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Computer modelling of electron paramagnetic resonance-active molybdenum(V) species in xanthine oxidase †

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Density Functional Theory calculations have been used to investigate structural models for the 'Very Rapid' and 'Inhibited' EPR signals ascribed to xanthine oxidase Mo^V species. Analysis of the observed hyperfine coupling tensors has suggested close Mo–C contacts in both cases and a side-on interaction between the substrate's carbonyl group and the Mo centre has been proposed. Attempts to confirm this for several model 'Very Rapid' species, based on a previous structure for the active site, either give short Mo–C contacts but too small a spin density on Mo or long Mo–C distances and a more reasonable Mo spin. Either the model system or the interpretation of the experimental data requires revision. In contrast, a good model can be developed for the 'Inhibited' species, which arises during reaction with formaldehyde, which is consistent with the EPR and other experimental data. However, rather than involving side-on co-ordination of the formaldehyde carbonyl group, the 'Inhibited' species forms a C–S bond between the formaldehyde and the sulfido ligand.

Interest in molybdoenzymes is booming.[1,2] Much of this is fuelled by recent single crystal X-ray diffraction studies on aldehyde oxidoreductase[3] (AOR) and dimethyl sulfoxide reductase.[4–7] The former is representative of the molybdenum hydroxylases of which xanthine oxidase (XnO) is a particularly well studied example.[8,9] There is enormous activity in trying to develop better mechanisms by correlating these new structural data with the wealth of other chemical, biochemical and physical measurements.

Electron paramagnetic resonance (EPR) spectroscopy is a powerful probe of the active site chemistry of molybdoenzymes.[8,10–18] Bray *et al.*[8] have made extensive use of EPR to follow the catalytic cycle of, among many other systems, xanthine oxidase. The technique is limited to paramagnetic Mo^V species but several distinct signals are obtained which have helped to unravel some of the mechanistic details.[1]

With xanthine as substrate, a signal labelled 'Very Rapid' is observed. This is believed to involve a Mo^V species lying on the catalytic pathway. The EPR data suggest a $Mo^VOS(OR)$ moiety where OR represents the bound product anion.[15] With formaldehyde, no 'Very Rapid' signal is seen, although formaldehyde is turned over. Instead, the EPR spectrum shows a new signal dubbed 'Inhibited'. As the name suggests, this signal corresponds to a Mo^V species which eventually stops catalysis. However, full activity can be restored by removing the excess formaldehyde. Less is known about the structure of this species although it is believed to contain a short Mo–C bond.[10]

In general, conventional EPR spectroscopy does not give definitive structural information. Some clues are afforded by comparison with model Mo^V complexes but the XnO active site is sufficiently unusual that relevant model systems are few and far between.[19–21] The problem is exacerbated since the EPR signals are observed during the reaction with substrates (catalytically or otherwise) and characterising intermediates is challenging even for simple reactions let alone in a complex enzyme system like XnO.

However, more sophisticated spectroscopic methods such as electron-nuclear double resonance (ENDOR) spectroscopy can provide much more information, particularly if isotope enrichment is employed to facilitate the observation of superhyperfine couplings. The ENDOR spectra for both the 'Very Rapid' and 'Inhibited' species exhibit coupling to the substrate carbon. An analysis of these data led to the suggestion that Mo–C bond formation is a key feature of the mechanism.[10–12]

We have been testing these proposals theoretically. Computer modelling is providing unique insights into metalloenzyme structure and catalysis.[22–27] High level quantum mechanical calculations are not limited by the constraints of synthetic chemistry. In principle, any model structure can be built and tested. Moreover, modelling is probably the only viable technique which can locate and characterise transition states.[22,28] However, powerful as it is, computer simulation is still far from perfect and must remain firmly rooted in experiment. Computer models must be consistent with the available experimental data in order for our predictions to have any validity.

This paper extends our previous theoretical studies on the reductive half-cycle of the oxygen-atom transfer reaction[22,23] to our most recent efforts to explore the oxidative half-cycle. We have focused particularly on developing structural models for the 'Very Rapid' and 'Inhibited' species seen by EPR spectroscopy for XnO.

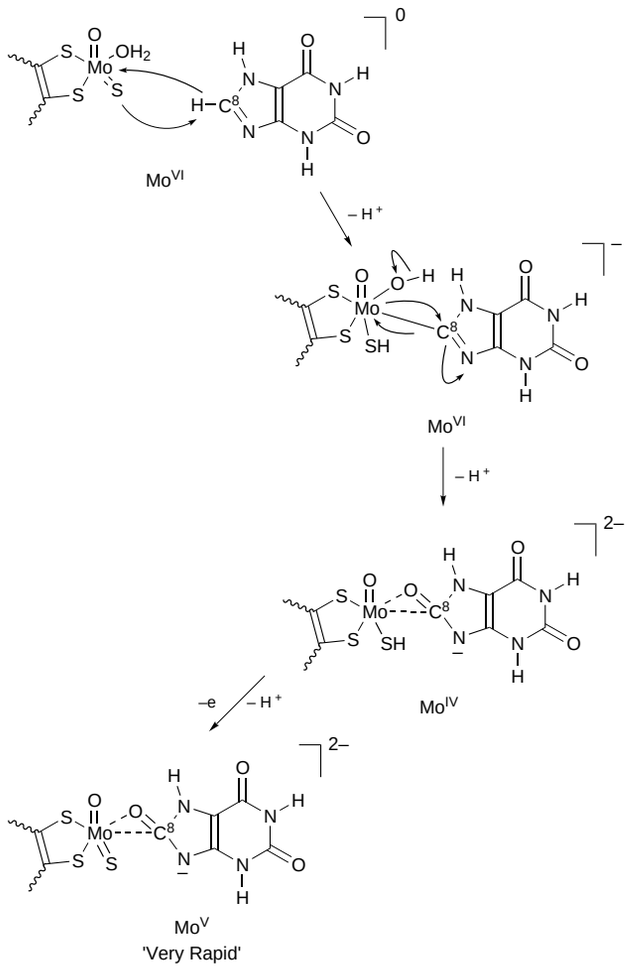
Computational Details

All calculations were performed with the Amsterdam Density Functional (ADF) program version 2.0.1 through 2.3.0.[29–31] The implementation of the local density approximation (LDA) uses the standard Slater exchange term[32] and the correlation term due to Vosko *et al.*.[33] Geometries were optimised using analytical energy gradients[34,35] usually within a spin-unrestricted formalism. Gradient corrections to the total binding energies were computed at the LDA-optimised geometries using the exchange correction of Becke[36] and the correlation functional of Perdew.[37]

Basis sets comprised uncontracted triple-ζ expansions of Slater type orbitals (ADF basis sets IV). All bases were augmented by additional functions (p on metals and H, d on the rest). The frozen core approximation[38] was used throughout:1s on carbon, nitrogen and oxygen; 1s–2p on S and 1s–3d on Mo.

No symmetry nor other constraints were applied during geometry optimisation. Default convergence criteria were used apart from the numerical integration factor which was set to 4 (the default value is 3).

† Based on the presentation given at Dalton Discussion No. 2, 2nd–5th September 1997, University of East Anglia, UK.



Scheme 1

Results and Discussion

Our calculations are based on density functional theory (DFT) which is remarkably successful especially for transition-metal systems[28,39,40] but has only recently been applied to bio-inorganic problems.

Our previous studies on XnO[22,23] have generated four important results. First, a five-co-ordinate model active site gave the best agreement with extended X-ray absorption spectroscopy (EXAFS) results.[41] The single-crystal X-ray diffraction study of aldehyde oxidoreductase has a five-co-ordinate active site.[3] Secondly, the fifth ligand was identified as hydroxide. Aldehyde oxidoreductase has a co-ordinated water but this is believed to deprotonate prior to reaction.[42] Thirdly, the calculations demonstrated the formation of Mo–C interactions at the beginning of the catalytic cycle which carry through to the reduction phase where they are detected by EPR spectroscopy.[11,12] Fourthly, the modelling was consistent with OH transfer being at least as competitive as oxo transfer and probably dominant. The prevailing view now favours OH transfer as the likely method for incorporating oxygen into the substrate.[42]

We turn now to the identity of the paramagnetic Mo^V species. The chemistry and the analysis of the ENDOR data place some quite tight electronic and geometric structural constraints on any model system. For the 'Very Rapid' species, a Mo–C distance of 2.2–2.35 Å is suggested by the ENDOR analysis with strong spin delocalisation onto the sulfido sulfur and a small coupling to the substrate carbon. A scheme consistent with these and other observations has been proposed by Lowe *et al.*[43] (Scheme 1) leading to a Mo–O–C metallacycle. For the 'Inhibited' species, the ENDOR analysis suggests a somewhat shorter Mo–C distance of 1.7–1.9 Å, a small spin delocalisation onto sulfur and the substrate carbon and a rather larger spin on one of the substrate oxygens (*i.e.* not the oxo ligand). Lowe *et al.*[43] again favour a side-on bound carbonyl group.

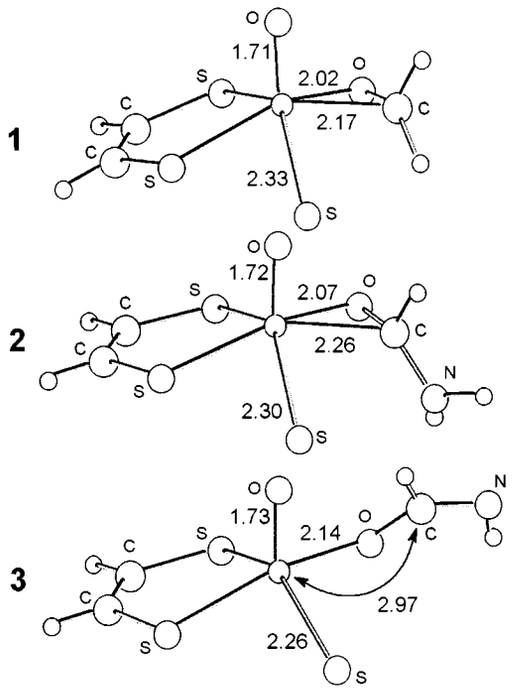


Fig. 1 Calculated structural models for the 'Very Rapid' species: **1** $[MoOS(H_2dt)(\eta^2\text{-}OCH_2)]^-$; **2** $[MoOS(H_2dt)(\eta^2\text{-}OCHNH_2)]^-$; **3** $[MoOS(H_2dt)(\eta^1\text{-}OCHNH)]^{2-}$. All distances in Å

The 'Very Rapid' species

The 'Very Rapid' species depicted in Scheme 1 has an η^2-carbonyl group from deprotonated uric acid [7,9-dihydro-1*H*-purine-2,6,8(3*H*)-trione]. The simplest model substrate is therefore formaldehyde itself. However, note that there is no sensible way of writing a catalytic cycle which would actually generate a co-ordinated formaldehyde in this way. This would imply $[CH_3]^+$ as the starting point. The calculations are merely designed to demonstrate whether this type of Mo–carbonyl interaction can be supported on an otherwise reasonable model for the XnO active site.

Full DFT geometry optimisation of formaldehyde bound to $[MoOS(H_2dt)]^-$ ($H_2dt = S_2C_2H_2^{2-}$) gives structure **1** shown in Fig. 1. The carbonyl group is co-ordinated in a side-on mode with a Mo–C distance of 2.17 Å, very close to the range (2.2–2.35 Å) derived from the ENDOR data. However, the theoretical spin distribution is not in such close accord. We compute 3.6% spin on the carbon atom, which seems reasonable, but 64% spin on the sulfido sulfur and only 2.6% spin on Mo. The remaining spin density is on the dithiolene with about 20% on the sulfur atoms (although most of this is concentrated on the sulfur *cis* to the sulfido ligand) and about 10% on the carbons. Experiment indicates the sulfido spin should be about 38%[44] and the Mo spin about 65%.[45]

Assuming that the Mo^V species shown in Scheme 1 is ostensibly correct, although this has been questioned,[1] the presence of a significant dithiolene contribution to the singly occupied molecular orbital may be the result of using such a simple model for the molybdopterin cofactor [a compound based on 2-amino-4(1*H*)-pteridinone (pterin)]. Alternatively, using formaldehyde instead of uric acid may perturb the electronic structure. The rather better results computed for the 'Inhibited' species (see below), which still use the model dithiolene but a chemically correct substrate, argue for the latter. To test this hypothesis, geometry optimisations were carried out for some other Mo–carbonyl species.

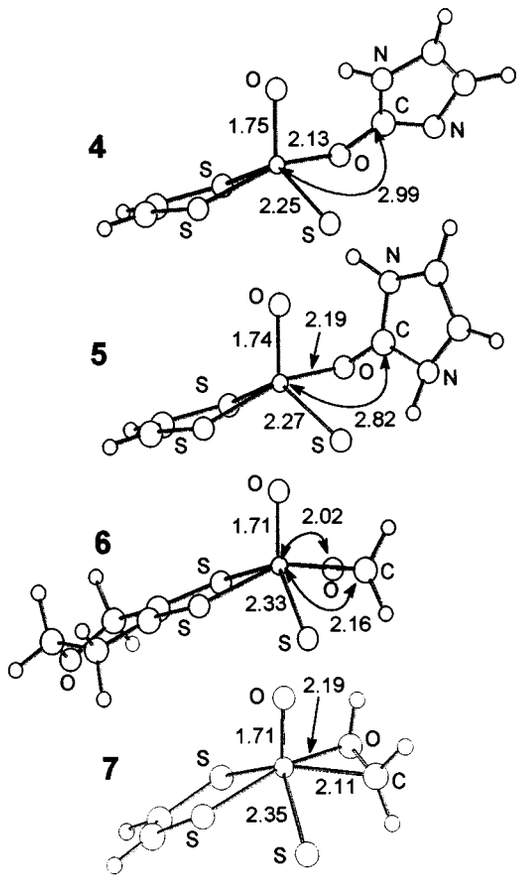


Fig. 2 Calculated structural models for the 'Very Rapid' species: **4** $[MoOS(H_2dt)(\eta^1\text{-Oim})]^{2-}$; **5** $[MoOS(H_2dt)(\eta^1\text{-OimH})]^{-}$; **6** $[MoOS(S_2R)(\eta^2\text{-}OCH_2)]^{-}$; **7** $[MoOS(H_2dt)(\eta^2\text{-}HOCH_2)]$. All distances in Å

Fig. 1 shows the geometry optimisation for formamide bound to $[MoO(H_2dt)]^{-}$, $[MoOS(H_2dt)(OCHNH_2)]^{-}$ **2**. Again, the side-on carbonyl motif appears with a computed Mo–C distance of 2.26 Å. However, the computed spin distribution is very similar to the formaldehyde model. Again, formamide itself is not a realistic candidate for a true 'Very Rapid' species. In contrast, the N-deprotonated species can be traced back to the parent imine $H_2C{=}NH$ which at least has the virtue of being a more reasonable substrate. However, the corresponding 'Very Rapid' model **3**, $[MoOS(H_2dt)(OCHNH)]^{2-}$ (Fig. 1), does not now show the side-on carbonyl co-ordination but does reinstate about 64% spin density on the Mo centre with 34% spin on the sulfido sulfur but only 0.6% on the substrate carbon.

Thus, either we obtain a molecular structure which is consistent with the ENDOR analysis, but with a theoretical spin distribution which is inconsistent, or *vice versa*. Given that the results vary dramatically with substrate, we conclude that our model system is inadequate. Accordingly, further calculations were attempted using imidazole as the notional substrate. Semi-empirical molecular orbital (MO) calculations comparing the frontier orbital compositions for imidazole and xanthine[46] show that the latter's are dominated by contributions from the imidazole fragment, suggesting that imidazole should be a reasonable electronic model. The optimised geometry of $[MoOS(H_2dt)(Oim)]^{2-}$ (**4**, Oim = $[O{=}\overline{CNHCHCHN}]^{-}$), which conforms to the proposal of Lowe *et al.*,[43] is shown in Fig. 2.

A long Mo–C bond is found. However, there are some clues in the structure which suggest new models. There is an obvious N–H···O interaction between the imidazole and the oxo group indicating that electrostatic interactions may be important. In the model system, there is formally a negative charge on the other imidazole nitrogen and we might therefore anticipate that this site is susceptible to protonation. This would allow for a N–H···S interaction which might force a shorter Mo–C distance. The optimised structure for the model $[MoOS(H_2dt)(OimH)]^{-}$ **5** is shown in Fig. 2.

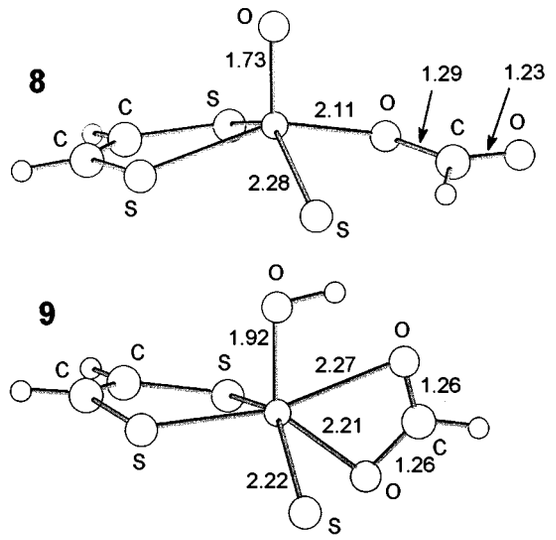


Fig. 3 Calculated structural models for the 'Inhibited' species: **8** $[MoOS(H_2dt)(\eta^1\text{-OCHO})]^{2-}$; **9** $[Mo(OH)S(H_2dt)(\eta^2\text{-}O_2CH)]^{-}$. All distances in Å

The intramolecular N–H···O and N–H···S interactions lead to a non-planar imidazole with both N–H bonds bent significantly towards the oxo and sulfido groups. There is evidently a strong competition between these interactions and the tendency for a large Mo–O–C angle which results in quite a strained system. Therefore, this model is probably not very reasonable. In any event, the Mo–C distance is only restrained to about 2.8 Å. Returning to formaldehyde but using an improved model for the molybdopterin with the entire pyran (R) ring included {$[MoOS(S_2R)(O{=}CH_2)]^{-}$ **6**, Fig. 2}, does not remedy the problem. Just as for the other formaldehyde model **1**, a short Mo–C distance and near zero Mo spin density result.

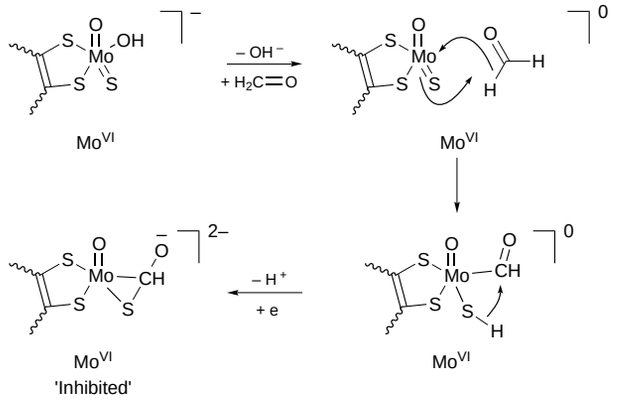
The final model we considered assumes that the formaldehyde oxygen is protonated. This should certainly have the effect of generating a strong Mo–C interaction since the C will now be formally sp^3 hybridised with a full Mo–C single bond. The computed structure of $[MoOS(H_2dt)(HOCH_2)]$ **7** is shown in Fig. 2. Unfortunately, while the Mo–C distance is short (2.1 Å), the Mo spin density is still too small (6.9%).

The 'Inhibited' species

During the investigation of likely candidates for the 'Very Rapid' species, one feature emerged which is relevant for modelling the 'Inhibited' moiety. Specifically, calculations on Mo^{V} systems with a terminal sulfido group generally give large spin delocalisation onto the sulfur (30–60%) while any with SH show only small S spin populations. However, experiment shows that the 'Inhibited' species does not have an SH group.

The ENDOR data suggest a short Mo–C interaction of around 1.9 Å and Lowe *et al.*[43] favour a side-on bound carbonyl structure similar to their 'Very Rapid' species proposal. This implies that no oxygen-atom transfer has occurred. Rather, formaldehyde co-ordinates directly and inhibits the enzyme. However, to test the possibility that oxygen-atom transfer might occur, our initial calculations focused on models based on formate (Fig. 3).

None of these species is satisfactory. Any unidentate formate, for example $[MoOS(H_2dt)(OCHO)]^{2-}$ **8**, leads to a large Mo–O–C angle such that the Mo–C distance is around 3 Å. A bidentate structure, such as $[Mo(OH)S(H_2dt)(\eta^2\text{-}O_2CH)]^{-}$ **9**, gives a shorter Mo–C bond but this is still some 2.6 Å. In any event, all these structures have sulfido groups with large spin populations which is at odds with experiment. The resolution



Scheme 2

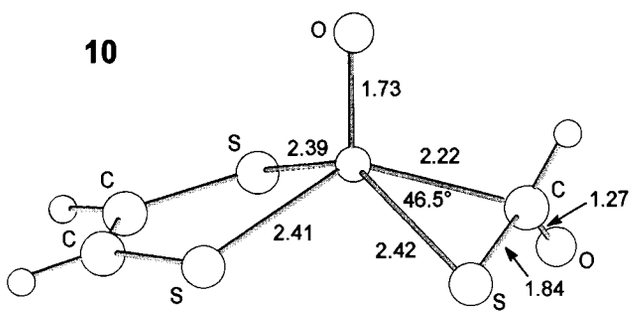


Fig. 4 Calculated structural model for the 'Inhibited' species: **10** $[MoO(H_2dt)(\eta^2\text{-SCHO})]^{2-}$. All distances in Å

of this dilemma is that the calculations suggest that the sulfur is bound to something in addition to Mo. If not H then why not C?

The proposal then is that the bound OH group of the Mo^{VI} resting state is lost. The formaldehyde attacks the metal directly *via* H transfer to sulfur or oxygen. This system is deprotonated, with concomitant formation of an S–C bond, and then picks up an electron to form the observed Mo^{V} species as shown in Scheme 2. The resulting structure of the 'Inhibited' species $[MoO(H_2dt)(SCHO)]^{2-}$ **10** is shown in Fig. 4.

This system has a relatively short Mo–C bond length of 2.2 Å. This is rather longer than the upper boundary derived from the ENDOR data but that analysis is only approximate and relies on a number of assumptions. The most critical one for us is the assumption of sp hybridisation on the formaldehyde carbon. This is required to convert the s spin density, derived from the isotropic hyperfine coupling, into a p spin density from which the dipolar correction can be estimated. The Fermi contact term gives an s density of about 1.2% which is quite close to our computed value of 0.93%. However, the calculated p spin density is 4.1% rather than the 1.2% which comes from the assumption of sp hybridisation. The calculations are more consistent with an approximately sp^3 hybridised carbon.

The rest of the spin is distributed among the Mo (63%) and the formaldehyde oxygen (18%) with much smaller contributions (less than 2.3%) from the oxo and sulfido groups. The calculations are therefore in good agreement with the ENDOR results.

The suggestion[43] that there is no oxygen atom transfer is also consistent with experiment. The 'Inhibited' signal appears during catalytic turnover when the Mo centre cycles from VI to IV to V and then to VI. In Scheme 2, the Mo centre goes directly from VI to V. A mechanism which does not go through Mo^{IV} fits in well with the species being formed during turnover but not at full reduction.[1]

The only remaining ambiguity, which does not materially affect our argument, is whether the oxo or the sulfido group is protonated in the first instance. In the parent resting state with co-ordinated OH^-, the calculated charges on oxo and sulfido groups are virtually identical. However, in the four-co-ordinate system, $[MoOS(H_2dt)]$, the oxo charge (− 0.57) is much larger than the sulfido charge (− 0.36). This is consistent with the oxo group being protonated by the incoming formaldehyde which seems to pave the way quite naturally for S–C bond formation.

Conclusion

The proposal of short Mo–C interactions in both the 'Very Rapid' and 'Inhibited' species, observed when XnO reacts with xanthine (3,7-dihydro-1*H*-purine-2,6-dione) and formaldehyde respectively, has been tested using *ab initio* Density Functional Theory calculations based on our active site model developed recently.[23] For the 'Very Rapid' species, we have not yet found a structure which is fully consistent with experiment. Either we obtain short Mo–C bonds (≈2.2 Å) but with too little spin density on Mo (≈6%) or a more reasonable Mo spin population (≈ 65%) but with long Mo–C bonds (> 2.8 Å). Adding the whole pyran ring to the dithiolene ligand to give a better representation of the pterin cofactor does not change this finding.

In contrast, very good agreement between the calculated and observed spin density distribution has been found for an 'Inhibited' species model. There is a short Mo–C interaction and large spin population on Mo. The spin is also localised on the formaldehyde oxygen (18%) and carbon (5%) atoms. The latter is rather larger than the value estimated from an analysis of the ENDOR spectra but this is largely due to the assumption of sp hybridisation. The DFT calculations indicate that the carbon atom is much nearer an sp^3 hybridised state. This also explains the somewhat longer Mo–C distance in the DFT structure compared to the estimate derived from the ENDOR analysis.

The significant feature of our 'Inhibited' species model is the observation of an S–C bond between the sulfido ligand and the formaldehyde. The possibility of sulfur-atom transfer has not, to our knowledge, been previously proposed although this does not play a role in catalysis since it leads to (reversible) inhibition. Sulfur would therefore never appear in any product.

From the modelling perspective, the successful reproduction of the experimental data by our 'Inhibited' species model gives some confidence that the theoretical procedures are valid. The inability to get as good agreement for our 'Very Rapid' species models would therefore be due to some flaw in the structures we have tested so far. Given that the calculations were guided by the scheme proposed by Lowe *et al.*,[43] aspects of this may need re-evaluation. Indeed, Hille[1] has questioned this scheme both with respect to the accuracy of the derived Mo–C distance and to its overall viability particularly with regard to the nature of the Mo^{IV} precursor. He notes that the η^2-carbonyl binding favoured by Lowe is usually associated with more electron rich centres (*e.g.* Mo^{III}) which can better accommodate π-back bonding. The evidence for Mo–C bond formation seems very strong. We must therefore look elsewhere for improved models for the 'Very Rapid' species.

Acknowledgements

We acknowledge the many enlightening discussions with Professor Bob Bray, University of Sussex, and Drs. David Lowe and Ray Richards, Nitrogen Fixation Laboratory, John Innes Centre.

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Received 23*rd June* 1997; *Paper* 7/04458E