A Theoretical Model for the Active Site of Nitrogenase

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Abstract: A model for the active site of nitrogenase is suggested and examined by means of the intermediate neglect of differential overlap (INDO) quantum mechanical method. The initial steps of the nitrogen fixation process are discussed within the framework of the present model, and it is shown that of several binding sites, initial location of the nitrogen molecule inside the MoFe cofactor is favored. Possible pathways for electron and proton delivery to the active site are also suggested on the basis of electrostatic potential calculations.

Introduction

In a recent study, Deng and Hoffmann[1] proposed a theoretical model of the nitrogen-fixing enzyme nitrogenase (N_2ase) based on the 2.7 Å resolution X-ray structure by Kim and Rees[2] and the extended Hückel (EH) method. Several possible configurations corresponding to the positioning of the N_2 molecule inside or outside the model system were studied.[1] The structure examined corresponds to the formula $[HFeS_3^*Fe_3(\mu\text{-}S^*)_3\text{-}Fe_3S_3^*MoH_4]^-$ where S^* stands for an inorganic, intracube sulfur atom, and μ indicates an intercube S^* atom. According to this structure the end transition-metal (TM) atoms—one Fe and Mo—are coordinationally saturated by H atoms.

We propose here a theoretical model for the active site of N_2ase based on the X-ray structural analysis of the enzyme at 2.2 Å resolution by Chan, Kim, and Rees[3] and the intermediate neglect of differential overlap (INDO) method as a theoretical tool that differs somewhat from that of Deng and Hoffmann.[1] The model also reflects experimental quantities obtained from ESR and Mössbauer spectra of N_2ase and suggests a reaction path for the initial stage of the nitrogen fixation process. In these calculations, we use the actual ligands attached to the end TM atoms to explore an electron count that reflects the charge distribution and the spin state inferred from magnetic experiments, and utilize a theoretical method which we have tested[4] and believe can represent the essential features of the electronic structure of the model N_2ase. In recent studies of iron–sulfur proteins[4] we have shown that proper ligand modeling and the INDO model can provide a good description of the magnetic and spectroscopic properties of such systems. Here we extend these considerations to the more complex MoFe–S cluster that is believed to be the active site of the enzyme N_2ase.[5–8]

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The Model

The model structure we utilize in this work is shown in Figure 1. In this model we preserve the basic structure as observed experimentally.[1] The ambiguous μ-Y atom in the cofactor with lower electron density than the ordinary sulfur atoms[2, 3] has been
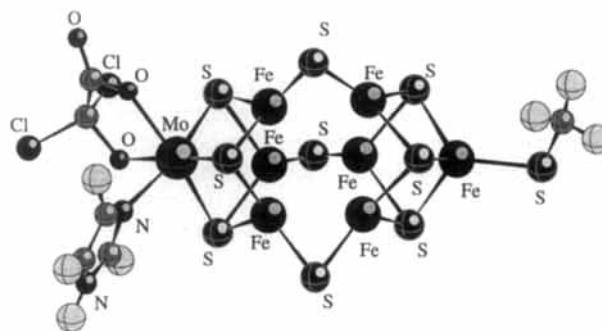

Fig. 1. The structure of the MoFe cofactor utilized in this work (see ref. [3]). The coordinates of the atoms were taken from the Protein Data Bank, Brookhaven National Laboratory.

assumed to be S in accordance with possible assignments suggested[2, 3, 9] and other X-ray examinations of N_2ase discussed elsewhere.[10] The ligand chain was truncated for Cys-α275 and His-α442 so as to involve the nearest and most influential parts of the ligating proteins to the end Fe and Mo atoms, respectively. The geometry of the homocitrate (HC) group has been preserved as reported[3] but with replacement of the two groups, -CH_2COO^- and -$(CH_2)_2COO^-$, attached to one of the C atoms by Cl^- ions, which carry essentially the same charge and electronegativity as the carboxyl group. This substitution is not expected to have a significant effect on the electronic structure, as the chlorine atoms are far from the oxygen binding site of the HC with the Mo atom; also, N_2ases with Cl-substituted HC groups still retain the enzyme's nitrogen-fixation properties.[11] At any rate, we expect that the field

created by the negatively charged groups may play a role in the eventual chemistry we wish to study.

Some averaged bond lengths and angles between the atoms in the MoFe–S cluster are: Fe–S 2.35 Å, Fe–S* 2.29 Å, Mo–S* 2.40 Å, Fe–μ-S 2.12 Å, Fe–Fe 2.73 Å, Mo–Fe 2.91 Å, Mo–O 2.30 Å, Mo–N 2.25 Å, C–Cl 2.00 Å; S*-Fe-S* angles 92.4°, Fe-μS*-Fe 71.2°, Mo-S*-Fe 93.4°.

The two sub-cubes in the cofactor are almost of the same size: the average bond length in the Mo sub-cube is 2.36 Å, compared with 2.35 Å in the Fe_4S_3 sub-cube. Given the larger size of the Mo atom compared with Fe, such a small difference could also indicate the charge distribution within the cluster: one might expect more reduced Fe forms on the Fe end of the cofactor than on the Mo end. Such a distinction is, however, difficult to observe experimentally because of the marked delocalization present in this system.[12]

The electron count for the structure must correlate with experiment. It is well-known from ESR experiments that the cofactor has a quartet ground state.[13, 14] This corresponds to three unpaired electrons out of 35 to 44 open-shell d electrons from 7 Fe and 1 Mo if we assume formal oxidation states + 2 and + 3 for the iron atoms and + 4, + 5, or + 6 for molybdenum. Mössbauer experiments[14, 15] strongly suggest that the oxidation state of the Mo atom in the native structure is + 4. This reduces the electron count range to 37–44. The fact that some of the Fe atoms are in a formal + 3 oxidation state, others in + 2, additionally limits the range of possible electron configurations. Furthermore, Mössbauer experiments[15] suggest that the average charge on the Fe atoms in the MoFe cofactor is + 2.66. Only two combinations of Fe^{II} and Fe^{III} can result in such an average charge: overall 39 electrons (2 from Mo, 25 from 5 Fe^{III} and 12 from 2 Fe^{II}), or 40 electrons (2 from Mo, 20 from 4 Fe^{III} and 18 from 3 Fe^{II}), with Fe charge averages of + 2.71 and + 2.57, respectively. Only the former, however, can give rise to a quartet. Other combinations, such as (4 Fe^{II} and 3 Fe^{III}) give an average charge + 2.43, which is far from experiment and is, therefore, not so likely, though such a combination seems reasonable from the point of view of balance between the Fe^{II} and Fe^{III} species. This latter charge distribution was suggested by Deng and Hoffmann;[11] this assumption should bring more negative charge to the system than the model we propose in this work. It would, therefore, be interesting to examine how such an electron-excessive model can be used to interpret the initial fixation of the nitrogen molecule, as compared with the 39-electron model adopted in this work. Such a comparison might also give us some ideas about the reactivity of the more reduced forms of the MoFe cofactor experimentally detected in the initial stages of the nitrogen fixation process.[12]

The core charge of the MoFe–S cluster we examine is calculated as + 5. Given that the HC ligand carries a formal negative charge −4, and the additional −1 charge that comes from the thiolate group modeling the Cys-α275 protein junction with the cofactor, the overall model structure is neutral. Besides other computational advantages (such as the possibility of carrying out effective electrostatic potential calculations), the zero charge obtained for the present model gives better representation of the native state, which is assumed to be neutral.

Results and Discussion

We report our initial findings here. Although we fully expect to examine this process further, we hope this model will stimulate others to pursue this approach also.

The INDO/1 model has been used in this work, as it has proven useful in a number of previous studies of TM in biological systems.[4, 17] The quartet spin state was calculated within the restricted open-shell Hartree–Fock (ROHF) approximation. Such a state with $S = \frac{3}{2}$ is singled out among 365 560 possible spin-flip states of 39 d electrons in 40 open-shell orbitals by means of a minimization procedure based on the configuration-averaged Hartree–Fock (CAHF) method[18] and a particular technique described in detail elsewhere.[19] This state has the lowest energy compared with other quartet states that stem from the above possibilities and is uniquely defined by the ROHF approximation.[20] We should also mention that other methods used to study similar TM systems, such as the X_α method or the unrestricted Hartree–Fock (UHF) model, either have difficulties with the proper description of the spin state or carry a significant amount of spin contamination in the calculations.[20] The EH method is useful in studies concerning one-electron properties but is typically unreliable for geometries, as two-electron interactions are not explicitly included in the Hückel model.

An important problem for the chemistry involved in the nitrogen fixation process is the specific reactivity of the MoFe cofactor. In order to shed light on this problem, we have performed electrostatic potential calculations on structure **1** with CAChe software[21] and the multiple moment approximation for the electrostatic potential using a 0.3 Å grid resolution. The result obtained is shown in Figure 2. The red color indicates positive, electron-attracting electrostatic potential. The blue zones are those which correspond to proton attraction; owing to the negative electrostatic potential in these regions, electron paths through such zones are unfavorable.

First, there is a clear indication that protons are likely to be supplied to the MoFe cofactor through the HC group, the Cys-α275 residue connected to the end Fe, or right in the middle of the cofactor (the blue zone around the μ-S* atoms). This proton could come, for example, from the histidine group His-α195, which has recently been found to be of crucial importance in the performance of the enzyme.[22] Similar observations have been made for the HC group, which is also a determining factor in enzyme performance.[11] Mutations in the Cys-α275 residue do not have a significant effect on the N_2ase activity, which might indicate that only a minor role is played by this residue in the overall process.

Secondly, the supply of electrons to the system is as important as that of protons, since both 8 electrons and 8 protons are needed to produce two molecules of ammonia and one
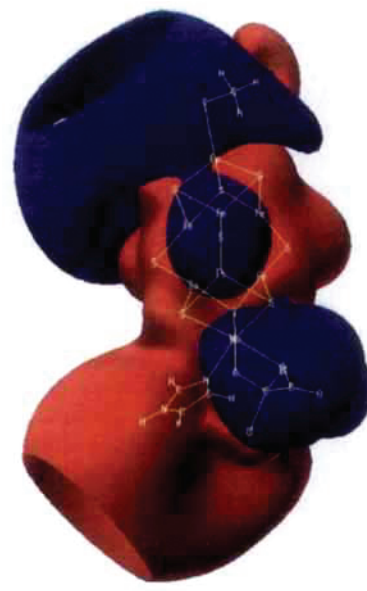

Fig. 2. The electrostatic potential of the MoFe cofactor obtained from the CAChe-ZINDO calculations, ref. [21]. The red zones indicate positive, electron-attractive potential; the blue areas are those corresponding to proton affinity. The electrostatic potential is calculated in a radius of 16.5 Å around the center of the cluster.

molecule of H_2 according to the basic stoichiometry of Equation (1).[20] Our electrostatic potential calculations show that

$$16\,MgATP + 8\,e^- + 8\,H^+ + N_2 \longrightarrow 16\,MgADP + 16\,P_i + 2\,NH_3 + H_2 \quad (1)$$
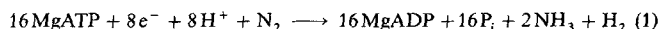
the most probable way for electrons to reach the cofactor is along His-α442 connected to the Mo atom. This idea is supported additionally by the facts that i) the distance between the P cluster, believed to be the source of electrons, and the Mo side of the cofactor is much shorter than the distance to the end-Fe side,[3] ii) the protein chains of the P-cluster approach the Mo side, as can easily be observed in the X-ray structure,[3] and iii) the addition of an electron to the model system and recalculation of the electrostatic potential turns all regions of Figure 2 to blue (electron-repulsive) except for the His-α442 group, which remains electron-attractive. This path for electron supply is again the most likely one after the attachment of the N_2 molecule to the cofactor (vide infra).

An important question is exactly *how* the MoFe cofactor succeeds in breaking apart such a very stable molecule as N_2. The cofactor we have modeled has a charge distribution inferred from the Mulliken population analysis, corresponding to Mo^{IV}, $5\,Fe^{III}$ and $2\,Fe^{II}$ ions in their formal oxidation states, consistent with our assumption. The dipole moment of this molecule is large (23.18 D), but we should note that this calculated value, though seemingly high, is quite reasonable given the length of the model structure—the distance between the end Fe and the HC group is approximately 12 Å, so that even higher values of the dipole moment might be possible on the basis of simple electrostatic considerations. If we insert the model active site into a reaction field, utilizing the self-consistent reaction field (SCRF)[23] model, the calculated dipole increases to 29.70 D. The SCRF, however, was found not to have a significant effect on the interaction between the cofactor and the N_2 molecule we describe here. These findings may indicate that the dipoles induced by the protein around the active site have no direct effect on the cofactor reactivity. The latter is, however, strongly dependent on intact supplies of reactants and electrons, the exclusion of external inhibitors (such as O_2), and the removal of the reaction products, factors which are managed in a complex way by the protein environment.[2, 3, 9]

The nitrogen molecule is small and light compared with the atoms that make up the N_2ase active site, so it can easily reach various places in the relatively open MoFe cofactor.[3] The actual positioning of the molecule with respect to the cluster is unknown, although various suggestions have been made.[1, 12] Instead of comparing different possible dispositions of the N_2 molecule on or in the MoFe cofactor, we have allowed complete freedom of the N_2 molecule to migrate through the structure starting from different initial N_2 positions in or on our model structure. Thus, the position of the N_2 molecule is judged on the basis of optimization of geometry (total energy) rather than from a comparison of guessed single points. A serious shortcoming of the present calculation, however, is the initial neglect of cofactor relaxation around the N_2 molecule which might be of importance in the advanced stages of the nitrogen fixation process. We therefore restrict ourselves to studies of the initial binding of the N_2 molecule to the MoFe cluster of Figure 1, and report the first step of this process here.

All the geometry optimizations we have performed have led us to the structure shown in Figure 3, which gives the most energetically favorable positioning of the N_2 molecule in the MoFe cofactor. Since the geometry optimization we have performed imposes no restrictions on the location of the N_2 molecule, this structure should be viewed as the structure preferred
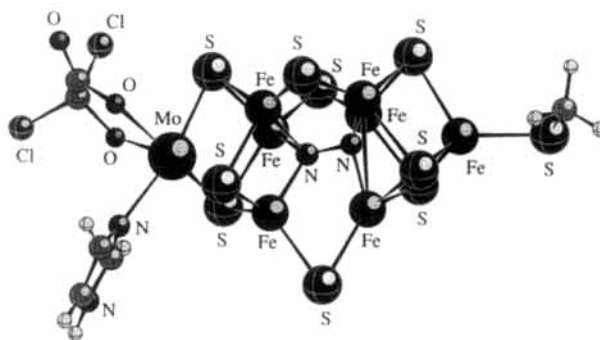

Fig. 3. The preferred attachment of the N_2 molecule within the MoFe cofactor. Note that the N atom closer to the Mo side is more tightly bound to the surrounding Fe atoms than the other nitrogen atom. The former N atom is suggested to be protonated first in the subsequent step of the nitrogen fixation process, see text.

according to our model calculations. Several interesting features are observed in Figure 3, and these can be commented upon as follows.

The first and perhaps most intriguing finding we wish to report is that the N_2 molecule was found to bind preferentially *within* the cluster, forming multiple bonds with the 6 central Fe atoms. This position of the N_2 molecule is independent of the starting conditions and yields a marked energy gain of nearly $150\ \text{kcal mol}^{-1}$ with respect to the sum of the energies of the isolated reactants. The INDO model we use here is known to overbind by about a factor of two, but the calculated relative energies are believed accurate. The idea that the initial N_2 attachment be to this internal position in the cofactor was recently put forward on the basis of steric factors, given the relatively open structure of the cofactor and the existence of 6 coordinatively unsaturated Fe atoms in the middle of the cofactor.[2, 3] Our calculations strongly support this suggestion as being the first step in the nitrogen fixation process. According to Figure 3, the N_2 molecule binds with the Fe atoms in a slightly asymmetric way—the N_2 molecule is not colinear with the end-Mo–end-Fe line but prefers a bent position: the Mo-N-N angle is 150.6°, the N-N-end-Fe 152.0°, and the dihedral angle Mo-N^1-N^2-end-Fe is 187.0°. The molecule is also found to be slightly displaced towards the end-Fe sub-cube, which carries more negative charge (see the Mulliken population analysis for more details) and is thus more reductive in character—the shortest Mo-N^1 distance is 3.46 Å, while the nearest N atom is 2.79 Å away from the end Fe^1 atom of the cofactor (see also Table 1). The binding pattern of the two N atoms is also different; a Wiberg analysis[24] indicates that the N atom on the Mo side is bound more strongly to the Fe manifold than the other one which is closer to the end Fe atom. This is reflected in the different charges the two atoms possess, as shown in Table 1. The nitrogen atom closer to the Mo atom is more negative and is, therefore, more promising as a binding site for an incoming proton in the next step of the nitrogen fixation process. This is also seen from our electrostatic potential calculations for the structure of Figure 3. A closer view of the N_2 molecule potential is shown in Figure 4. The difference in the electron density on the two N atoms is readily seen in this figure, where the blue regions of the N^1 potential surface indicate that this atom is prone to further H^+ bonding. This effect is much more pronounced for the reduced form of the cofactor (see below) where larger polarization of the N_2 molecule is observed (Table 1). We can, therefore, suggest that the first incoming proton should be more likely to attack the N atom closer to the Mo atom.

Similar results were obtained for the alternative 41-electron model of the active site already discussed. The differences in the

Table 1. Atomic charges from the Mulliken population analysis and geometric properties for the two alternative electron counts examined, the 39 and 41 open-shell electron models. The charges on the N atoms, the N–N bond length in the optimized structure, the N–N bond index (b.i.), and interatomic distances and angles are also given. The first column shows data for the MoFe cofactor prior to attachment of the N_2 molecule, the second column, after the optimization of the N_2 molecule position. The first four lines correspond to the end-Fe sub-cube, the next four to the $MoFe_3S_4$ sub-cube.

	39 Electrons		41 Electrons	
end-Fe[1]	0.984	0.819	0.846	0.855
Fe[2]	0.776	0.977	0.737	0.933
Fe[3]	0.940	1.267	0.808	1.019
Fe[4]	0.698	1.023	0.640	1.066
Fe[5]	0.754	0.873	0.680	0.663
Fe[6]	0.787	0.946	0.669	0.700
Fe[7]	1.028	1.007	0.967	1.014
end-Mo[8]	1.159	1.100	0.964	0.895
N[1]		− 0.486		− 0.510
N[2]		− 0.385		− 0.280
$r(N^1-N^2)$ (Å)		1.26		1.40
N^1-N^2 (b.i.) [a]		0.999		0.856
$r(Mo^8-N^1)$ (Å)		3.46		4.01
$r(Fe^1-N^1)$ (Å)		2.79		1.97
$r(Fe-N)$ (Mo side) (Å)		1.90±0.10		2.20±0.10
$r(Fe-N)$ (Fe side) (Å)		1.90±0.20		1.90±0.10
$r(Fe^1-N^1)$ (Å)		2.79		1.97
$⋆Mo^8-N^1-N^2$		151°		176°
$⋆N^1-N^2-Fe^1$		146°		170°
$⋆Mo^8-N^1-N^2-Fe^1$		70°		150°

[a] The bond index for triple-bonded N_2 is 3.0, that for double-bonded O_2, 2.0, etc. See also refs. [24, 26].

optimized geometries, charge distributions, and bonding parameters can be seen from the comparison given in Table 1. The only significant deviation observed from the structure of Figure 3 is the slightly larger displacement of the N_2 molecule towards the end-Fe sub-cube, which results in an increased Mo–N^1 interatomic distance (4.01 Å). Again partial negative charges are observed at the N atoms, a charge distribution originating predominantly from Fe-to-N d → π^* charge transfer (see Table 1). As in the 39 open-shell electrons model, the larger negative charge is detected on the N atom closer to the Mo atom, and this N atom is again the more probable candidate for

Fig. 4. A closer view of the electrostatic potential on the two N atoms within the MoFe cofactor. The blue areas on the N atoms indicate the negative, proton-attractive potential.

the initial protonation. Interestingly, we find that the electron donors are predominantly the Fe atoms that belong to the end-Fe sub-cube: the charge supplied to the N_2 molecule by the end-Fe sub-cube is 3.5 times larger than that provided by the $MoFe_3$ sub-cube. This difference becomes even more pronounced for the reduced 41-electron model, where the charge-transfer is dominated by the end-$FeFe_3$ sub-cube. If this is the case, this finding gives a hint as to why the native enzyme does not necessarily need Mo atoms, but can operate with a vanadium substitute, or even with all-Fe cofactors.[6 − 8, 12]

Finally, we would like to mention that the first step of the N_2 fixation that we examined here was found to have an enormous effect on the N–N bond strength. The N–N bond index, initially 3 (a triple bond) is reduced to almost 1 (a single bond) as follows from our calculations for the first step (see Table 1). As already noted, this effect is mostly caused by d → π^* charge transfer from the Fe atoms to the N_2 molecule. The population of the N_2 antibonding π^* orbitals leads to a marked extension of the N–N bond to 1.26 Å in the 39-electron case and 1.40 Å in the case of 41 open-shell electrons, compared with the 1.1 Å bond length observed in diatomic N_2. A bond length of 1.4 Å between N atoms is very large indeed, and might suggest that reduction of the cofactor might precede the proton addition, although this is certainly conjecture at this stage of our examination.[25]

Conclusions

We report here on a model that we think reliably mimics the active site for nitrogen fixation in nitrogenase. It is consistent with the quartet spin state and with the available Mössbauer information. We have explored various positions for the nitrogen attachment and, although the potential energy surface is reasonably flat for a wide range of similar structures and has many local minima, found the most stable one to be the one in which the N_2 molecule is internal to the cluster. This attachment leads to a considerable extension of the N–N bond, and leaves both ends of the N_2 molecule with a net negative charge. Although most of this d → π^* charge transfer originates from the Fe-ended cube, we speculate that the first proton binds to the nitrogen atom on the Mo-end side, as suggested by the electrostatic potential. We are now examining further steps in this process.

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[25] Typically the same results are obtained for more reduced forms of the cofactor containing, for example, 42 open-shell electrons, in which case the N–N bond length is increased slightly to 1.405 Å. In these latter reduced forms of the cofactor, one can expect greater metal–sulfur bond lengths which result in expansion of the cofactor, thus facilitating the access of the N_2 molecule to the interior of the active site; see refs. [2, 3].

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