Chemical Evolution of a Nitrogenase Model. 18. Reduction of Molecular Nitrogen with Molybdoinsulin Catalysts

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Abstract: Complexes of oxomolybdate with the reduced peptides of bovine insulin, generated in situ, catalyze the reduction of molecular nitrogen to ammonia with $NaBH_4$ as the reducing agent. The reduction of nitrogen is stimulated by ATP and significantly inhibited by CO. The six Cys-SH moieties of the reduced insulin peptides are the primary binding sites of molybdenum in these extended "molybdothiol" model systems of nitrogenase. This study reaffirms that substrate reductions of nitrogenase are typical of mononuclear molybdenum active site and that nonheme Fe-S or Mo-S-Fe clusters are not required for the simulation of nitrogenase reactions in artificial systems.

We have recently demonstrated[1] that oxomolybdate has a strong "natural" binding affinity for Cys-SH residues of peptides. The resulting complexes as a rule possess catalytic activity for the reduction of typical substrates of nitrogenase (N_2-ase). Using the peptide chains A and B of reduced *bovine insulin* as the ligands of molybdenum, some of the steric effects that have been observed under enzymatic conditions could be simulated with alkynes and nitriles as the substrates.[1] The new molybdoinsulin model systems of N_2-ase were furthermore found to reduce C_2H_2 as efficiently, albeit with somewhat lower selectivity, as the "*Iron-molybdenum cofactor*" (FeMo-co)[2,3] under comparable conditions. It was concluded from the results of these studies that the substrate reducing site of N_2-ase consists of a mononuclear molybdenum ion in a pocket-like environment created by the apoprotein; this environment can be partially simulated by attaching oxomolybdate to the Cys-SH residues of the reduced insulin peptide chains.[1]

Initial experiments revealed that the new model systems also reduce molecular nitrogen. The formation of detectable amounts of NH_3 was initially demonstrated from reactions with $NaBH_4$ as the reductant in borate buffered solutions in the presence of ATP.[1] In later work, Tris (2-amino-2-hydroxymethyl-1,3-propanediol) was employed; this buffer is frequently used in in vitro assays with N_2-ase.[4]

Consistent with our philosophy of modeling enzymatic reactions,[5] which demands that the *initial systems must have maximum simplicity and contain only essential components*, we omitted the addition of cofactors containing iron and inorganic sulfide. Our previous work[1,5] has shown that these cofactors are not needed as long as $NaBH_4$ is used as the reducing agent. In the following, we therefore describe the results of N_2-reduction experiments with iron- and sulfide-free molybdoinsulin catalysts. Such studies are a prerequisite for the future development of model systems containing nonheme-iron electron transfer chains.

Experimental Section

Reagents and Chemicals. A more complete compilation of the reagents and chemicals used for the experiments is given in ref 1. Cylinder nitrogen (99.998%) and argon (99.995%) were both obtained from National Cylinder Gas Co.; carbon monoxide (CP, 99.9%) was purchased from Matheson. Cylinder acetylene (Matheson) was purified by washing with concentrated H_2SO_4; the remaining gases were used without further purification.

$^{30}N_2$ was purchased from Monsanto Research Corp., Miamisburg, Ohio. ATP and Tris buffer were purchased from Calbiochem, San Diego, Calif., and used as received. Bovine insulin was purchased from Sigma.

Performic acid oxidized insulin was prepared as described in ref 1.

Reduction of Isotopically Labeled Nitrogen. Rubber serum capped reaction flasks of 38-mL capacity (from Pierce Chemical Co., Rockford, Ill.) were evacuated and filled with $^{30}N_2$ at 1 atm of pressure. Subsequently, aliquots of 10^{-4} or 10^{-5} M solutions of bovine insulin (freshly prepared), and of Na_2MoO_4 in 0.2 M pH 9.6 borate buffer, were injected in amounts to produce solutions containing Mo and insulin in defined molar ratios; in most experiments, Mo:insulin was 6:1. The reactions were initiated by injecting 0.5 mL of a freshly prepared, pH 9.6 buffered, 0.6 M solution of ATP, immediately followed by 0.5 mL of a 1.45 M solution of $NaBH_4$. The pH adjustment of the ATP solution must be done with care to avoid ATP hydrolysis. It is preferable to add the predetermined amount of NaOH to the buffer prior to the dissolution of the ATP. After specified reaction times, 2 mL of 1 M HCl was injected to stop the reactions. The flasks were thoroughly flushed with pure argon to remove all $^{30}N_2$. The yields of $^{28}N_2$ and $^{29}N_2$ (and possibly of $^{30}N_2$) were determined mass spectrographically after hypobromite oxidation as outlined in ref 6. The $^{29}N_2$ mass peak heights were corrected for natural abundance and converted into yields of NH_3 by comparison with the corrected peak height of $^{29}N_2$ released on hypobromite oxidation from a solution containing a known amount of ^{15}N-enriched NH_4Cl. Typical results are summarized in Table I.

Reduction of N_2 and Colorimetric Determination of NH_3. The experiments with natural-abundance N_2 as the substrate were performed as those described above for $^{30}N_2$. Since the product NH_3 had to be determined colorimetrically and the reaction solutions had to be distilled before the assay it is important to point out experimental details to facilitate the repetition of our experiments by other workers. The HCl-stopped reaction solutions were transferred into a micro-Kjeldahl apparatus and were made *mildly* alkaline (pH 10, with dilute NaOH). Steam was passed through the Kjeldahl flask at a rate that allowed distillation without the application of external heat to the flask. In this manner release of NH_3 from insulin and the adenosine moiety (in experiments with ATP) can be largely suppressed. Argon blanks were run simultaneously with the N_2 experiments. All yields of NH_3 shown in Figures 1-6 are corrected for background. The ammonia was in all cases determined according to Kruse and Mellon.[7] Aliquots of some of the reaction solutions were also routinely tested for N_2H_4, employing the assay of Watt and Chrisp[8] as modified in ref 10. Thus far, only traces of N_2H_4 were detected and are therefore not recorded. The results of the N_2 reduction experiments involving colorimetric assays for NH_3 are presented graphically in Figures 1-6.

Results

Reduction of Nitrogen with Molybdoinsulin Catalysts. The experiments with $^{30}N_2$ as the substrate prove that the molybdoinsulin catalysts generated *in situ* reduce N_2 both in the absence and presence of ATP (see Table I). However, with our assay technique (see Experimental Section), the formation of NH_3 can also be demonstrated with natural-abundance N_2 under various conditions. These results are summarized in Figures 1-6.

Figure 1 reveals a near-linear dependence of the yields of NH_3 on the partial pressure of N_2 from 0 to 1.3 atm. In Figure

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Table I. Reduction of $^{30}N_2$ with Molybdoinsulin (6:1) Catalysts

no.	conditions[a]	mass peak heights (arbitrary units) $^{28}N_2$	$^{29}N_2$	$^{29}N_2$ (cor)	NH_3 yields,[b] μmol
1	without ATP, 1 atm Ar	826	7.14	0	0
2	with ATP, 1 atm Ar	869	7.52	0	0
3	without ATP, 1 atm $^{30}N_2$	880	8.38	0.64	0.30
4	with ATP, 1 atm $^{30}N_2$	869	9.23	1.58	0.75
5	as in 4, with fumarate	1008	8.96	0.24	0.11
6	as in 4, with succinate	961	8.74	0.43	0.20
7	NH_3 standard, 3.12 μmol	840	13.88	6.61	(3.12)

[a] Reactions solutions contained, in a total volume of 5.0 mL, 114 nmol of bovine insulin, 686 nmol of MoO_4^{2-}, in 0.2 M pH 9.6 borate buffer. At $t = 0$, the solutions were 0.14 M in $NaBH_4$ and 0.066 M in ATP. N_2 contained 99% $^{30}N_2$. [b] Yields measured at t_∞ (after 240 min of reaction).

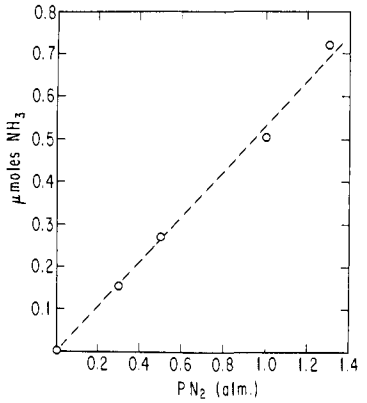


Figure 1. Dependence of the yields of NH_3 as a function of the partial pressure of N_2. Each reaction vessel contained, in a total solution volume of 5 mL, MoO_4^{2-} (0.686 μmol) and bovine insulin (0.114 μmol) in 0.2 M pH 9.6 borate buffer. At $t = 0$, the reaction solutions were 0.18 M in $NaBH_4$ and 0.066 M in ATP. The reactions were stopped after 60 min; the reaction temperature was 23 °C.

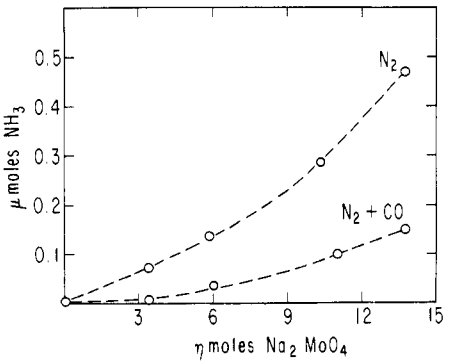


Figure 2. Dependence of the yields of NH_3 on the catalyst concentration. The reaction vessels contained, in a total solution volume of 5.0 mL, MoO_4^{2-} (as indicated) and insulin, in 1/6 of the molar amount of MoO_4^{2-}. The reactions were run in 0.2 M pH 9.0 Tris buffer. At $t = 0$, the reaction solutions were 0.14 M in $NaBH_4$ and 0.066 M in ATP. The reactions were stopped after 60 min; the reaction temperature was 23 °C. Carbon monoxide inhibition was determined under identical conditions except that the gas phase contained 1.0 atm of N_2 and 0.25 atm of CO.

2, the yields of NH_3 are plotted against the concentration of catalyst (expressed in nanomoles of Na_2MoO_4 in the reaction solutions). Although the yields increase up to the catalyst concentration of about 3 nmol/mL (corresponding to 15 nmol per incubation in the experiments in Figure 2), a decline of the yields is observed at higher concentrations. A similar behavior has been seen[5,10] in experiments with other N_2-ase model systems and may be attributed to the formation of catalytically inactive Mo dimers. In Figure 3, a time dependence of the reaction is shown. The S shape of the curve suggests that the reaction occurs after a short induction period, although no effort was made to estimate it accurately. With C_2H_2 as the substrate, an induction period has been noted particularly in experiments in the absence of ATP.[1] From the data in Figure 3, the apparent turnover number of N_2 reduction was estimated as 2.5 (moles of N_2 reduced to NH_3 per mol of Mo per min).

In Table I we describe the result of an experiment with $^{30}N_2$ as the substrate which was performed in the presence of fumarate. Fumarate reacts with N_2H_2 to yield succinate and thus may be used to detect the intermediacy of N_2H_2 in N_2-reduction experiments. The yield of NH_3 in the presence of fumarate was quite low (see Table I). However, succinate also caused a lowering of the amount of NH_3 generated; hence the result of the N_2H_2 trapping experiment in Table I is not yet necessarily conclusive, unlike in our previous experiments with molybdothiol and other N_2-ase model systems.[5,10]

The catalytic variant of the method of continuous variation (CCV method[1]) was applied to the molybdoinsulin systems with N_2 as the substrate. The CCV plot in the system MoO_4^{2-}–chain A exhibits a maximum at the Mo:ligand ratio of 4:1 (see Figure 4). In the corresponding plot of the system with chain B as the ligand, the maximum occurs at the Mo:ligand ratio of 2:1 (Figure 5). With insulin itself, the CCV plot in the system with MoO_4^{2-} and with N_2 as the substrate is shown in Figure 6 and reveals a sharp maximum at the Mo:ligand ratio of 6:1. These results indicate that only two of the four possible molybdenum-binding sites of chain B are utilized or give rise to catalytically active complexes for N_2 reduction. To demonstrate that the Cys-SH residues of the insulin peptides are the actual molybdenum-binding sites, *performic acid oxidized* insulin was tested as the ligand in N_2-reduction experiments in the presence of MoO_4^{2-} under conditions identical with those of the experiment in Figure 6. However, the yields of NH_3 were virtually zero at all Mo:ligand ratios tested.[9]

Discussion

Reduction of Molecular Nitrogen with Molybdoinsulin Catalysts. The present study shows that complexes of oxomolybdate with the reduced peptides of bovine insulin as the ligands are catalysts of the reduction of N_2 to NH_3. The reduction of N_2 is stimulated by ATP and inhibited by CO. The main features of the substrate reactions studied are identical with those observed in experiments with molybdothiol and related catalysts. Hence it is not necessary to discuss these in any great detail; they are summarized in Scheme I instead, and ref 5 and 10–12 should be consulted for further information.

The molybdoinsulin catalysts reduce N_2 with a turnover number of about 2.5 (moles of N_2 reduced to NH_3 per mol of Mo per min), which corresponds to 5% of the activity of N_2-ase at the same per-molybdenum basis. However, such high turnover numbers of N_2 reduction can only be realized at low

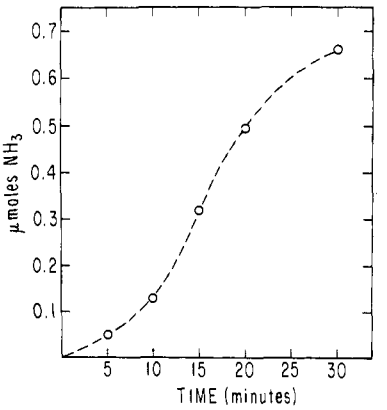


Figure 3. Dependence of the yields of NH_3 as a function of time. The reaction vessels contained, in a total volume of 5.0 mL, MoO_4^{2-} (10.3 nmol) and insulin (1.7 nmol) in 0.2 M pH 9.6 borate buffer. At $t = 0$, the reaction solutions were 0.14 M in $NaBH_4$ and 0.066 M in ATP. The reaction temperature was 23 °C; the partial pressure of N_2, 1 atm.

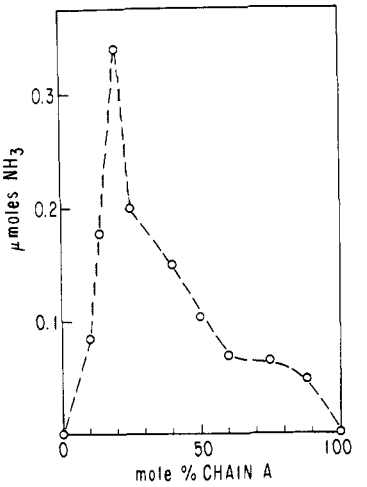
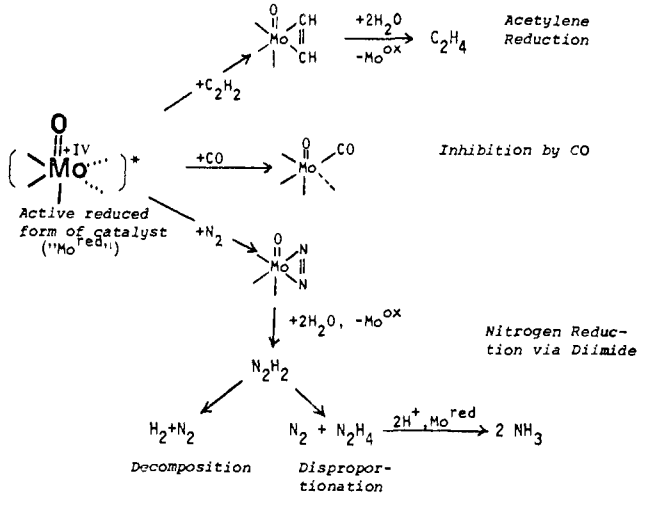


Figure 4. CCV plot for the catalytic system: MoO_4^{2-}-insulin (chain A) with N_2 as the substrate. Each experimental point was obtained from an individual measurement at the given molar ratio of insulin (chain A) and MoO_4^{2-}. The total solution volume was 5.0 mL; in each reaction vessel the sum of the concentrations of insulin (chain A) and of MoO_4^{2-} was 1.6 μM. At $t = 0$, the solutions were 0.18 M in $NaBH_4$, 0.066 M in ATP, and 0.2 M in pH 9.0 Tris buffer. The yields of NH_3 were determined after 25 min of reaction at 23 °C.

Scheme I. Main Mechanistic Features of the Reduction of Nitrogen and Acetylene with Molybdothiol Catalysts



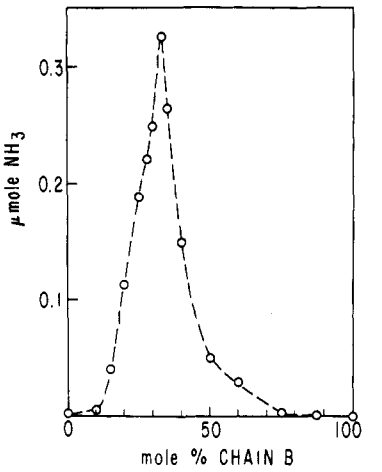


Figure 5. CCV plot for the catalytic system MoO_4^{2-}-insulin (chain B) with N_2 as the substrate in the presence of ATP and $NaBH_4$. Experimental conditions are the same as described in the legend of Figure 4, except that insulin chain B was employed.

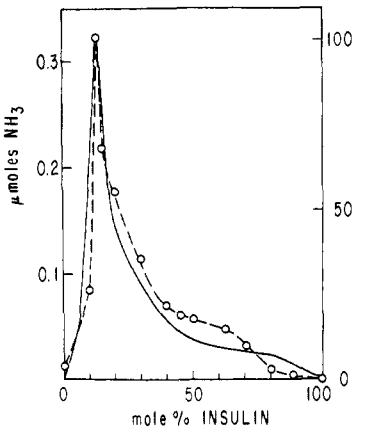


Figure 6. CCV plot for the catalytic system MoO_4^{2-}-insulin, with N_2 as the substrate. Experimental conditions as outlined in the legend of Figure 4 except that whole bovine insulin was used as the ligand component. The solid line is the predicted relative yield curve calculated for the CCV data for the separate chains A and B, assuming that all six Cys-SH groups have equal affinity for molybdenum.

catalyst concentrations and with $NaBH_4$ as the reductant. With $Na_2S_2O_4$ in place of $NaBH_4$ no reduction is observed; this reductant is an efficient reductant in molybdothiol systems only in the presence of electron transfer catalysts, notably, of ferredoxin model compounds.[5,10]

Our work demonstrates that CO is an inhibitor of N_2 as well as of C_2H_2 reduction.[1] The blockage of one coordination site of molybdenum may be envisaged to prevent the side-on attachment of substrates and is shown in Scheme I. The same was previously assumed on the basis of studies with the simple molybdothiol model systems,[5] but at that time such strong inhibitory effects could not be verified because the experiments were run at higher catalyst concentrations. The blockage of one coordination site of Mo by CO does not prevent H_2 evolution, however, consistent with observations with N_2-ase; an example for analogous behavior of a transition metal compound is given by the $V(OH)_2$-$Mg(OH)_2$ N_2-reducing system. The reduction of N_2 and of C_2H_2 in this case is also inhibited by CO, while the H_2 evolution [from the reaction of $V(OH)_2$ with protons of the medium] is unaffected or even stimulated by CO.[13]

Although molybdoinsulin catalysts reduce N_2 even in the absence of ATP, a significant stimulation of the reduction of N_2 occurs in the presence of substrate amounts of ATP. We

interpret the effect of ATP as an acceleration of the conversion of the oxidized forms of the catalysts to the active reduced form, as has been outlined elsewhere.[5]

The application of the CCV method to the system MoO_4^{2-}-insulin with N_2 as the substrate revealed that insulin provides a total of six catalytically near-equivalent sites of which four are located on chain A and two on chain B. These sites must be the Cys-SH residues in view of the fact that performic acid oxidized insulin does not produce catalytically active complexes. With C_2H_2 as the substrate, insulin exhibits a molybdenum-binding capacity of 8, with attachment sites of four on each of the chains A and B.[1] With C_2H_2 the six Cys-SH residues as well as the two His-imidazole groups (located on chain B) bind molybdenum to form catalytically active complexes. With N_2 as the substrate, the complexes generated through interaction of molybdate with the His-imidazole moieties are catalytically silent. Independent experiments aimed at testing L(+)-histidine complexes of oxomolybdate for catalytic activity in the reduction of N_2 similarly produced only negative results.[9]

Figure 6 indicates that the CCV plot of the system MoO_4^{2-}-insulin can be reproduced satisfactorily from the CCV plots of the systems with the isolated insulin chains A and B in Figures 4 and 5, provided that the assumption is made that molybdenum occupies the Cys-binding sites preferentially over the His sites.

The molybdoinsulin complexes are more active catalysts for the reduction of N_2 than the simple molybdothiol systems and can be employed at high dilution. The decomposition of N_2H_2 into N_2 and H_2 has previously been shown responsible for the low efficiency of the simple molybdothiol model systems of N_2-ase.[5,10] It is possible that the attachment of molybdenum to a peptide chain produces catalysts which in some manner protect N_2H_2 from decomposing into the elements. In a previous paper[13] we have shown that ethanolamine increases the yields of N_2H_4 in the $V(OH)_2$-$Mg(OH)_2$-N_2 reducing system and suggested that this was due to a similar "protecting effect". An analogous role of the peptide ligands may be envisaged. Last but not least, even the choice of buffer influences the yields of NH_3 in N_2-reducing systems. The experiments in Figures 3-6 were all performed in Tris buffer, for example, which was found to give higher yields of NH_3 than when borate buffer was used under otherwise identical conditions. We attribute this to "diimide protecting effects" of the type discussed above.

Comparisons with Nitrogenase and with "FeMo-co". The present work supports our concept that *the substrate chemistry of N_2-ase is molybdenum chemistry*. It also reaffirms our previous mechanistic conclusions derived from the study of the simpler molybdothiol and other model systems.[5,10] The molybdoinsulin model systems begin to simulate some of the steric hindrance effects at the substrate-reducing site of N_2-ase and thus permit the conclusion that the enzymic active site is located in a partially obstructed "pocket" which prevents the access of certain bulky substrates to the reducing site, as has been discussed elsewhere.[1] This special feature of N_2-ase could well be of decisive importance because it could mean that N_2H_2 is continuously generated at the active site and undergoes all of its subsequent reactions without leaving the pocket. This would explain why only traces of N_2H_4 are detectable in the enzymatic reduction of N_2[14] and why N_2H_4 does not accumulate significantly during the reaction. The comparatively low efficiency of the model systems could in turn be attributed to the fact that they do not, or do not yet, contain the molybdenum active site(s) in similarly well-protected environments. It would be of interest to study the reduction of various substrates, especially of nitriles, with FeMo-co. This would show whether the molybdenum active site in the cofactor is still in its original environment or whether some changes in the tertiary structure of the protein occurred during its isolation.

Lyophilized Fe-Mo protein, as well as preparations of FeMo-co, has been subjected to EXAFS measurements.[15,16] The results of these measurements have been interpreted to suggest that the molybdenum atom is part of a Mo-Fe-S cluster. Our model studies clearly showed that iron-sulfur clusters are efficient catalysts of electron transfer from external reductant ($Na_2S_2O_4$) to the molybdothiol complexes.[17,18] A similar role of the nonheme iron complexes in N_2-ase holoenzyme may thus be logically assumed. If this transfer of electrons is to occur with any degree of efficiency, at least one Fe-S cluster must be able to approach the molybdenum site rather closely. As has been emphasized some time ago,[17] this would *not* require the assumption of the existence of a Mo-Fe-S cluster and would still be compatible with the available experimental evidence.[19]

So far as FeMo-co is concerned, it is of interest to note that partly oxidized (colorless) forms of the cofactor catalyze the reduction of C_2H_2 (with $NaBH_4$ as the reductant) as efficiently as the fully reduced (brown) modifications. The partial oxidation of FeMo-co evidently destroys the iron-sulfur components, but this has no effect on the ability of the cofactor to catalyze the reduction of C_2H_2 with $NaBH_4$.[16]

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(19) This includes the results of EXAFS measurements, even though these have thus far indicated the presence of a Mo=O bond only in partly oxidized FeMo-protein preparations. In lyophilized, fully reduced FeMo-protein this bond was not detectable.[15] Perhaps a consensus should be reached as to when to use the term "active complex". We prefer to employ it solely to describe the state of the catalyst in its actual functional form. Measurements on lyophilized preparations of isolated enzyme constituents such as the Fe-Mo-protein, according to our term usage, would not provide information on the state of molybdenum in functional, actively turning over N_2-ase during catalysis. In contrast, our studies[12,20-22] with Mo(IV) complexes with various ligands and containing a Mo=O bond clearly demonstrated stoichiometric reductions of typical N_2-ase substrates that were closely analogous to the behavior of the catalytic systems.
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