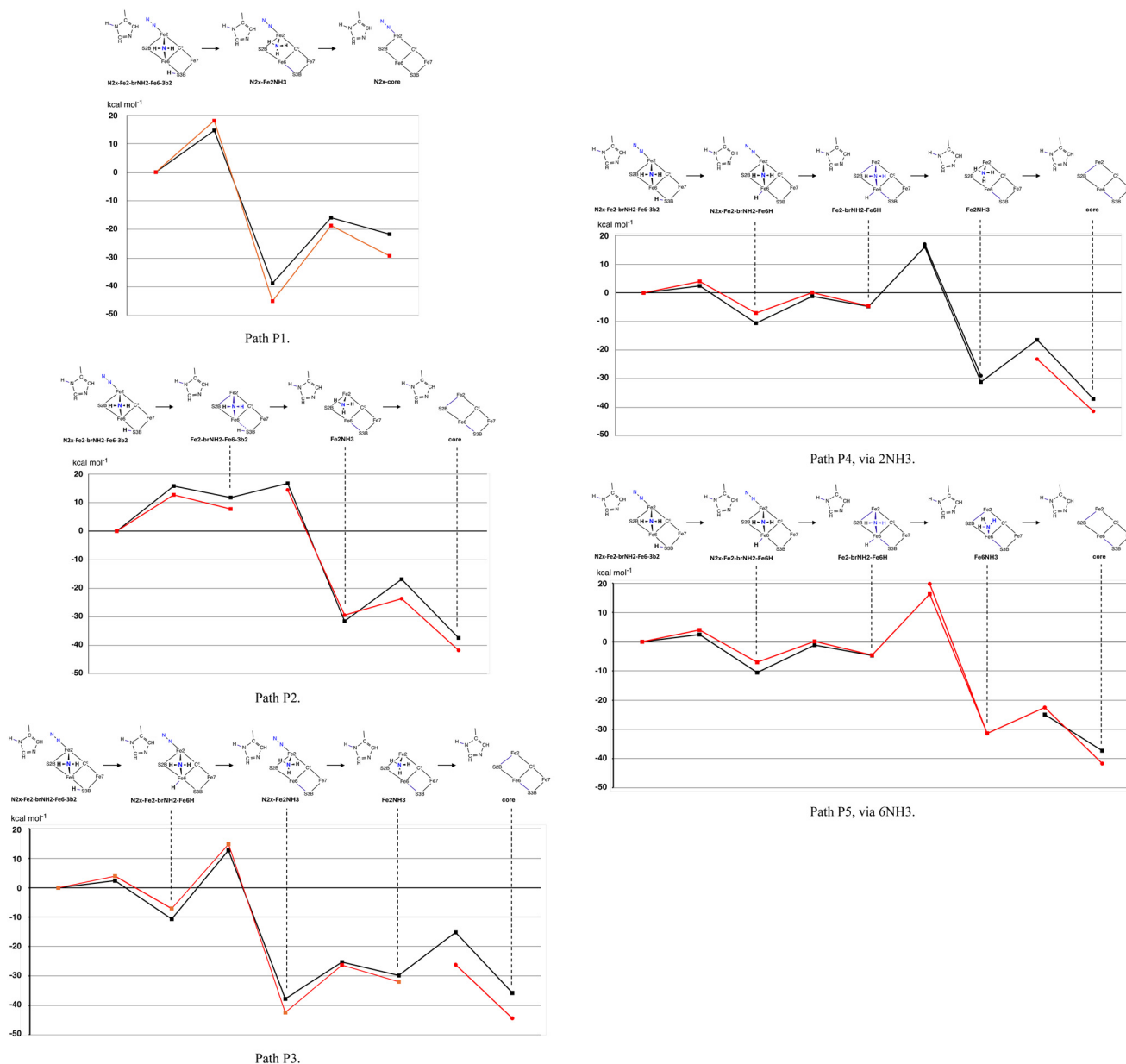


Chart 1 Potential energy profiles for five reaction pathways from $N2x-Fe2-brNH2-Fe6-3b2$ to $core$. Circles signify $S = 0$, squares $S = 1$, black = 35, red = 47. Discontinuities in the traces occur where a linked intermediate or transition state was not calculated due to electronic structure uncertainties.

$Fe2-brNH2-Fe6H$, possess small energy changes and barriers. $NH3$ formation, at either Fe2 (P4) or Fe6 (P5) involves barriers 21 to 23 $kcal\ mol^{-1}$, and is exergonic at $-25\ kcal\ mol^{-1}$. Final dissociation of $NH3$ is also exergonic, -6 to $-12\ kcal\ mol^{-1}$.

In summary at this point, pathways P1 and P3 appear to be unfavourable, P2 is feasible with no large barriers, and pathways P4 and P5 are feasible except for the relatively large barrier to $NH3$ formation. However, another factor is to be considered in the kinetic analysis, namely the possibility of H atom tunnelling. This is investigated in the following section.

3.7 H atom tunneling possibilities

Quantum mechanical tunneling through a reaction barrier is maximized for the smallest atom encountering the barrier, hydrogen. Tunneling is often evaluated in terms of the de Broglie wavelength, which for a hydrogen atom with ambient (300 K) thermal energy of 2.5 $kcal\ mol^{-1}$ is *ca.* 0.5 Å: the basic concept is that as the H atom ascends a barrier it can tunnel through it when the barrier width is of similar dimension.^{95–99} Many of the steps in the mechanism of nitrogenase involve H atom transfer, and I have suggested that H atom tunneling might occur in some H transfer steps, and should be investigated.⁴³



In a previous trajectory analysis of an S3B-H to N (N_2) hydrogen atom transfer in nitrogenase¹⁰⁰ I noted similarities with the trajectories for proton transfer in the enzyme aromatic amine dehydrogenase, where a detailed investigation concluded that the reaction is dominated by quantum tunneling over a distance of *ca.* 0.6 Å, at ~ 10 kcal mol⁻¹ below the top of the classical potential energy barrier.¹⁰¹ Experimental and theoretical investigation of hydrogen atom tunneling is complicated, even for reaction systems much more conventional than those in the mechanism of nitrogenase. In the present context the assessment of possible tunneling adopts the basic criterion that the potential energy profile for H transfer be symmetrical about the classical transition state, for a narrow (~ 0.6 Å) section of the H atom transfer coordinate, during which heavier atoms do not move. I am not aware of other reports of H tunneling involving S-H or Fe-H bonds.

First I examine the 6Hx $\rightarrow$ NH₂ component of NH₃ formation, which occurs in reaction paths P3, P4 and P5. Fig. 7 plots the relevant geometrical properties in the vicinity of the transition state (TS) on the potential energy surface for the **Fe2-brNH₂-Fe6H** $\rightarrow$ **Fe6NH₃** step, in the $S = 1$, 47 electronic state. The calculation used energy minimisation with small energy intervals from the TS towards reactant and towards product. The 6Hx atom moves by swinging from the *exo*-Fe6 position towards N^{br} (Fig. 4), with hardly any change in the H-Fe6 distance until beyond the TS. As the potential energy climbs towards the TS there is only one major geometry change, the shortening of the H-N distance, from near 2.5 Å to 1.5 Å at the TS. The two Fe-N bonds of the NH₂ bridge are essentially unchanged near the TS, and differentiate in favour of Fe6-N only after the H-N bond and NH₃ are almost fully formed and the H-Fe6 bond is weakening. This reaction is atypical of other H atom transfer reactions that are believed to involve quantum

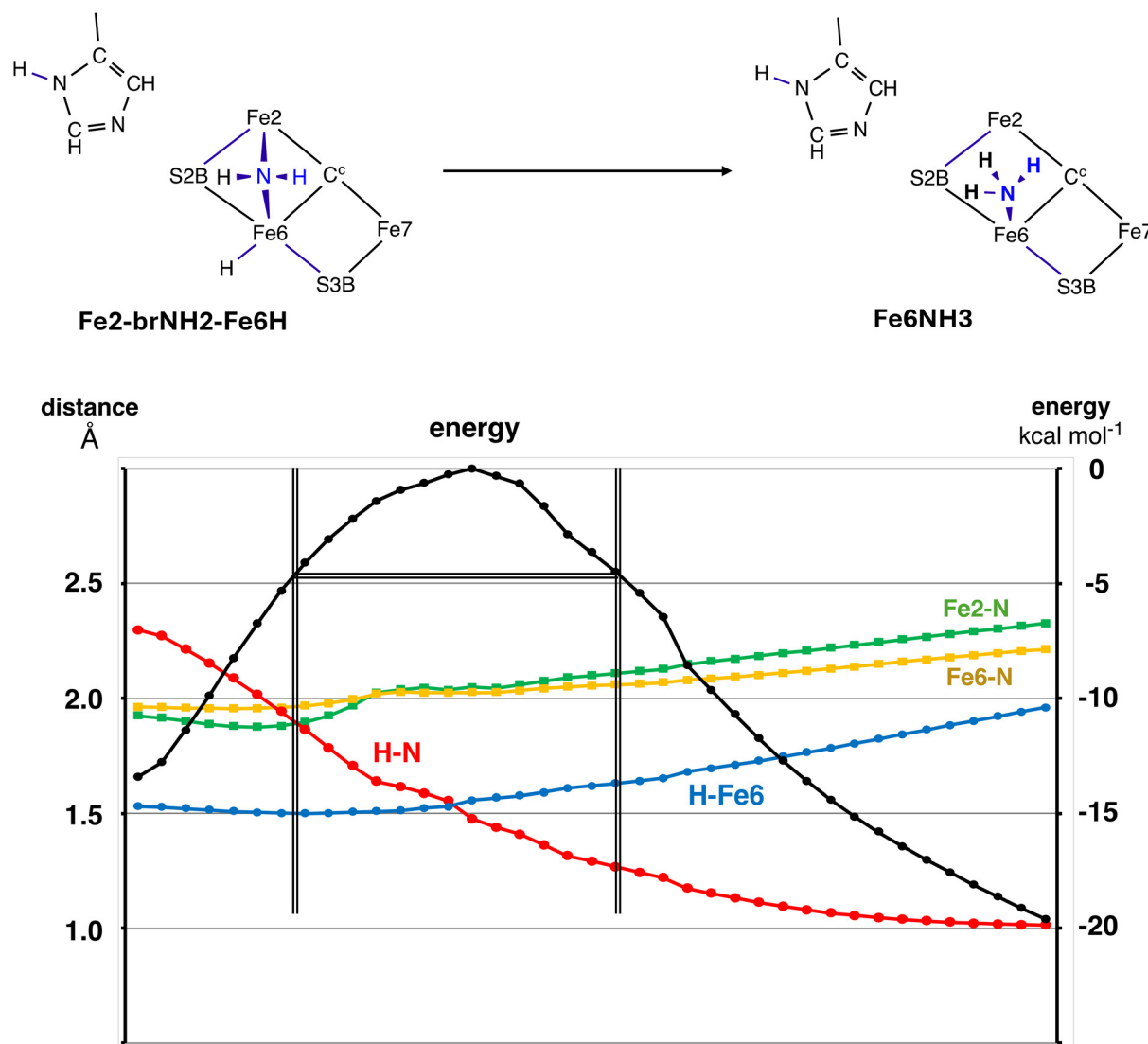


Fig. 7 Potential energy profile near the transition state for the **Fe2-brNH₂-Fe6H** $\rightarrow$ **Fe6NH₃** reaction step in the $S = 1$, 47 electronic state. The 'goal posts' symmetrically bracket the TS energy potential, and encompass movement of H by *ca.* 0.5 Å.



tunneling, because here the geometry bracketing the TS involves a change mainly in only one H–X distance. The majority of H atom transfer reactions involve synchronous bond breaking and bond making. Conventionally understood H atom tunneling systems are usually *intermolecular*, between distinctly separate H donor and acceptor atoms, and tunneling occurs when there is a shortening of the distance between them:^{98,102} in the present *intramolecular* reaction the H atom donor (Fe6) and acceptor (N) atoms are instead strongly bonded.

To assess of the possibility of a tunneling contribution to this **Fe2-brNH₂-Fe6H** → **Fe6NH₃** step, consider the ‘goal posts’ on Fig. 7. They are placed to bracket symmetrically the energy potential about the TS (as required for quantum tunneling), and to include an H atom displacement of ~0.5 Å. These goal posts cross the potential energy trace ~4 kcal mol^{−1} below the TS, and so suggest that H atom tunneling here could occur ~4 kcal mol^{−1} below the classical barrier. In a different electronic state, *S* = 1 35, **Fe2-brNH₂-Fe6H** forms NH₃ at Fe2 instead of Fe6 (Scheme 3 panel D). During this step the potential energy profile as it extends 10 kcal mol^{−1} either side of the TS is very similar to that shown in Fig. 7. Therefore the involvement of H quantum tunneling is similarly indicated for the **Fe2-brNH₂-Fe6H** → **Fe2NH₃** step. The differentiation of products, Fe2–NH₃ in the 35 electronic state and Fe6–NH₃ in the 47 electronic state, appears late in both trajectories, after the H–N bond has formed. Results for the formation of NH₃ by H transfer from 6Hx but with N2x present, **N2x-Fe2-brNH₂-Fe6H** → **N2x-Fe2NH₃** resemble those in Fig. 7.

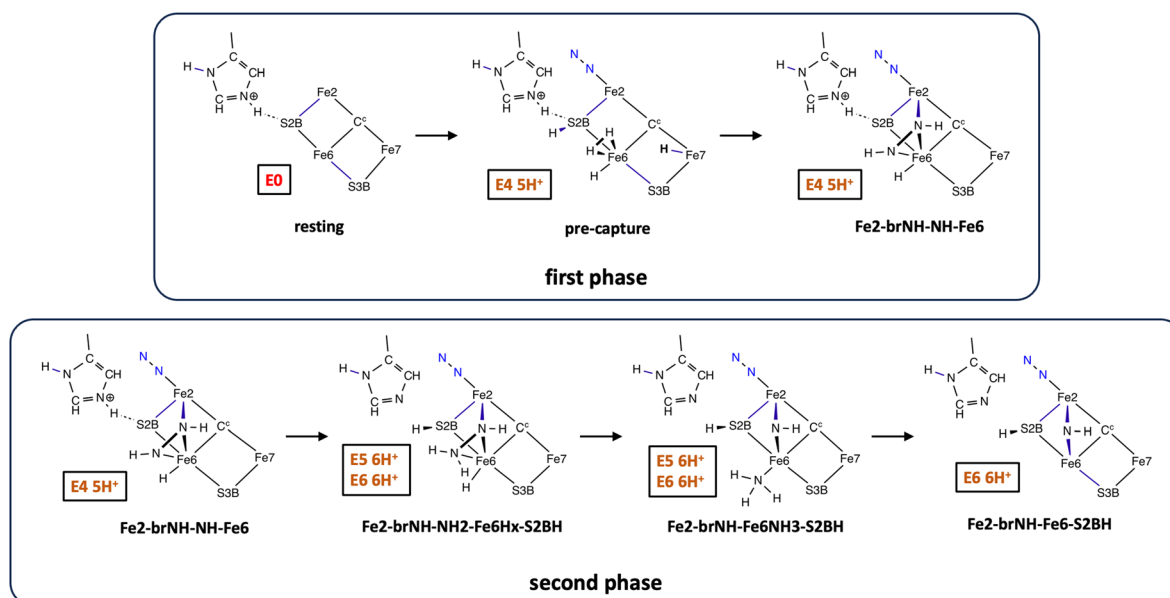
The alternative step forming NH₃ by H transfer from 3b2 to N, is different because the S3B...Fe6 distance is variable, and movements of S3B during passage over the potential energy barrier for H transfer could obviate quantum tunneling by H. In the reaction trajectory for **N2x-Fe2-brNH₂-Fe6-3b2** → **N2x-**

Fe2NH₃, the S3B...Fe6 distance is initially long, *ca.* 2.9 Å, decreases to *ca.* 2.6 Å at the transition state, and continues towards 2.3 Å in the product. Although not analysed in detail it appears that this reaction is not a candidate for possible H tunneling. However, the trajectory for corresponding reaction with N2x absent, **Fe2-brNH₂-Fe6-3b2** → **Fe2NH₃**, is different because the S3B–Fe6 separation is almost invariant through most of the H transfer. The trajectory plot (see ESI Fig. S4†) shows minor heavy atom movement through the TS, and suggests that there could be H tunneling about 2 kcal mol^{−1} below the TS. For this reaction step (in path P2) the classical potential energy barrier is small, 5–7 kcal mol^{−1}, and so H any tunneling would have minor kinetic influence.

Note that although these putative tunneling systems appear to be complex, a small 22 atom model such as that of Fig. 3G embodies essential features of **Fe2-brNH₂-Fe6H** → **Fe6NH₃** and could be amenable to higher level calculations of the tunneling characteristics.¹⁰³

4. Discussion

Prior reports described how the active site of nitrogenase can capture N₂ using a propitious array of H atoms bound at S2B and Fe7, to generate an intermediate with bound HNNH, **Fe2-brNH-NH-Fe6**.⁶⁵ Then, in a second phase, two sequential hydrogenations can convert this HNNH first to HNNH₂, then to NH and NH₃, after which this first NH₃ is released.⁶⁷ These two phases of the reaction are reviewed in outline in Scheme 4. The results presented in this report, the third and final phase, describe how the intermediate at the end of the second phase, **Fe2-brNH-Fe6-S2BH** with status E6 6H⁺, can continue by adding the seventh electron + proton, generate and release the



Scheme 4



second NH_3 , and after adding the eighth electron + proton complete the cycle and revert to the resting state.

At the beginning of this third phase the transfer of S2BH to bridging NH ($\text{N2x-Fe2-brNH}_2\text{-Fe6}$), and introduction and placement of the seventh H at the 3b2 position, intermediate $\text{N2x-Fe2-brNH}_2\text{-Fe6-3b2}$, are straightforward and energetically accessible (Scheme 3, panel A). Thereafter five pathways can proceed from this intermediate, forming and then releasing NH_3 , finally reaching the intermediate **core**. There is no change in the electron–proton status of the system during this sequence. These pathway possibilities (Chart 1) are now evaluated according to their potential energy profiles, the possibility of H atom tunneling, and inclusion of entropic effects.

4.1 Entropy contributions

This last phase of the mechanism involves two dissociations of small molecules, N2x from the *exo* position of Fe2, and NH_3 from the *endo* position of Fe2 or Fe6. My previous assessment of the entropic component of N2x binding at *exo*-Fe2 leads to the conclusion here that $T\Delta S$ for dissociation is ca +4 kcal mol⁻¹ and no more than +6 kcal mol⁻¹.⁶⁴ This estimate was based on experimental entropy measurement for the binding of N_2 in comparable model complexes in solution, experimental measurements of the entropy of dissolution of N_2 , and an assumption that the environments, solution vs. protein, are similar in their encapsulation of small molecules.

Estimation of the entropic component of NH_3 dissociation from Fe is less certain. Despite abundant knowledge of metal–ammonia complexes and measurements of ligand substitution reactions in aqueous solution, I have not been able to find experimental entropy data for dissociation of NH_3 from a suitable model complex into a medium comparable with the non-hydrogen bonding and weakly polar domain between Val70 and Arg96. There are gas phase data for adduct formation between trimethylgallium (and trimethylindium) and ammonia: $\Delta S_{\text{association}} = -32.4 \text{ cal mol}^{-1} \text{ K}^{-1}$. Despite an apparent dissimilarity of this model and the enzyme, Me_3Ga is chemically comparable with *endo*-Fe in FeMo-co: both are fully coordinated except for one vacant position, both have soft coordination, and Fe and Ga are similar size. This $\Delta S_{\text{association}}$ is for NH_3 in the gas phase; adjustment to the liquid phase with the standard entropy of vaporisation of NH_3 (+23 cal mol⁻¹ K⁻¹), results in eqn (2).



In search of entropy data for $\text{Fe-NH}_3 \rightarrow \text{Fe} + \text{NH}_3$ as it occurs in *proteo*, another approximation is $\text{NH}_3(\text{s}) \rightarrow \text{NH}_3(\text{l})$, i.e., immobilised NH_3 to fluid NH_3 . The standard entropy of fusion of NH_3 is 6.92 cal mol⁻¹ K⁻¹. In the absence of more direct data I average this and the numerically similar eqn (2), to yield 8 cal mol⁻¹ K⁻¹, or $T\Delta S = +2.4 \text{ kcal mol}^{-1}$ for the NH_3 dissociation steps in the nitrogenase mechanism.

4.2 Quantum tunneling contributions

The analyses described in section 3.7 indicate that three steps $\text{Fe2-brNH}_2\text{-Fe6H} \rightarrow \text{Fe6NH}_3$, $\text{Fe2-brNH}_2\text{-Fe6H} \rightarrow \text{Fe2NH}_3$, and

$\text{N2x-Fe2-brNH}_2\text{-Fe6H} \rightarrow \text{N2x-Fe2NH}_3$, could involve some H atom tunneling, up to 4 kcal mol⁻¹ below the classical barrier. The steps that involve hydrogen atom transfer between S2B and Nε of His195, occurring at $\text{N2x-Fe2-brNH-Fe6-S2BH} \rightarrow \text{N2x-Fe2-brNH}_2\text{-Fe6}$ (Scheme 2, grey enclosure) and **core**-S2BH $\rightarrow$ **resting** (Scheme 2, lavender-yellow enclosures), and $\text{Fe2-brNH}_2\text{-Fe6-3b2} \rightarrow \text{Fe2NH}_3$, appear to fit the criteria for intermolecular H atom tunneling, but a detailed analysis is not available. These tunneling contributions are included in the following analyses.

4.3 Corrected energy profiles

The reaction pathways described in section 3.6 are described as their potential energy profiles. These can now be adjusted, to include the favourable entropy contributions to the dissociation of N2x (4 kcal mol⁻¹) and of NH_3 (2.4 kcal mol⁻¹), and the predicted H atom tunneling kinetic advantage (4 kcal mol⁻¹) when NH_3 is formed by transfer of 6Hx. These adjustments have been applied to pathways P2, P3, P4 and P5, and the results, P2*, P3* and P5*, are shown in Chart 2. Because the dependence of potential energy profile on electronic and spin state is small (Chart 1), the potential energies at each intermediate and TS are averaged in Chart 2 to yield a single energy profile. The adjusted path P4* is very similar to P5* and is not shown.

Each of the pathways P2*, P3*, P4* and P5* is thermodynamically and kinetically feasible. They are exergonic by more than 40 kcal mol⁻¹, and kinetic barriers do not exceed 20 kcal mol⁻¹. However there is a significant differentiating datum, which is the substantially lower barrier, ca. 5 kcal mol⁻¹, for formation of NH_3 at Fe2 from 3b2 when 2N2x is absent, in path P2*. Barriers for the other NH_3 formation steps at Fe2 or Fe6 are larger, 13 to 18 kcal mol⁻¹. As described in section 3.7 (and Fig. S4†) the Fe2– NH_3 formation step in P2* probably benefits from H-tunneling. Pathway P2* encounters an initial barrier of ca. 12 kcal mol⁻¹ for dissociation of 2N2x, followed by barriers of ca. 5 and 8 kcal mol⁻¹ in two strongly exothermic steps, and is the favoured pathway from intermediate $\text{N2x-Fe2-brNH}_2\text{-Fe6-3b2}$ to intermediate **core**.

4.4 Completion of the mechanism

The final part of the mechanism is from intermediate **core** to the resting state, shown on the salmon, lavender and yellow enclosures of Scheme 2. The migration of H from **core-3b5**, and its terminus on S2B (intermediate **core-S2BH**) as the most stable location have been described previously.^{42,62,104} The final step, **core-S2BH** to **resting**, is exergonic with a small barrier (Scheme 3, Panel F). This concludes description of the third and final phase of the complete mechanism, from $\text{N2x-Fe2-brNH}_2\text{-Fe6-3b2}$ back to **resting**.

4.5 Overall kinetics

Harris *et al.* reported steady-state kinetic studies on Mo-nitrogenase, monitoring formation of H_2 and of NH_3 using a full range of variables, and deriving key kinetic parameters.⁶ These results revealed a previously unrecognised slow step in the



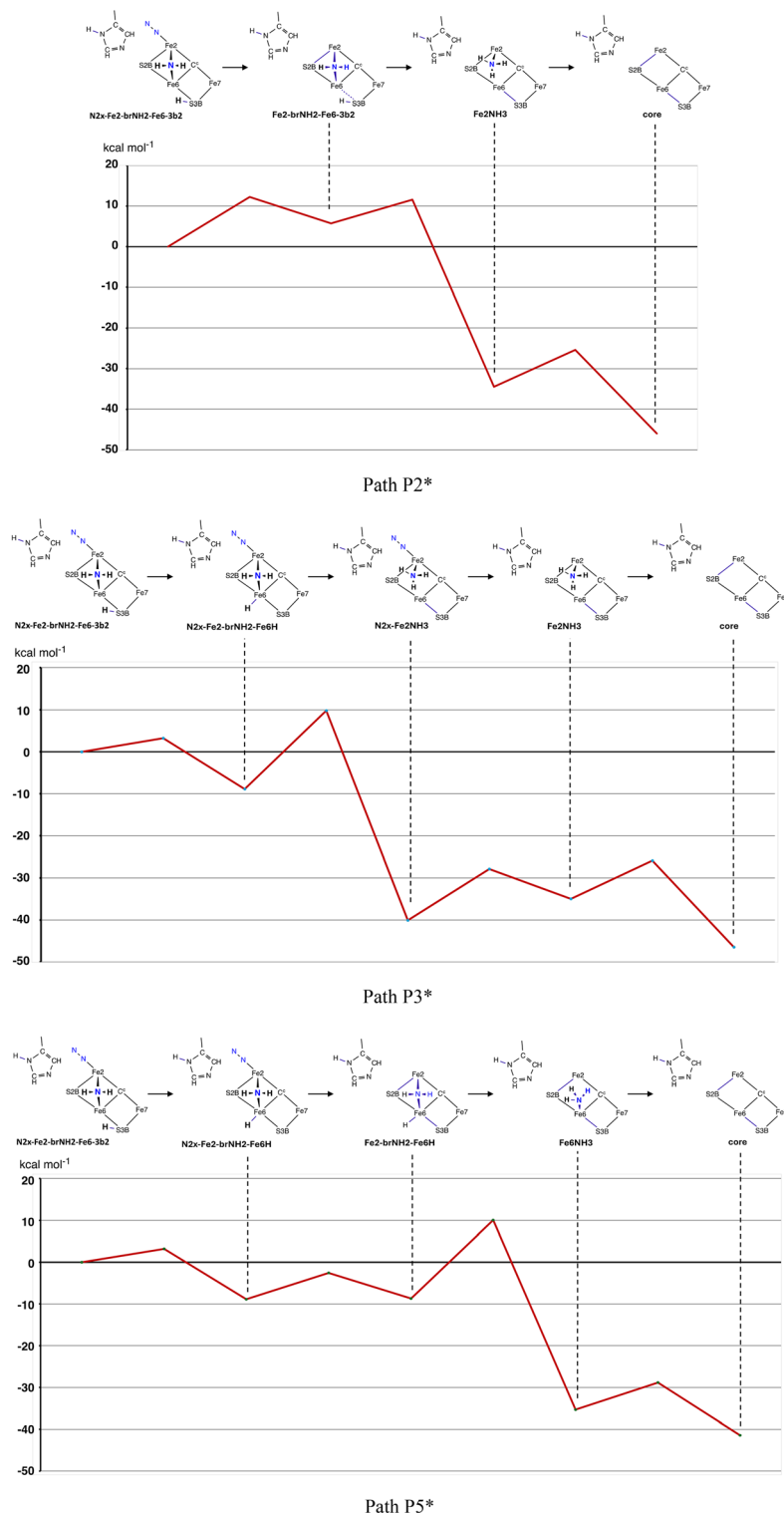


Chart 2 Energy plots for three pathways, with the estimated $T\Delta S$ entropy component applied to the potential energies in the steps where $N2x$ or NH_3 dissociate, and with the estimated tunneling contribution included to decrease the reaction barrier in the steps where $6Hx$ forms NH_3 .

route from $HNNH$ (labelled $E4(N2; 2H)$ in ref. 6) to product NH_3 . Specifically, the rate constant for conversion of the $HNNH$ intermediate to NH_3 was less than the rate of electron accumulation during these steps. The rate of N_2 binding and

formation of the $HNNH$ intermediate could not be explicitly determined. However, the conclusion was that a slow step *followed* the formation of the $HNNH$ intermediate. In the previous paper I provided an explanation of this.⁶⁷ In my calcu-



lated mechanism the formation of the HNNH intermediate (**Fe2-brNH-NH-Fe6** in Scheme 1) involved classical potential energy barriers of 19 to 29 kcal mol⁻¹, while the later N–N breaking step in which HNNH₂ converts to NH + NH₃ (lavender enclosure in Scheme 1) faces a larger potential energy barrier in the range 30 to 36 kcal mol⁻¹. Therefore I proposed that the post-HNNH slow step identified in the kinetic analysis by Harris *et al.* is the N–N breaking step. This argument depends on the absence of larger barriers in all of the subsequent steps to the conclusion of the mechanistic cycle, and the present report confirms this and the validity of the overall kinetic interpretation.

4.6 The role of S2B

The complete mechanism that I propose involves a catalytic site, FeMo-co, that has some structural plasticity without major disruption of intra-cluster bonding. In recent years the structural integrity of FeMo-co during catalysis has been questioned by the crystal structures and reactivities of Mo-nitrogenase isoforms in which S2B has been replaced by Se,¹⁰⁵ or CO,^{106,107} or, controversially, by N₂.^{108–111} The mechanistic relevance of these findings is unresolved, because the apparent displacement or replacement of S2B could reflect different dynamics between the time scales of substrate reduction and crystal growth.⁴ CryoEM diminishes this limitation. A recent cryoEM investigation of Mo-nitrogenase under pH 9.5 turnover conditions in the presence of acetylene recorded time dependent depletion of EM density in the vicinity of S2B, and changes near the S5A–Fe7 bond.³⁹ Hopefully further experiments will provide greater certainty about the presence or absence of S2B during turnover.

In the mechanism I propose S2B is retained within intact FeMo-co, and in that position has crucial roles in which an H atom on S2B is used to form an H–N bond. One role is the N₂ capture step forming **Fe2-brNH-NH-Fe6**, and the second is the **Fe2-brNH-Fe6-S2BH** → **Fe2-brNH₂-Fe6** step. In both steps S2BH is needed to form an H–N bond on the His195 side of intermediates in the reaction zone, a space which cannot be accessed by H atoms coming from the S3B source. Throughout the mechanism His195 functions as proton reservoir and proton buffer, using a hydrogen bond with S2B at a distance (*ca.* 3.2 Å) very similar to that in the resting state. Key components of the proposed mechanism would be invalidated if S2B were absent or unable to function as H donor.

The cryoEM images of Warmack and Rees mentioned above also showed other substantial changes in FeMo-co, interpreted as HCA depletion and His442 rearrangement.³⁹ There is clear distinction between any mechanism involving disruptive chemistry (S2B displaced, cofactor distorted, HCA rearranged or displaced) and the conservative chemistry of FeMo-co underlying the mechanism I have constructed.

5. Conclusions

5.1 The final phase of the mechanism

This report describes the third and final phase of the mechanism, starting at the intermediate with NH bridging Fe2 and

Fe6 and with H on S2B, then proceeding to form and dissociate the second NH₃, and return the catalytic site to its resting state. First S2BH is used to form NH₂ bridging Fe2 and Fe6. This is strongly exergonic, in part reflecting the greater strength of an N–H bond relative to S–H. Here the system has E6 6H⁺ status. The seventh electron + proton is used to generate NH₃, bound at the *endo* coordination position of either Fe2 or Fe6. Calculated reaction trajectories and potential energy profiles generate five mechanistic pathways through this NH₃ formation phase of the reaction. These pathways are evaluated with incorporation of entropic components and possible kinetic contributions by H atom tunneling, leading to identification of P2* as the most favourable pathway for generation of NH₃ and its subsequent dissociation. Addition of the eighth electron + proton straightforwardly regenerates the resting state. All steps in this third and final phase of the mechanism are thermodynamically and kinetically feasible.

5.2 The complete mechanism

In conjunction with the first⁶⁵ and second⁶⁷ phases, this report completes a trilogy describing a total mechanism for the intrinsic chemistry of Mo-nitrogenase. The mechanism contains mainstream steps and auxiliary steps. Mainstream steps involve the generation and release of H₂, the H₂/N₂ exchange, the concerted capture and hydrogenation of N₂ to form the central HNNH intermediate, further hydrogenation to H₂NNH followed by concerted breaking of the N–N bond and generation of the first NH₃, then after release of this NH₃ further hydrogenation to form the second NH₃, and its dissociation. The auxiliary steps include introduction of protons, triggered by electronation,⁶³ and migration of the resulting H atoms to positions on FeMo-co where they are required for the mainstream steps. These auxiliary steps replenish the H atom gallery, and occur without influential energy barriers.^{43,70,112,113}

5.3 Architecture and function

This proposed chemical mechanism is founded on the architecture of Mo-nitrogenase as the performance space for the choreography of catalysis by nitrogenase. Fig. 8 shows the macro-structural components that support the mechanism, and Fig. 9 shows the functional components in and adjacent to the reaction domain. With reference to these cartoons and the two prior papers of the trilogy,^{65,67} the key features and principles of the complete mechanism are summarised here.

1. All eight protons in the reaction are derived from water at the protein surface and supplied by a Grotthuss proton wire along the conserved water chain to S3B.^{58,114} This could be triggered by electron addition to FeMo-co.⁶³ In all stages except one at the beginning, proton addition to FeMo-co is accompanied by electron addition. The proton + electron combination occurs on FeMo-co as a slightly polarised H atom.¹¹⁵



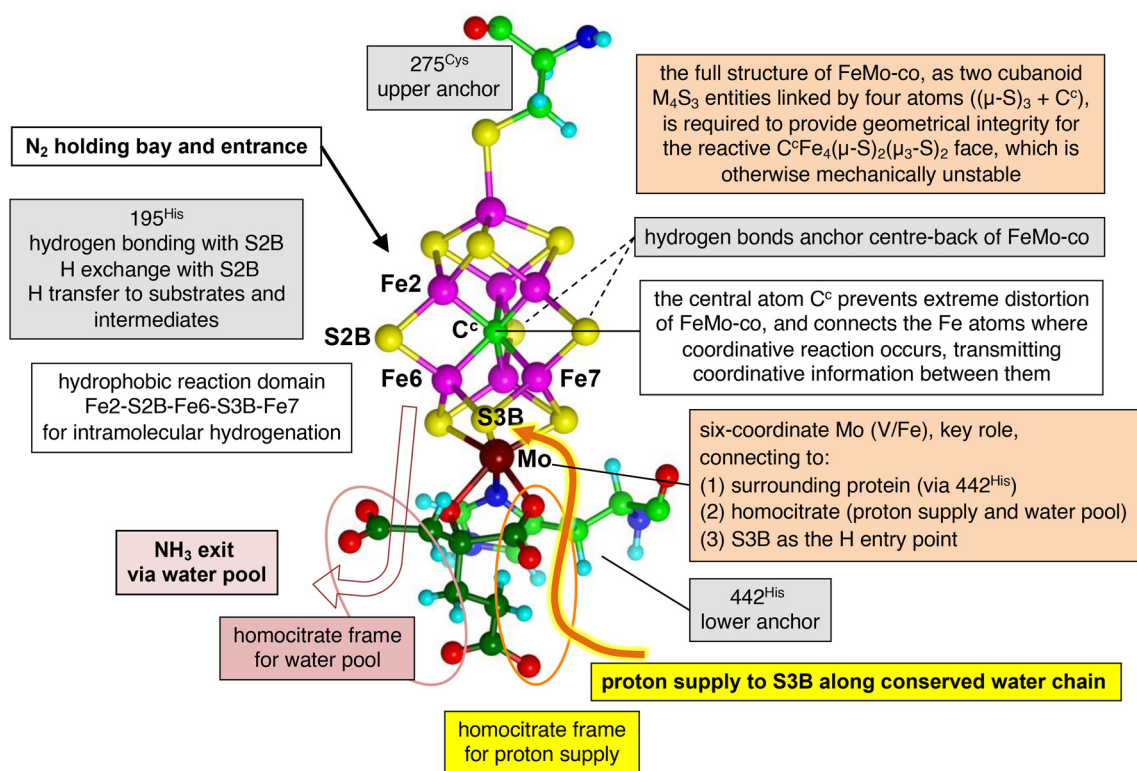


Fig. 8 Structural components of the nitrogenase active site, and their mechanistic roles supporting the proposed mechanism.

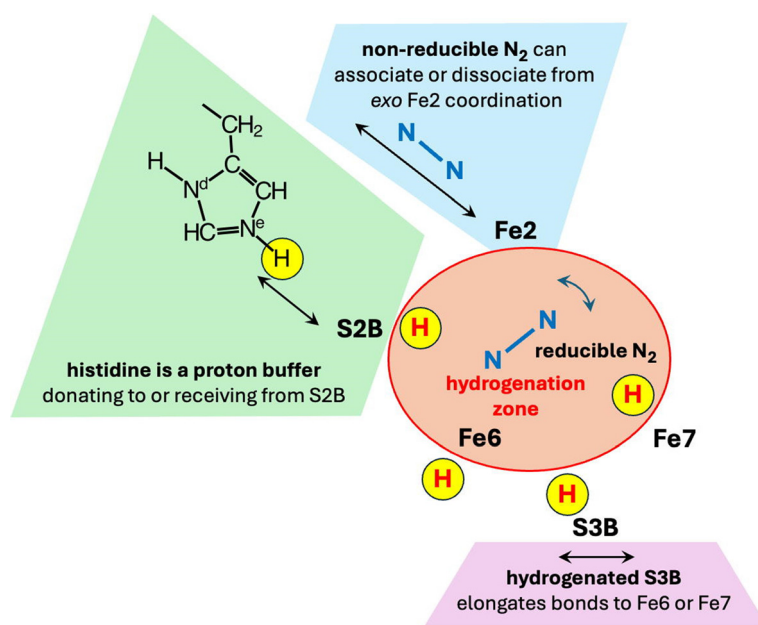


Fig. 9 Functional components of the nitrogenase mechanism. The red H atom sites, S2B-H, exo-Fe6-H, S3B-H and endo-Fe7-H, effect the intramolecular hydrogenation of N₂ and of subsequent intermediates in the hydrogenation zone.

2. An H atom on S3B can migrate to other sites on FeMo-co, with reaction barriers generally <10 kcal mol⁻¹.⁵⁸

3. From an early stage in the mechanism, until status E6 6H⁺ after N₂ capture, the number of added protons is one greater than the number of added electrons.

4. All hydrogenation steps are intramolecular, enabled by a strategic gallery of H atoms located at S2B, exo-Fe6, S3B or endo-Fe7.

5. The sidechain of His195 acts as a proton buffer, *via* Ne, donating to S2B or receiving from S2B. This is influenced



(perhaps controlled) by a hydrogen bonding network that extends from N δ of His195 through water molecules to the protein surface.¹¹⁶

6. There is no hydrogenation of intermediates directly from His195: S2B is the intermediary transfer agent between His195Ne and the N atoms of mechanism intermediates.

7. Resting state His195 is protonated at Ne and N δ . After use of this NeH *via* S2B it is replenished *via* S2B to regenerate the resting state.

8. Homocitrate has an essential role in maintaining a framework for the penultimate sections of the water chain that constitutes the proton wire, and in supporting the water pool that absorbs product NH₃ at the commencement of its egress along a defined pathway.⁹³

9. The distinctive CH₂CH₂COOH arm of homocitrate maintains a necessary separation of the acidic chemistry of the proton wire and the basic chemistry of departing NH₃.

10. H₂ is formed at the *endo* position of Fe6, from which it dissociates, providing space for reducible N₂ to enter. This is the H₂/N₂ exchange property of the enzyme.

11. Reducible N₂ entering the reaction zone is able to tumble freely (*ca.* 4 kcal mol⁻¹ (ref. 65)) and orient obliquely to Fe2 and Fe6, favouring the N₂ capture step.

12. The N₂-capture step is a concerted double hydrogenation combined with bonding to both Fe2 and Fe6. H atoms at S2B and *endo*-Fe7 are the key reactants. Two N-H bonds and three Fe-N bonds are formed in one reaction trajectory, producing bound HNNH.

13. The singular N₂-capture step allows the enzyme to bypass the notoriously difficult initial hydrogenation of unreactive N₂.⁶⁵ It is different from the conventional view of the activation of N₂ by metal systems, in which the binding of N₂ initiates activation, with subsequent hydrogenation.

14. The orientation of bound HNNH oblique to Fe2-Fe6 provides excellent stereochemistry at N for subsequent hydrogenation to form bound HNNH₂.⁶⁷

15. H at *exo*-Fe6 is well placed to break the N-N bond in the HNNH₂ intermediate and form Fe-bound NH₃, in a single step.⁶⁷ This step is proposed as the slow step identified in the kinetic analysis of Harris *et al.*⁶

16. All intermediates bond to Fe2 and Fe6 only at their *endo* positions, except the first NH₃ which is formed at *exo*-Fe6, from which it dissociates.

17. Two of the hydrogenation steps (capture and formation of bridging NH₂) are, necessarily, from the left side of the reaction zone. The first uses S2BH, and then HisNeH replenishes S2BH for the second.

18. S2B must be in its position bridging Fe2 and Fe6 to effect its two hydrogenation steps. There is ample space between Fe2 and Fe6 for both the S2B bridge and bridging intermediates.

19. Non-reducible N₂, sliding easily between the N₂ entry pocket (see Fig. 1) and ligation of Fe2 at the *exo* position,⁶⁴ modulates the coordination capacity of Fe2 and the dynamics of NH₃ dissociation from Fe2.

20. Hydrogenation steps that involve very little movement of atoms heavier than H might benefit kinetically from H atom tunneling. Further investigation is required.

21. To facilitate some reaction steps the reaction space is expanded slightly by *ca.* 1.4 Å movements of Val70 and Arg96, consistent with the secondary structure around Val70,⁶⁵ the hydrogen bond to S5A by Arg96 (Fig. S1†), and experimental reactivities of Val70 mutants.^{8,54,57}

The crucial test of this proposed mechanism is its ability to accommodate and account for *all* experimental data, and this will be elaborated in a future publication.

Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This research was undertaken with the aid of resources from the National Computational Infrastructure (NCI Australia), an NCRIS enabled capability supported by the Australian Government. My research is funded by UNSW Sydney.

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