The yellow binuclear Fe(II) complex derived from decomposition of the red nitrosyl defied characterization for some time. Consequently, the actual details of the decomposition reaction were not extensively studied. Air (presumably water or O_2) is probably necessary for the dark (thermal) reaction since solutions of the complex kept in an inert atmosphere did not deposit ligand salt. Specific photochemical studies were not made. The low, single pK_{a_1} value observed for this complex is a bit surprising, but since the only "communication" between the two protonated amine nitrogens is through space, the presence of the large, charged substituents on the other two nitrogen atoms [Fe(TMC) units] may force a conformation on the bridging TMC moiety such that this interaction is too small to observe two, separate deprotonation steps.

Acknowledgments. Financial support for this research from the National Institutes of Health (D.N.H., Grant HL13652) and the National Science Foundation (E.K.B., Grants MPS 73-08709 and CHE 76-21758) is gratefully acknowledged. We also thank Dr. R. D. Feltham for his helpful comments concerning the manuscript.

Supplementary Material Available: Structure factor tables for $[Fe(TMC)NO](BF_4)_2$ and $[Fe(TMC(NO)(OH)](ClO_4)_2 \cdot CH_3CN$ and magnetic susceptibility data for one sample of $[Fe(TMC)NO](BF_4)_2$, isomer A and two samples of isomer AB (30 pages). Ordering information is given on any current masthead page.

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The Chemical Evolution of a Nitrogenase Model. 17. Simulation of Steric and of Inhibitory Effects at the Enzymic Active Site with Acetylenes and Nitriles as the Substrates, and "Molybdoinsulin" Catalysts

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Abstract: Stoichiometric combinations of the reduced peptide chains A and B of bovine insulin with MoO_4^{2-} reduce C_2H_2 at virtually the same rate but with lower selectivity, as the "iron-molybdenum cofactor" of nitrogenase, with $NaBH_4$ as the reducing agent. The reduction of C_2H_2 is inhibited by CO, and under certain conditions also by N_2. These "molybdoinsulin" model systems of nitrogenase simulate some of the steric hindrance effects in the reduction of substituted *acetylene* and of saturated and unsaturated *nitriles* observed under enzymatic conditions. The results of binary systems studies with a catalytic variant of the method of continuous variation are consistent with the formation of catalytically active complexes through the interaction of molybdenum with the six Cys-SH and the two His-imidazole residues of the reduced insulin peptides.

Recently, Shah and Brill[1a] reported the isolation of an "iron-molybdenum cofactor" (FeMo-co) from *Azotobacter vinelandii* nitrogenase (N_2-ase). It consists[2b] of a small peptide (or peptides) containing one Mo, eight Fe, and six labile S^{2-}. This cofactor reduces C_2H_2 to C_2H_4 at 8% of the rate of N_2-ase (on the same per-molybdenum basis), with $NaBH_4$ as the reductant.[2] The reduction of C_2H_2 was found to be inhibited by CO, but is apparently not stimulated by ATP. Nitrogen could not be reduced detectably under the conditions employed thus far.

Independently, we have been investigating new versions of N_2-ase model systems in which small peptides are the ligands of molybdenum. The initial aim of our studies was to increase the activity and selectivity of the "molybdothiol" model sys-

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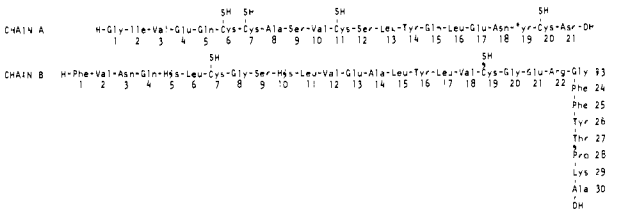


Figure 1. Amino acid sequences of the reduced chains A and B of bovine insulin.

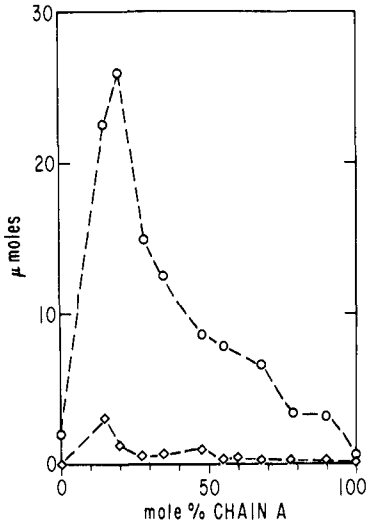


Figure 2. Catalytic continuous variation (CCV) plot for the system MoO_4^{2-}–insulin (chain A) with C_2H_2 as the substrate and $NaBH_4$ as the reducing agent. The product yields for C_2H_4 (- O - O -) and C_2H_6 (- ◊ - ◊ -) were determined after 1 h of reaction at 25 °C. In each reaction vial the sum of the molar concentrations of Na_2MoO_4 and chain A was 0.09 mM, in a total reaction volume of 4.5 mL of 0.2 M borate buffer (pH 9.6). The initial concentration of $NaBH_4$ was 0.18 M in all cases, $p_{C_2H_2}$ = 1 atm.

tems of N_2-ase[3] and to increase their sensitivity to CO, since C_2H_2 reduction with molybdothiol catalysts was previously shown to be inhibited by CO only weakly, in contrast to the behavior of N_2-ase. With the enzyme, $CH_3C_2CH_3$ is not a substrate. Nonenzymatically, molybdothiol catalysts reduce this alkyne, albeit more slowly than C_2H_2, to *cis*-$CH_3CH{=}CHCH_3$. Obviously, the steric obstruction at the enzymic active site was not present in the early model systems. Analogous conclusions were drawn from studies of the reduction of different aliphatic and olefinic nitriles. With N_2-ase, a reactivity sequence "acrylonitrile ≫ *cis*-crotononitrile > acetonitrile ≃ *trans*-crotononitrile > methacrylonitrile ≃ propionitrile" was observed.[4,5] Nonenzymatically, this reactivity sequence was not duplicated. It was found instead that unsaturated nitriles were reduced more rapidly than saturated nitriles, indicating that electronic effects dominated over steric factors.[6] With *cis*- and *trans*-crotononitrile as the substrates it was noted, however, that the replacement of the ligand *cysteine* by *glutathione* caused an improvement of stereochemical selectivity.[6] We thus became interested in developing new N_2-ase model systems that would duplicate steric effects at the enzymic active site even more closely. This necessitated a search for suitable peptides as possible ligands of molybdenum. Ultimately, *bovine insulin* was selected because it is well characterized[7,8] and contains three Cys-*S*-*S*-Cys moieties which on reduction could provide six possible molybdenum attachment sites. On reductive cleavage of the Cys-*S*-*S*-Cys bonds, the reduced chains A and B of insulin are formed whose amino acid sequences are shown in Figure 1. Each chain was also studied separately in catalytic "molybdoinsulin" model systems of N_2-ase.

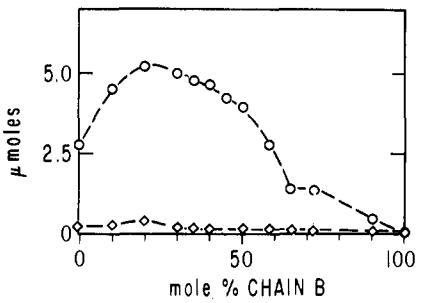


Figure 3. CCV plot for the system MoO_4^{2-}–insulin (chain B) with C_2H_2 as the substrate and $NaBH_4$ as the reducing agent. Product yields of C_2H_4 (- O - O -) and of C_2H_6 (- ◊ - ◊ -) determined after 1 h of reaction at 25 °C. All other experimental conditions are the same as in the legend of Figure 2 except that chain B was used as the ligand of molybdenum.

To determine the nature of the catalytically active complexes in the functional systems under reducing conditions, a catalytic variant of the method of continuous variation[9] (CCV method) was applied. The value of this technique in the study of binary catalytic systems will be illustrated not only for insulin but also for a number of other ligands. We subsequently report the results of experiments with C_2H_2, CH_3C_2H, and $CH_3C_2CH_3$ as the substrates to test the new model systems for catalytic selectivity. The inhibitory effects of CO and of N_2 on C_2H_2 reduction will be described as well, and brief mention will be made of the effects of ATP and of Mg-ATP on C_2H_2 reduction and inhibition. The reduction of N_2 will be reported only qualitatively because it is the subject of the next paper of this series. Instead, we report the results of reduction experiments with the six nitriles whose enzymatic reactivity sequence was given above. Where possible, comparisons with N_2-ase and with FeMo-co will be made. We will demonstrate that the molybdoinsulin model systems resemble N_2-ase more closely than any other known models of this enzyme and that the turnover numbers for C_2H_2 reduction by FeMo-co can be obtained with artificial systems under nonenzymatic conditions.

Results

Peptides and Thiols as Ligands of Molybdenum in Nitrogenase Model Systems. The reduced chains A and B of bovine insulin were used as the ligands of molybdenum under conditions of substrate reduction. To determine the number of molybdenum atoms which is bound by the peptides to yield catalytically active complexes the CCV method was applied with C_2H_2 as the substrate and $NaBH_4$ as the reducing agent. Mixtures of the insulin peptides with Na_2MoO_4 in buffered aqueous solutions were prepared at various defined molar ratios and tested for catalytic activity in the reduction of C_2H_2. The reactions were initiated by injecting aliquots of a freshly prepared solution of $NaBH_4$. All reaction systems remained homogeneous and essentially colorless before, during, and after the C_2H_2 reduction experiments. The yields of C_2H_4 and C_2H_6 were measured after specified reaction times and are shown in Figures 2 and 3 for chain A and chain B as a function of the molar MoO_4^{2-}:ligand ratio. From the observed maxima of the CCV plots the molar Mo:ligand ratios of the catalytically active complexes can be determined provided that the catalytic reactions occur in homogeneous solution and the catalytically active complexes are formed reversibly from catalytically inactive components. These conditions are fulfilled in the molybdoinsulin systems. In the system MoO_4^{2-}–chain A (see Figure 2), the catalytic maximum is observed at the Mo:ligand ratio of 4:1, suggesting that the four Cys-SH groups of chain A are the "catalytic molybdenum binding sites"; these should not be confused with nonspecific molybdenum–ligand interactions of a physical nature which do not give rise to catalyti-

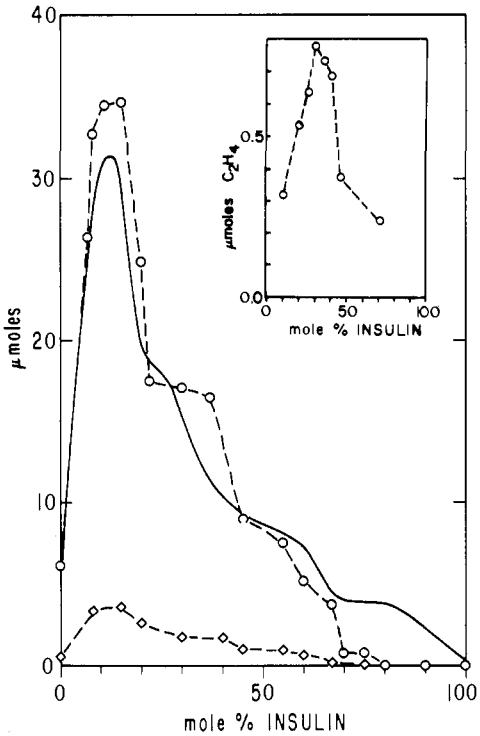


Figure 4. CCV plot for the system MoO_4^{2-}–insulin with C_2H_2 as the substrate and $NaBH_4$ as the reducing agent. Product yields of C_2H_4 (- O - O -) and of C_2H_6 (- ◊ - ◊ -) determined after 1 h of reaction at 25 °C; experimental conditions as in legend for Figure 2. The solid line represents the predicted yield curve calculated by summation of the CCV data for the separate chains A and B, assuming that all six Cys-SH sites have equal affinity for molybdenum and are saturated before binding to the His-imidazole groups occurs. Insert shows CCV plot of C_2H_2 reduction in the system MoO_4^{2-}–performate oxidized insulin.

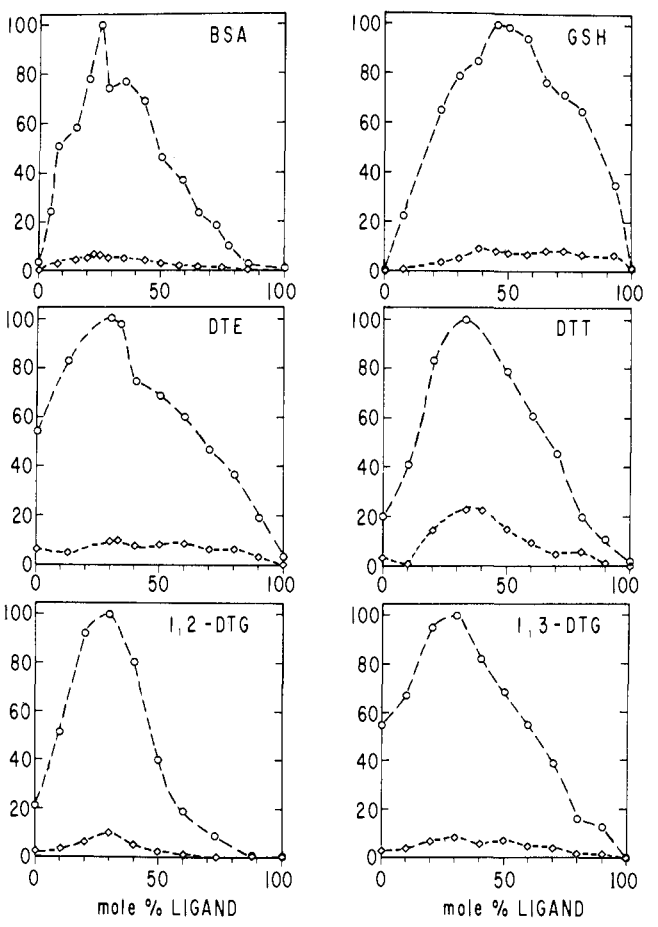


Figure 5. CCV plots for six different catalytic systems of MoO_4^{2-} with thiol ligands. The relative yields of C_2H_4 (- O - O -) and of C_2H_6 (- ◊ - ◊) are shown in each case as determined after 1 h of reaction. The sum of the molar MoO_4^{2-} and ligand concentrations was 0.009 mM for the systems with BSA, 0.9 mM for the systems with BSA, and 0.9 mM for GSH. For the remaining four dithiols (DTT, DTE, 1,2-DTG, and 1,3-DTG) the sum of the MoO_4^{2-} and ligand was 0.09 mM. The initial concentration of $NaBH_4$ was 0.18 M in all cases; the total solution volume was 4.5 mL. The experiments were performed at $p_{C_2H_2}$ = 1 atm, in 0.2 M pH 9.6 borate buffer. The absolute yields of C_2H_4 at the maxima for the six thiols follow (in µmol): BSA, 7.9; GSH, 38; DTE, 48.9; DTT, 46.2; 1,2-DTG, 39.1; 1,3-DTG, 57.6.

cally active complexes. In the system MoO_4^{2-}–chain B, the CCV-plot maximum also occurs at the Mo:ligand ratio of 4:1 (Figure 3), even though it contains only two Cys-SH residues. From the shape of the CCV plot it appears that the two other molybdenum binding sites give rise to only weakly active complexes. Inspection of the amino acid sequence of both insulin chains reveals that the two additional catalytic Mo binding sites of chain B must be the imidazole groups of His-5 and His-10 (see Figure 1), since we have previously shown[10] that histidine can replace cysteine in the molybdothiol model systems with C_2H_2 as the substrate; these "molybdohistidine" catalysts have lower activity than the molybdothiol complexes.

In Figure 4, the CCV plot for C_2H_2 reduction in the system MoO_4^{2-}–insulin is shown. This plot can be reconstructed from the CCV data for the corresponding systems with the A and B chains (see Figures 2 and 3), since insulin under the reducing conditions is converted to a molar 1:1 mixture of chains A and B.

Confirming evidence for the involvement of Cys-SH groups in the binding of molybdenum was obtained by oxidizing insulin with performic acid. This produces peptides in which all Cys moieties are specifically oxidized to the cysteic acid derivatives.[11] As expected CCV studies of the systems MoO_4^{2-}–performate oxidized insulin with C_2H_2 revealed a residual catalytic maximum at the Mo:ligand ratio of 2:1 (see insert of Figure 4), indicating the presence of two catalytic Mo-binding sites.

For comparative purposes, a variety of other thiol ligands were tested by the CCV method. The results with six different ligands are given in Figure 5 with C_2H_2 as the substrate.

Molybdoinsulin Complexes as Catalysts of C_2H_2 Reduction. The reduction of substrates in the molybdoinsulin system has been studied at various Mo:insulin ratios. Where necessary, the ratios employed are indicated in parentheses. Figure 6 shows the results of measurements with molybdoinsulin (1:1) systems at different catalyst concentrations as well as the effect of CO. A very similar illustration of the catalytic activity of FeMo-co with C_2H_2 as the substrate is given in ref 2a. In Figure 7, the dependence of C_2H_4 production on reaction time is given. The reactions with *insulin* exhibit a lag period of about 5-min duration which presumably reflects the slow rate with which the active catalysts are produced. Brief lag periods were also observed in experiments with reduced chains A and B as the ligands. In Table I, turnover numbers for C_2H_2 reduction and the C_2H_4:C_2H_6 product ratios are compiled for FeMo-co (data from ref 2a), and for different molybdoinsulin and molybdothiol catalysts. The C_2H_4:C_2H_6 product ratios are variable and depend on the total concentration of catalyst, reaction time, and buffer used. Table I indicates that under comparable conditions, Mo-insulin catalysts reduce C_2H_2 to C_2H_4 with higher selectivity than Mo-cysteine or Mo-glutathione catalysts. Of the two insulin chains, B produces more selective catalysts than A. In all cases only traces of C_4 hydrocarbons are formed under the experimental conditions chosen and are not shown.

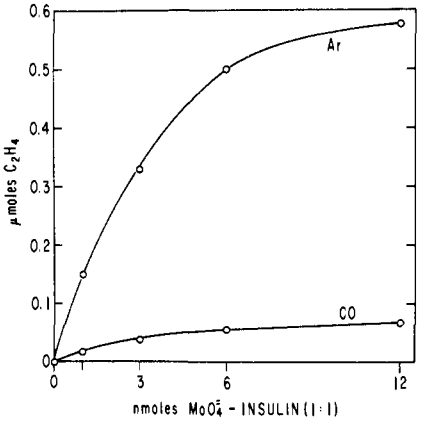


Figure 6. Reduction of C_2H_2 (0.25 atm) under argon (0.75 atm) or CO (0.75 atm) at different catalyst concentrations. The molar ratio of MoO_4^{2-} and insulin was 1:1. The initial concentration of $NaBH_4$ was 0.24 M in all cases. The total solution volume was 3.0 mL (pH 9.6 borate buffer, 0.2 M). Yields of C_2H_6 are not shown. (The experimental conditions for these experiments were chosen to be similar to those of C_2H_2 reduction with FeMo-co as described in ref 2a.)

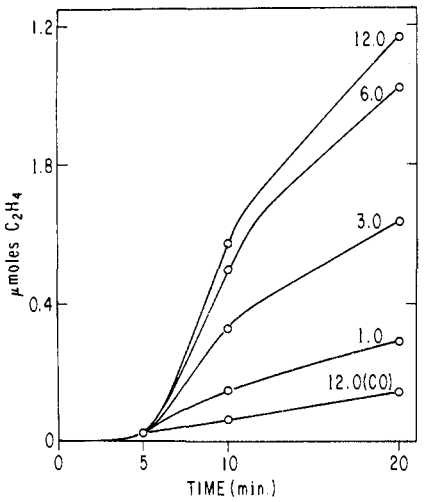


Figure 7. Time dependence of C_2H_2 reduction to C_2H_4 (initial pressure of C_2H_2 = 0.25 atm) under argon or CO, both at 0.75 atm, with Mo-insulin catalysts (1:1). Conditions as given in legend to Figure 6. The numbers identifying the lines indicate nanomoles of catalysts in 3 mL of solution.

Effects of ATP, Mg-ATP, CO, and N_2 on C_2H_2 Reduction. The addition of excess ATP or of Mg-ATP to the reaction solutions causes the disappearance of the lag period and produces a noticeable stimulation of C_2H_2 reduction which persists for about 20 min. Eventually the product yields from runs without ATP exceed those with ATP because $NaBH_4$ is conserved and utilized more effectively for substrate reduction in the absence of ATP. The results of typical experiments are shown graphically in Figure 8.

Carbon monoxide is an inhibitor of C_2H_2 reduction by molybdoinsulin catalysts. However, even with N_2, consistent inhibitory effects of C_2H_2 reduction have been observed, provided that the inhibition experiments are conducted in the presence of a large excess of N_2 relative to C_2H_2. Results of inhibition experiments under these conditions are summarized in Table II. Additional results are shown in Figure 6.

Reduction of N_2. In view of the demonstrated inhibitory effects of N_2 on C_2H_2 reduction described above and in Table II, the ability of the molybdoinsulin catalysts to reduce N_2 was tested as well. Under the conditions given in Table II (but in the absence of C_2H_2), 0.2-0.6 μmol of NH_3 was reproducibly detected with molybdoinsulin catalysts in borate buffer in the presence of ATP. A more detailed study of the reduction of N_2 will be described in the next paper of this series.

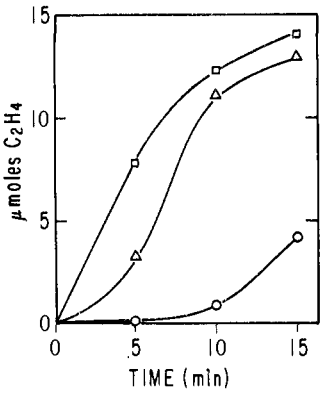


Figure 8. Effects of ATP and of Mg-ATP on the initial yields of C_2H_4 from C_2H_2 (1 atm) in solutions containing MoO_4^{2-} and insulin at the molar ratio of 6:1 and the total concentration of 0.064 mM: - O - O -, C_2H_4 yields with $NaBH_4$; - Δ - Δ, with 0.062 M ATP; - □ - □ -, with 0.062 M Mg-ATP (1:1). The total reaction volume was 5.3 mL in pH 9.6 borate buffer (0.2 M).

Table I. Turnover Numbers and C_2H_4:C_2H_6 Product Ratios for C_2H_2 Reduction with $NaBH_4$ and Different Catalysts under Comparable Conditions in the Absence of ATP

system	pH, buffer	total molybdenum, nmol[a]	turnover numbers[b]	C_2H_4: C_2H_6
FeMo-co	9.6 borate (0.2 M)	1.2	36.7	*c*
		2.8	32.6	*c*
		5.6	32.1	*c*
		11.2	25.3	*c*
Mo-insulin (1:1)	9.6 borate (0.2 M)	1.0	30.1	7.1
		3.0	27.6	3.1
		6.0	23.4	2.1
		12.0	12.3	2.9
Mo-insulin (chain A, 1:1)	9.6 borate (0.2 M)	1.0	30.0	2.6
		3.0	29.8	2.9
		6.0	18.3	1.5
		12.0	17.6	1.9
Mo-insulin (chain B, 1:1)	9.6 borate (0.2 M)	1.0	41.3	14.7
		3.0	16.7	3.5
		6.0	15.4	2.7
		12.0	7.2	1.7
Mo-insulin (1:1)	9.0 Tris (0.2 M)	1.0	25.0	12.1
		3.0	11.6	6.0
		6.0	7.6	3.3
		12.0	4.4	2.5
Mo-GSH (1:1)	9.6 borate (0.2 M)	12.0	15.4	1.3
Mo-Cys (1:1)	9.6 borate (0.2 M)	12.0	11.3	1.0

[a] Reaction solutions contained the catalysts in a total solution volume of 3.0 mL. The initial concentration of $NaBH_4$ was 0.24 M; C_2H_2 = 0.25 atm. [b] Moles of C_2H_2 reduced per mol of Mo per min, corrected for lag period where necessary. Turnover numbers for FeMo-co were calculated from Figure 2 in ref 2. [c] No C_2H_6 detectable above background. Data from ref 2.

Reduction of Substituted Acetylenes. Table III shows the relative reactivities of Mo-insulin, Mo-glutathione, and Mo-cysteine catalysts (all at the Mo:ligand ratios of 1:1) in the reductions of C_2H_2, CH_3C_2H, and $CH_3C_2CH_3$ from experiments under identical conditions. It may be seen that $CH_3C_2CH_3$ is reduced at still considerable rates with Mo-cysteine but only very slowly with Mo-insulin.

Reduction of Saturated and Unsaturated Nitriles. The reduction of acrylonitrile, *cis*- and *trans*-crotononitrile, methacrylonitrile, acetonitrile, and propionitrile was studied in the presence of substrate amounts of ATP with molyb-

Table II. Inhibition of C_2H_2 Reduction by CO and N_2 in the Absence and Presence of ATP with Molybdoinsulin and Molybdocysteine Catalysts in Borate and Tris Buffer

buffer	catalyst[a]	inhibitor	percent inhibition without ATP C_2H_4	without ATP C_2H_6	with ATP C_2H_4	with ATP C_2H_6
borate 0.2 M, pH 9.6	Mo-insulin (1:1)	N_2	40	29	21	9
		CO	96	97	95	87
	Mo-cysteine (1:1)	N_2	33	60	18	0
		CO	96	95	91	95
Tris 0.2 M pH 9.0	Mo-insulin (1:1)	N_2	48	41	24	15
		CO	97	92	70	55
	Mo-cysteine (1:1)	N_2	21	23	9	17
		CO	95	97	79	80

[a] Each reaction bottle contained, in a total solution volume of 3.0 mL: MoO_4^{2-}, 10 nmol; ligand, 10 nmol. ATP (initial concentration), 0.055 M; $NaBH_4$, 0.24 M. The partial pressures of N_2 or CO were 0.97 atm; the initial partial pressure of C_2H_2 was 0.03 atm. Yields of products were measured after 10 min of reaction at 23 °C.

Table III. Relative Rates of Reduction of C_2H_2, CH_3C_2H, and $CH_3C_2CH_3$ with Mo-Insulin, Mo-Glutathione, and Mo-Cysteine Catalysts in the Absence of ATP, in 0.2 M pH 9.6 Borate Buffer, with $NaBH_4$ as the Reductant (Mo:Ligand Ratios 1:1 in All Cases)

catalyst[a]	rel rates of redn[b] C_2H_2	CH_3C_2H	$CH_3C_2CH_3$
molybdoinsulin	1.00	1.05	0.05
molybdoglutathione	1.00	0.85	0.10
molybdocysteine	1.00	0.80	0.20

[a] All reaction solutions contained 400 nmol of MoO_4^{2-} and of the respective ligands in a total volume of 4.5 mL. The initial concentration of $NaBH_4$ was 0.24 M. Acetylenic substrates were added at identical amounts (1.33 mmol). [b] Calculated from total electrons transferred to substrate after 18 h of reaction. The predominant product of reduction of CH_3C_2H was C_3H_6 (propylene), of $CH_3C_2CH_3$ *cis*-2-butene.

doinsulin catalysts at the molar Mo:ligand ratio of 8:1. This metal:ligand ratio was chosen in order to saturate all molybdenum binding sites of chains A and B. The presence of ATP was necessary because of the low yields of hydrocarbon products if the reactions are run in the absence of ATP. Because of the slow rates of reduction, particularly of propionitrile, methacrylonitrile, and acetonitrile, the hydrocarbon yields per incubation rather than initial rates are quoted in Table IV together with similar data taken or calculated from published results with N_2-ase.[4,5]

Discussion

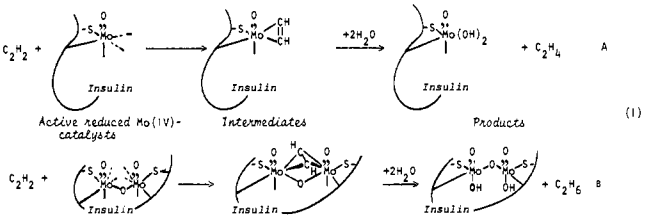
Insulin Peptides as Ligands in Nitrogenase Model Systems. Studies of metal-ligand interactions under static conditions produce data which cannot always be extrapolated to catalytic systems since complexes that can be isolated or detected by conventional spectroscopic methods are usually not the catalytically active species. The catalytic variant of the method of continuous variation (CCV method) has the advantage over other methods in that it allows the study of catalytic reactions in functional systems. Using this method in conjunction with other criteria we previously concluded that the catalytically active species in the molybdothiol model systems of N_2-ase are 1:1 complexes of thiols with oxomolybdate. The present investigations of the systems involving the insulin peptides as the ligands show that molybdate has a natural affinity for peptide-SH groups. Under reducing conditions, the Cys-SH groups of insulin are clearly the primary binding sites of molybdenum, and the resulting complexes are active catalysts of the reduction of typical N_2-ase substrates. The only other molybdenum binding sites in the systems with insulin peptides as the ligands are the imidazole groups of His-5 and His-10 of chain B, but these interactions give rise to only weakly active catalysts.

The attachment of oxomolybdate to the Cys-SH groups of reduced insulin-chain A produces a sharp catalytic maximum at the molar Mo:ligand ratio of 4:1 (Figure 2). Inspection of the CCV plot in Figure 2 reveals that the attachment of only one, two, or three oxomolybdate ions to chain A also produces active catalysts whose activity on a per-molybdenum basis diminishes only slightly as chain A becomes fully "loaded" with molybdenum. All active sites thus are approximately equal and independent.

The CCV measurements do not permit conclusions as to which of the four Cys-SH groups of reduced chain A interacts with oxomolybdate preferentially. All four groups are evidently accessible, but Cys-20 could possibly be more reactive in view of its near-terminal position. The two Cys-SH groups of the reduced insulin chain B are also accessible. The fact that the catalytic maximum for C_2H_2 reduction appears at the Mo:chain B ratio of 4:1 is suggestive of the involvement of the imidazole groups of His-5 and His-10 in the formation of molybdenum complexes, but these complexes give rise to lower catalytic activity. Previous studies in which L(+)-cysteine was replaced by L(+)-histidine in C_2H_2 reduction experiments with molybdenum catalysts have already been mentioned above and support this conclusion.

Since insulin undergoes reductive S-S-bond cleavage under the reducing reaction conditions the CCV plot for C_2H_2 reduction is essentially identical with the plot expected for a system consisting of a molar 1:1 mixture of insulin chain A and B as the ligand component. Indeed, the insulin CCV plot can be reconstructed from the plots obtained for chain A and B separately (see Figures 2-4).

Reduction of C_2H_2, CH_3C_2H, and $CH_3C_2CH_3$. The reduction of C_2H_2 to C_2H_4 with molybdoinsulin catalysts is formulated in eq 1A in analogy to previous work with molyb-



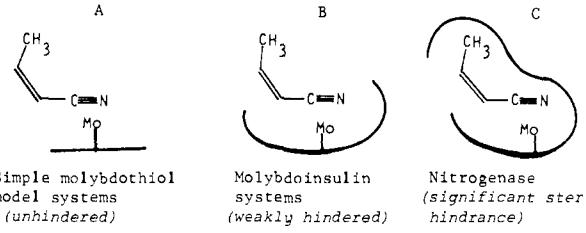
dothiol catalysts.[3] The reduction of C_2H_2 to C_2H_6 is typical of binuclear catalytic sites and shown in eq 1B. Presumably, such binuclear species are formed intermolecularly at high catalyst concentrations since the C_2H_4:C_2H_6 product ratios decline as the total concentration of catalyst is increased (see Table I). The *intramolecular* formation of dimeric species as indicated in eq 1B appears to occur to a greater extent with

Table IV. Product Hydrocarbon Yields and Ratios in the Reduction of Unsaturated and Saturated Nitriles by Nitrogenase, Molybdoinsulin (8:1), and Molybdocysteine Catalysts in the Presence of ATP

substrate	total hydrocarbons produced, nmol per incubation			hydrocarbon products	av hydrocarbon product ratios		
	N_2-ase[a]	Mo-insulin[b]	Mo-Cys[b]		N_2-ase	Mo-insulin	Mo-Cys
CH_2=CHCN	670	483	321	C_3H_6, C_3H_8	(6-10):1	1:1	(1-6):1
cis-CH_3CH=CHCN	23	435	338	1-C_4H_8, *cis*-2-C_4H_8, *trans*-2-C_4H_8, C_4H_{10}	10:5:1:1	13:2:1:17	7:0.9:1:7
CH_3CN	2-4	322	133	C_2H_6	1	1	1
trans-CH_3CH=CHCN	2.3	407	682	1-C_4H_8, *cis*-2-C_4H_8, *trans*-2-C_4H_8, C_4H_{10}	0.5:0.1:1:0.1	8:0.8:8:1:6	5:0.4:1:4
CH_2=$C(CH_3)$CN	1	159	261	*i*-C_4H_8, *i*-C_4H_{10}	1:0	1.4:1	1.5:1
C_2H_5CN	1	139	160	C_3H_8	1	1	1

[a] Data from ref 4-6. [b] The total molybdenum concentration was 0.14 mM in all cases. The Mo:insulin ratio was 8:1, the Mo:cysteine ratio 1:1. The initial concentrations of the other systems components were as follows: ATP = 0.03 M; $NaBH_4$ = 0.14 M (in 0.2 M, pH 9.6 borate buffer). The added amount of nitrile substrates was 2.6 mmol in all cases; the total reaction solution volume was 5.0 mL. Yields of hydrocarbons were measured after 20 h of reaction, corresponding to t_∞.

Scheme I. Schematic Representations of Steric Hindrance Effects



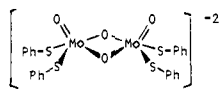
chain A than with chain B, as judged from the observed C_2H_4:C_2H_6 ratios. In chain A, the SH groups of Cys-6 and Cys-7 are sufficiently close to suggest that intramolecular dimer formation occurs to some extent. The molybdoinsulin catalysts reduce C_2H_2 to C_2H_4 with higher selectivity than the Mo-Cys or Mo-GSH systems under identical conditions (Table I). Compared to FeMo-co, the selectivity is still lower, however, since the formation of binuclear catalytic species apparently cannot be fully eliminated.

The higher catalytic selectivity of molybdoinsulin catalysts as compared to the simple molybdothiol complexes also follows from the results of the reduction experiments with CH_3C_2H and $CH_3C_2CH_3$ as the substrates. Table III reveals that the molybdoinsulin catalysts reduce both alkynes, but N_2-ase apparently reduces $CH_3C_2CH_3$ not at all. This shows that the dimensions and geometry of the "pocket" in which the Mo center of N_2-ase resides possesses features that have not been duplicated. Although such details are unimportant as long as only the chemical aspects of the reduction of the substrates are discussed, they may ultimately provide the explanation why N_2-ase reduces N_2 so efficiently. If it is considered that N_2-ase reduces *cis*- and *trans*-crotononitrile only at about 3 and 0.3% of the rate of acrylonitrile (see Table IV) while the molybdoinsulin catalysts reduce these substrates at 90 and 84% of the rate of acrylonitrile, this can only mean that the molybdenum center of N_2-ase sits in a deeper and somewhat asymmetric "pocket" than is the case in the molybdoinsulin systems. By analyzing the relative rates of reduction of all alternate substrates of N_2-ase it may be concluded that the environment of molybdenum at the active site of the enzyme is more nearly as shown in Scheme I, formula C, than B, and certainly not as in A, in which no large steric effects would be expected. Structure A reflects the environment in the simple molybdothiol model systems of N_2-ase; structure B may be a fair description of the active site in the molybdoinsulin systems.

C_2H_2 Reduction in Catalytic Systems with Other Thiol Ligands. The CCV method was applied to other binary systems primarily to illustrate its value in the study of binary catalytic systems but also to reaffirm the previous conclusions in the molybdoinsulin system. The results with *bovine serum albumin* (Figure 5) are of interest because they show that in this case only a few of the theoretically possible molybdenum-binding sites are utilized. The catalytic maximum for C_2H_2 reduction occurs at the Mo:BSA ratio of 3:1. If all 17 Cys-*S*-*S*-Cys moieties of BSA had been reduced and would interact with molybdate, the catalytic maximum would have been expected at the Mo:BSA ratio of 35:1 (BSA contains 17 Cys-*S*-*S*-Cys bonds and one isolated Cys-SH[12]). However, the shoulder in the CCV plot occurs at a Mo:BSA ratio suggesting that only two or three of the Cys-*S*-*S*-Cys bonds are reduced and/or interact with Mo under the conditions of C_2H_2 reduction. An alternative interpretation is that all Cys-*S*-*S*-Cys bonds are reductively cleaved but only at most five to seven give rise to catalytically active complexes; this, however, appears to be less likely.

With reduced glutathione (GSH) as the ligand, the CCV plot in Figure 5 shows a maximum at the Mo:GSH ratio of 1:1, consistent with the formation of one catalytically active complex involving the Cys-SH group as the primary site of attachment of molybdenum. Huang and Haight[13] have reported a complex $Na[Mo_2O_4(glutathione)]\cdot 4H_2O$. In the absence of an X-ray structural analysis these authors proposed a structure in which an oxo-bridged Mo_2O_4 unit is coordinated to the Cys-S^-, glycine, and the terminal glutamyl moieties. Our CCV study indicates, however, that a 1:1 Mo-GSH complex is the most active catalyst in this system.

The four dithiol ligands for which results of binary systems CCV studies are reported (see Figure 5) are *dithioerythritol* (DTE), *dithiothreitol* (DTT), *1,2-dithioglycerol* (1,2-DTG), and *1,3-dithioglycerol* (1,3-DTG). Recently, Dance et al.[14] characterized several binuclear Mo(V) complexes of thiols and dithiols and reported on the structure of the thiophenolato complex $(R_4N)_2[Mo_2O_4(SPh)_4]$. In these complexes two RS^- anions are in the coordination sphere of each oxomolybdate(V) ion (see formula **1**) and both 1,3-propanedithiol and DTE act



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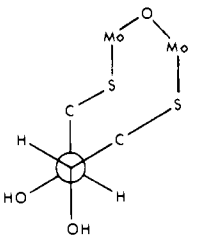
as bidentate chelating sulfur donor ligands. Our CCV studies indicate that all dithiols in Figure 5 form complexes which exhibit the highest catalytic activity at the Mo:ligand ratio of 2:1, suggesting that these thiols act as monodentate ligands under catalytic conditions. The complexes of the type described in ref 14 are not the actual catalysts in molybdothiol model systems.

The ligands DTT and DTE differ structurally only slightly but give rise to molybdothiol catalysts with significantly different product selectivity with C_2H_2 as the substrate. Thus, Figure 5 shows that DTT yields much more C_2H_6 relative to C_2H_4 than DTE, at Mo:ligand ratios of 2:1, suggesting that DTT has a greater tendency to form catalytically active binuclear molybdenum complexes. We have verified, with molecular models of DTT and DTE, that the formation of a μ-oxo-bridged dimolybdenum complex would be more facile with DTT than with DTE because of an unfavorable *gauche* C-OH interaction across the C_2-C_3 bond in DTE which is absent in DTT (see formulas **2** and **3**). We believe that this effect explains the different catalytic selectivity of DTT and DTE in molybdothiol-catalyzed reductions of C_2H_2.



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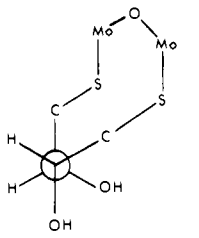

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Effects of ATP, Mg-ATP, and CO and N_2. In the presence of substrate amounts of ATP or of Mg-ATP, the overall electron transfer efficiency increases as has been observed in other molybdothiol studies. A detailed discussion of the ATP or Mg-ATP effects is not required in view of our previous work.[3,15] However, the effects of ATP are of interest in relation to inhibition experiments with CO and with N_2. Table II and Figures 6 and 7 show that CO is a powerful inhibitor of the molybdoinsulin-catalyzed reduction of C_2H_2, both in the presence and absence of ATP. The degree of inhibition is almost as strong as observed with FeMo-co under similar experimental conditions.[2] Table II reveals that the reduction of C_2H_2 is also inhibited by CO in experiments with molybdocysteine catalysts. We have previously not observed[10] such strong inhibitory effects because of differences in the experimental conditions (higher catalyst and lower inhibitor concentrations were used and are mainly responsible).

Inhibition of C_2H_2 reduction by N_2 has also been observed; it is generally weaker than the effect of CO under comparable conditions. Our results clearly show that CO interacts with molybdenum and in this manner prevents interactions of C_2H_2 with the catalyst. It is noteworthy that this inhibitory effect occurs in *iron-free* systems. The effects of N_2 on C_2H_2 reduction are also reassuring in that they demonstrate that N_2 and C_2H_2 compete for the same catalytically active species.

Reduction of Nitriles. The reduction of nitriles by N_2-ase provides examples of reactions in which six or more electrons and protons are transferred to the bound substrate and C=N bonds are hydrolytically cleaved. The overall reaction can be expressed in terms of reaction eq 2, if R is an *alkyl* residue. Olefinic nitriles are reduced to mixtures of alkenes and alkanes with the transfer of six and eight electrons and protons, respectively.

$$RCN + 6e^- + 6H^+ \xrightarrow[-ADP,\ -P_i]{N_2\text{-ase},\ +ATP,\ H_2O} RCH_3 + NH_3 \quad (2)$$

Model studies[6] have previously indicated that the nitrile substrates interact with the molybdenum center in the side-on fashion. In the simple model systems, acrylonitrile is reduced more rapidly than propionitrile, indicating a predominance of electronic over steric effects. Under enzymatic conditions, steric effects at the molybdenum active site are also important, as has been pointed out already (see Scheme I). With the molybdoinsulin catalysts the reactivity sequence for the six nitriles is nevertheless almost the same as that observed on reduction of these substrates with N_2-ase (Table V). Only the position of CH_3CN relative to *trans*-crotononitrile is reversed. This shows that the molybdoinsulin catalysts at least begin to simulate the steric hindrance effects at the active site of the enzyme. The abnormally high reactivity of acrylonitrile relative to the other nitriles has yet to be duplicated. This may be difficult because effects of the secondary or tertiary structures of the apoprotein may be involved. There are also differences in the hydrocarbon product distribution between N_2-ase and the nonenzymatic models, but these are of lesser concern because they are mainly dependent on the electron transfer efficiency in individual enzyme preparations.

Table V. Reactivity Sequences in the Reduction of Saturated and Unsaturated Nitriles by Nitrogenase, Molybdoinsulin, and Molybdocysteine Catalysts

system	sequence of declining reactivity[a]
N_2-ase	$CH_2{=}CHCN \gg$ *cis*-$CH_3CH{=}CHCN >$ $CH_3CN \simeq$ *trans*-$CH_3CH{=}CHCN >$ $CH_2{=}C(CH_3)CN \simeq C_2H_5CN$
Mo-insulin	$CH_2{=}CHCN >$ *cis*-$CH_3CH{=}CHCN >$ *trans*-$CH_3CH{=}CHCN > CH_3CN >$ $CH_2{=}C(CH_3)CN \simeq C_2H_5CN$
Mo-cysteine	*trans*-$CH_3CH{=}CHCN \gg$ *cis*-$CH_3CH{=}CHCN$ $> CH_2{=}CHCN > CH_2{=}CH(CH_3)CN >$ $C_2H_5CN > CH_3CN$

[a] Compiled from ref 4-6 and the present work.

Conclusions

The present study indicates that molybdate forms catalytically active complexes primarily with protein sulfhydryl groups. Accordingly, a Cys-SH residue is expected to be the natural binding site of molybdenum in N_2-ase. The fact that the reduction of C_2H_2 with molybdoinsulin catalysts proceeds at rates nearly equal to those observed with FeMo-co under similar experimental conditions is of particular interest since we deliberately investigated *iron-free* systems. This shows that the nonheme iron components in FeMo-co do not participate effectively in the electron transfer from external reductant to the molybdenum active site, and that iron centers are not involved in the reduction of this substrate. The inhibitory effect of CO in the reduction of C_2H_2 with FeMo-co[2a] has also been satisfactorily simulated with the molybdoinsulin catalysts. Obviously, CO interacts with the molybdenum site and thus inhibits C_2H_2 reduction.

The molybdoinsulin model systems in addition simulate some of the finer details of the steric accessibility of the molybdenum active site. Hardy and Burns[4] have previously alluded to the topography of the active site of N_2-ase on the basis of the reduction of bulky substrates, especially of olefinic nitriles. They concluded that the active site of N_2-ase is "pocketed", imposing certain steric restrictions to reactions with substrates. Our work shows that appropriate molybdenum peptides could eventually be found which simulate these effects even more closely than the molybdoinsulin catalysts. Since N_2-ase reduces acrylonitrile 670 times faster than propionitrile while molybdoinsulin catalysts reduce acrylonitrile only 3 times more rapidly it follows that the active site of N_2-ase is specifically designed to accommodate N_2 but can obviously accommodate a number of somewhat larger alternate substrates selectively. The "pockets" of the molybdoinsulin catalysts are evidently more "open", and we suspect that this is also the case in FeMo-co. This could explain why FeMo-co is not a good N_2-reducing catalyst.

Experimental Section

Reagents and Chemicals. Insulin, crystalline, from bovine pancreas, with a zinc content of ca. 0.5%, and the isolated chains A and B of

bovine insulin were purchased from Sigma Chemical Co. A generous gift of bovine insulin from E. Lilly Corp. is also gratefully acknowledged. The insulin samples were used without further purification. Also employed as received were the following: L(+)-cysteine hydrochloride, reduced glutathione (96%), L(+)-histidine hydrochloride hydrate, dithioerythritol, dithiothreitol, dithioglycerol(1,2), dithioglycerol(1,3), and crystalline bovine serum albumin (all from Aldrich). Na_2MoO_4 (Baker Analyzed Reagent) was also used without further purification. Crotononitrile, a mixture of the cis and trans isomers, was subjected to fractional distillation at a high reflux ratio using a column of 1 M length packed with glass helices. The isomers thus isolated were analyzed for purity by ^1H NMR. All other nitriles were also fractionally distilled before use to remove impurities. The substituted acetylenes CH_3C_2H and $CH_3C_2CH_3$ were purchased from Farchan Laboratories. The compressed gases argon (National Cylinder Gas), 99.995%, and carbon monoxide (Matheson) were used straight from the tank; C_2H_2 (Matheson) was washed with concentrated H_2SO_4.

Because of the high dilution at which most of the experiments were performed, care must be taken to minimize the contamination by trace element impurities in the reaction solutions. Accordingly, borate buffer (0.2 M, pH 9.6) was prepared from ultrapure H_3BO_3 and NaOH, as marketed by Alfa-Ventron. Sodium borohydride, also from Alfa-Ventron, was analytical grade, 99%. Tris [tri(hydroxymethyl)aminomethane] was 99.9% Gold Label from Aldrich.

Standard Gas Chromatographic Technique. Hydrocarbons (C_2–C_4) were measured by GLC using a Varian 1440 Aerograph laboratory gas chromatograph equipped with a 6-ft phenyl isocyanate–Porasil C, 80–100 mesh column, using FID detection. The identity of the individual gases was checked by measurement of the retention times at several operating temperatures and by coinjection, and, where required, confirmed by mass spectrographic measurements.

CCV Studies with C_2H_2 as the Substrate. Up to 12 reaction bottles of 38-cm^3 capacity with rubber seals (from Pierce Chemical Co., Rockford, Ill.), were enumerated and flushed with pure argon at 1 atm. Measured amounts of solutions of Na_2MoO_4, e.g., 10^{-4} M, in 0.2 M pH 9.6 borate buffer, and of the respective peptide or ligand at the same concentration were injected in amounts to produce mixtures of the two components at identical total concentrations and molar ratios ranging from 0 to 100% in each component. The bottles were subsequently either flushed with C_2H_2, or, for measurements at C_2H_2 pressures of less than 1 atm, a known volume of C_2H_2 was injected into each bottle by means of a gas-lock syringe. At $t = 0$, 0.5 mL of a freshly prepared aqueous 1.45 M solution of $NaBH_4$ was injected. For yield measurements, gas-pressure relieve syringes of 50-mL capacity were inserted into the rubber seals to allow the pressure to reach 1 atm, and 0.5-mL gas samples were withdrawn for hydrocarbon determinations by GLC. Typically, yield measurements were made after 5, 10, 20, and 60 min of reaction. The yields of hydrocarbon products were calculated by correcting for background hydrocarbons produced in runs without added MoO_4^{2-} and for the total volume of gases evolved (after expansion to 1 atm of pressure) in each reaction bottle. The experiments in the presence of substrate amounts of ATP or Mg–ATP were performed analogously except that 0.5 mL of a freshly prepared 0.6 M solution of ATP (or of a solution containing 0.6 M ATP and 0.6 M $MgSO_4$) was injected immediately before adding the $NaBH_4$ solution. The ATP solutions were prepared by dissolving 0.725 g of ATP in 1.3 mL of precooled borate buffer. This causes a drop of pH which is corrected by the dropwise addition of 0.65 mL of 4 N NaOH. (It is often more convenient to add the previously determined amount of NaOH to the borate buffer prior to the dissolution of the ATP.)

Individual Measurements and Reduction of Substituted Acetylenes. Individual measurements of C_2H_2 reduction with molybdoinsulin, molybdoglutathione, or molybdocysteine catalysts were performed in reaction bottles of 38-mL capacity. The solutions of the catalysts were prepared by combining buffered solutions of Na_2MoO_4 and the ligands at defined molar Mo:ligand ratios. Typically, 2×10^{-4} M borate buffered stock solutions of Na_2MoO_4 and of bovine insulin were prepared. Before the experiments, a 1:1 mixture of Na_2MoO_4 and the insulin solution was made and diluted tenfold with pH 9.6 borate buffer. This produces a solution which is 10^{-5} M in terms of a 1:1 Mo–insulin catalyst. This solution (1 mL) was injected into the 38-mL, argon-filled and rubber serum capped reaction bottles. A measured volume of C_2H_2 was injected next and the reaction initiated by the injection of 0.5 mL of a freshly prepared 1.45 M $NaBH_4$ solution. Descriptions of specific experiments are given in the legends or footnotes of figures and tables. For the reduction of $CH_3C_2CH_3$, 0.4 mL of the liquid alkyne cooled to 5 °C was dissolved in 3.6 mL of C_2H_5OH. Since d_l^{278K} of $CH_3C_2CH_3$ is 0.691 g/cm^3, 1 mL of this solution corresponds to 1.33 mmol of $CH_3C_2CH_3$, or 38 cm^3 of the gas at STP. The ethanol solvent addition was chosen to improve the solubility of the alkyne in water. For the reduction of CH_3C_2H the reaction bottles were flushed with the gaseous alkyne.

Performic Acid Oxidation of Insulin. Bovine insulin (100 mg) was dissolved in 3.6 mL of 88% formic acid and 0.4 mL of 30% (w/w) H_2O_2. The reaction mixture was stirred at room temperature for 15 min and subsequently diluted with 4.0 mL of H_2O. The volume of the solution was reduced to ca. 2 mL by evaporation in vacuo, and 40–50 mL of pure acetone was added, causing precipitation of the oxidized insulin product. It was isolated by centrifugation, washed twice with acetone, and dried. A CCV study under the conditions of the experiment in Figure 4 but with performate-oxidized insulin was performed and revealed the absence of catalytic maxima at all Mo:ligand ratios. Although C_2H_4 was formed, the yields were lower than with no added insulin ligand, suggesting some inhibitory effects of performate-oxidized insulin.

CO- and N_2-Inhibition Experiments of C_2H_2 Reduction. The C_2H_2 reduction experiments in the presence of the inhibitors CO and N_2 were performed in the 38-cm^3 reaction bottles which were filled first with CO or N_2 at 1 atm of pressure. Subsequently, 1.0 cm^3 of C_2H_2 at 1 atm was injected and the pressure in the bottles reduced back to 1.0 atm. Measured amounts of Na_2MoO_4–ligand stock solution were injected and the reactions initiated as described above for experiments in the absence or presence of ATP.

Reduction of Nitriles. The MoO_4^{2-}–insulin mixtures at defined molar ratios (usually Mo:ligand was 8:1) were prepared in the 38-mL reaction bottles. They were filled with pure argon, and measured amounts of the nitriles corresponding to 2.6 mmol were injected. Immediately before the start of the experiments, 0.725 g of ATP was dissolved in 1.3 mL of precooled pH 9.6 borate buffer and the pH of the solution was carefully increased back to 9.6 through the dropwise addition of 0.65 mL of 4.0 N NaOH solution. Of this ATP solution, 0.5 mL was injected shortly before beginning the reaction. At $t = 0$, 0.5 mL of a freshly prepared 1.45 M solution of $NaBH_4$ was injected. After specified reaction times at 25 °C, 0.5-cm^3 gas samples were withdrawn for hydrocarbon analysis by GLC. The results are summarized in Table IV.

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