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Catalytic Behavior of the Nitrogenase Iron–Molybdenum Cofactor Extracted from the Enzyme in the Reduction of C_2H_2 under Nonenzymatic Conditions

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Abstract—To compare the catalytic effect of the active center of nitrogenase (iron–molybdenum cofactor (FeMoco)) under nonenzymatic conditions with the behavior of FeMoco incorporated in a protein, the kinetics of C_2H_2 reduction with Zn and Eu amalgams was examined in the presence of the cofactor extracted from the MoFe protein of nitrogenase (the specific activity of the extracted FeMoco after its integration into the cofactor-deficient MoFe protein of *Kp 5058* was 200 ± 20 mol of C_2H_4 (mol of Mo)$^{-1}$ min^{-1}. It was found that under exposure to reducing agents of different strength—Zn amalgam (**I**) (–0.84 V with respect to a normal hydrogen electrode (NHE)) and Eu amalgam (**II**) (–1.4 V with respect to NHE)—different reduction states of FeMoco were produced. They differed in the number and properties of substrate- and inhibitor-coordinating active sites. For **I**, the rate of ethylene formation was described by a hyperbolic function of substrate concentration (K_M = 0.045 atm). Carbon monoxide reversibly inhibited the reduction of acetylene (K_i = 0.05). For **II**, a sigmoid relationship between the rate of accumulation of C_2H_4 or C_2H_6 and substrate concentration was found. This relationship was explained by the occurrence of three interrelated sites of acetylene coordination and reduction with the apparent constant K_M = 0.08 atm in the FeMoco cluster reduced by europium amalgam. In this case, the specific activity was 40–60 mol of C_2H_4 (mol of Mo)$^{-1}$ min^{-1}. For the system with Eu (Hg), the CO inhibition constants were 0.004 and 0.009 atm for the formation of ethylene and ethane, respectively. The behavior of FeMoco as a catalyst for acetylene reduction and the inhibition of this reaction by carbon monoxide in various reducing protein and nonprotein media were compared. This comparison demonstrated that typical features of the catalytic behavior of FeMoco depend primarily on its composition and structure and only secondarily on the type of the reducing agent and on the reaction medium.

INTRODUCTION

One of the principal unsolved problems on the mechanism of biological nitrogen reduction catalyzed by the enzyme nitrogenase is the problem concerning a real chemical mechanism of substrate reduction at the active center of the enzyme—iron–molybdenum cofactor (FeMoco). Although intensive biochemical, structural, and kinetic studies of nitrogenase and biomimetic nitrogen-fixing model systems were performed, the structure of the center and the nature of nitrogen binding to the FeMoco cluster remain unclear. As a result of X-ray diffraction (XRD) analysis of single crystals, the three-dimensional structures of all protein components of the nitrogenase system were found [1, 2]. On this basis, the molecular structures of metal clusters incorporated into the enzyme were determined at a near-atomic resolution [3]. In particular, the XRD data supported that the active center of the enzyme has the composition $MoFe_7(S^{2-})_9$ (homocitrate). Nine sulfide groups play the role of bridges that join molybdenum and iron to form a prismatic cluster, which includes six coordinatively unsaturated iron atoms and bears two additional metal atoms on each side: on one side, a molybdenum atom in an octahedral environment and, on the other, an iron atom in a tetrahedral environment. Only these two atoms are responsible for the association between the cofactor and protein: the iron atom, by a sulfide bond via SH groups of cysteine and the molybdenum atom, via the imidazole of histidine [4].

FeMoco was first isolated from protein by Shah and Brill in 1977 [5]. They used N-methylformamide (NMF) as a solvent for the extraction of FeMoco from denatured protein. In this solvent, the FeMoco cluster separated from the protein matrix is soluble and stable for a long time. More recently [6, 7], it has been found that the cofactor can also be extracted from denatured protein as quaternary ammonium salts $[R_4N]^+[FeMoco]^-$ using other organic solvents, such as N,N-dimethylformamide (DMF), tetrahydrofuran (THF), and acetonitrile. Intensive studies of the cofactor extracted into NMF demonstrated that the separation of FeMoco from the protein matrix only slightly affected the spectral properties and electrochemical behavior of the cluster, which are much like the properties of the M-center (FeMoco inside a protein globule of MoFe protein) [8, 9]. The biological activity of FeMoco after the separation from protein was also changed only slightly: the reverse incorporation of FeMoco into MoFe protein deficient

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with respect to the cofactor completely restored the ability of the protein to catalyze the reduction of nitrogenase substrates (see the review [9] and references therein). There is almost no data on the effect of separation from the protein environment on the catalytic behavior of the cofactor, i.e., on the catalytic properties of extracted FeMoco toward the reduction of nitrogenase substrates as compared with the properties of the M-center. To close this gap, we attempted to find nonenzymatic conditions for the operation of FeMoco as a catalyst and then to examine the mechanism of cofactor-catalyzed reactions under potentially simpler nonprotein conditions. Using this approach, we hoped to find answers to the following questions, which are of principal importance for the understanding of the nitrogenase catalysis:

(1) Is the enzyme active center outside the protein matrix capable of catalyzing the reduction of particular nitrogenase substrates?

(2) What is the difference between the catalytic effects of FeMoco inside a protein globule and outside the protein, and which properties of FeMoco and to what extent are reproduced outside the protein?

(3) Is it possible to obtain such a state of FeMoco, in which it can catalyze N_2 reduction under mild conditions and nonenzymatic conditions?

A comparison of the catalytic behaviors of FeMoco inside protein and under nonprotein conditions provides was insight into the role of the protein environment (both amino acids nearest to the cofactor and the protein matrix as a whole) and its contribution to the reduction of a difficult-to-reduce substrate such as N_2 under mild conditions and sheds light on the fundamental difference between enzymatic and purely chemical nitrogen-fixing systems.

A positive answer to the first question was received in previous publications [10–12]. It was found that FeMoco can in principle be used as a catalyst for the reduction of a number of nitrogenase substrates (acetylene, azide, and nitriles) in nonprotein systems under special conditions, namely, in the presence of a reaction medium (DMF, NMF, or THF) that does not damage the cluster structure of FeMoco, a rather strong reducing agent (Zn, Eu, and Na amalgams), and a protonating agent of appropriate acidity (for example, thiophenol).

To obtain information concerning the mechanism of the catalytic effect of FeMoco in nonenzymatic systems, we started a kinetic study of the reactions found. In this work, we report data on the kinetics of C_2H_2 (a nitrogenase substrate) reduction and on the inhibition of this reaction by carbon monoxide. The reduction of acetylene is a common test for nitrogenase activity, both *in vivo* and *in vitro*. A great body of experimental data on the reduction of acetylene by nitrogenases was accumulated. These data make it possible to perform a comparative analysis of the catalytic behaviors of FeMoco in various protein environments and in purely chemical systems.

EXPERIMENTAL

The following chemicals were used in this study: tris(hydroxymethyl)aminomethane (Tris) and benzylviologen (Serva), sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES), creatine phosphate disodium salt, europium, tetra-*n*-butylammonium bromide, sodium dithionite, and thiophenol (Fluka); diethylaminoethyl (DEAE) Sepharose CL-6B and Sephadex LH-20 (Pharmacia); creatine kinase (Sigma); magnesium chloride, trichloroacetic acid (TCA), zinc, and mercury R0 (Reakhim); tetra-*n*-butylammonium chloride (Alfa); tetra-*n*-butylammonium hexafluorophosphate, disodium salt of adenosine triphosphate (ATP), and lithium aluminum hydride (Aldrich); 2,2'-dipyridyl of analytical grade (Reanal); and high-purity nitrogen and pure argon without additional purification.

Tetrabutylammonium dithionite was synthesized according to the procedure [6].

Molecular sieves 4 Å (Fluka) were activated by evacuation on heating and stored in argon.

Pure DMF (Reakhim) and NMF (Fluka) were dried and distilled in a vacuum (15 torr) over molecular sieves 4 Å and then degassed by evacuation; pure THF (Reakhim) was dried by distillation over $LiAlH_4$; pure acetone (Reakhim) was distilled over potassium carbonate. After evacuation, all of the solvents were stored in an argon atmosphere.

Buffer solutions (Tris · HCl (pH 7.4) and HEPES (pH 7.5)) were prepared using triply distilled water.

Acetylene (pure grade) was additionally purified as follows: it was frozen in liquid nitrogen and then evacuated to a residual pressure of 5×10^{-3} torr in an alcohol bath (–95°C) to remove trace oxygen; next, C_2H_2 was evaporated into a glass vessel by increasing the bath temperature to –50°C.

Carbon monoxide was prepared by the reaction of sodium formate with concentrated sulfuric acid.

All manipulations with air-sensitive materials (including chromatographic procedures) were performed under strictly anaerobic conditions using Schlenck techniques. All aqueous buffer solutions and organic solvents contained 5×10^{-3} M sodium dithionite and $(2–5) \times 10^{-3}$ M tetrabutylammonium dithionite, respectively. The presence of dithionite was monitored using a benzylviologen indicator.

The samples of FeMoco in different solvents and solutions of Fe protein and MoFe protein[1] were kept frozen in liquid nitrogen.

[1] Fe protein is a small ($M \sim 60\,000$) protein component of nitrogenase, and MoFe protein is a large ($M \sim 240\,000$) protein component of nitrogenase containing FeMoco.

Preparation of FeMoco

FeMoco was isolated from the MoFe protein of nitrogenase from *Azotobacter vinelandii* (the protein solution concentration was 20–40 mg/ml in 0.25 M NaCl–25 mM Tris · HCl). The isolation was performed according to the procedure described in [6, 7] by extracting the cofactor from DMF-denatured MoFe protein bound to a DEAE Sepharose anion-exchange support. The cofactor was eluted with Bu_4NCl/Bu_4NBr solutions in DMF, acetone, or NMF.

Removal of the Excess Salt $(R_4N)X$ (X = Cl or Br) from FeMoco Samples

The desalting of cofactor samples was performed according to the procedure [6, 7]. A concentrated FeMoco solution was passed through a column packed with Sephadex LH-20 in DMF or acetone, and the cofactor was eluted with a corresponding solvent.

Analysis of FeMoco

The quality of FeMoco after extraction (the retention of the cluster skeleton and the presence of homocitrate in its composition) was checked by the biological activity of FeMoco, i.e., by its ability to reconstruct the catalytic activity of MoFe protein of *Klebsiella pneumoniae* (mutant strain *Kp 5058* [13]) defective with respect to the cofactor toward acetylene reduction (protein reactivation). The assay was performed according to the procedure [13, 14]. A sample of the desalted cofactor was incubated with a cell extract of *Kp 5058* in a 50 mM Tris buffer (protein concentration of 10 mg/ml). Then, the reconstructed MoFe protein formed was added to a reaction mixture containing acetylene, Fe protein of *A. vinelandii*, ATP, $MgCl_2$, creatine phosphate, and creatine kinase in a HEPES buffer solution. After 15 min, the reaction with acetylene was stopped by adding TCA, and the amount of ethylene formed was measured as described below. The specific activity of FeMoco samples used in this study was 200 ± 20 mol of C_2H_2 (mol of Mo)$^{-1}$ min^{-1}.

Electrochemical Measurements

Voltammetric measurements were performed in a dry argon atmosphere in a DMF medium using a 3-ml electrochemical cell. A glassy carbon electrode (3 mm, Tokaii, Japan) sealed in glass and polished with diamond paste (particle size <1 μm) was used as a working electrode. The voltammetric measurements were carried out using a PAR 175 signal generator and a PAR 173 potentiostat with the compensation of ohmic losses. The voltammograms were recorded on an RE0074 chart recorder.

A 3-ml portion of a FeMoco solution (1.46×10^{-4} M) in DMF containing 0.05 M $[Bu_4N][PF_6]$ as a supporting electrolyte was placed in an electrochemical cell at a constant temperature of 18°C, and cyclic voltammograms were measured in the potential range from +0.5 to –1.5 V at a potential sweep rate of 200 mV/s.

All the potentials measured are given with respect to a saturated calomel electrode (SCE) by referring the potential of a reference electrode (Ag/AgCl/4 M aqueous LiCl solution), which was separated from the test solution with an electrolytic bridge filled with a supporting electrolyte solution, to the bis(diphenyl)chromium$^{0/+}$ redox potential (E^0 = –0.68 V with respect to SCE in DMF).

Preparation of Metal Amalgams

Europium amalgam was prepared by dissolving Eu metal in mercury heated to 80°C in an argon atmosphere. Zinc amalgam was prepared according to the procedure [15] by dissolving Zn in mercury upon heating with the addition of dilute sulfuric acid. The concentrations of amalgams were determined by titrimetry. They were found to be 0.9 wt % (0.785 M) and 2 wt % (4.27 M) for Eu(Hg) and Zn(Hg), respectively.

The potentials of amalgams in DMF and DMF/0.04 M PhSH media were measured using a P5837M potentiostat in a constant-temperature three-electrode cell (Ag/AgCl (1 M KCl) as a reference electrode and a platinum working electrode) in an inert atmosphere.

Experiments on the Catalytic Activity of FeMoco outside Protein

Experiments were performed in a specially designed constant-temperature flat-bottomed glass vessel [16] equipped with a magnetic stirrer for operations with metal amalgams. The vessel was evacuated and filled with argon; then, 0.7 ml of Zn(Hg) or 0.5 ml of Eu(Hg) was introduced into a side tumbler vessel in an argon flow, and 4 ml of a $(1–2) \times 10^{-5}$ M FeMoco solution and 0.2 ml of a 0.2 M thiophenol solution in DMF were added to the main vessel. The liquid phase was frozen, and the reaction vessel was connected to a circulation unit, evacuated, and then filled with a required gas mixture. In the course of an experiment, the gas phase was forcibly mixed in the circulation unit, and the liquid phase was stirred with a magnetic stirrer, choosing conditions under which the amalgam was maximally disintegrated. The gas-phase composition was varied depending on the problem to be solved: while studying acetylene reduction, the vessel was filled with an acetylene–argon mixture in different ratios; in experiments on H^+ reduction, with argon; and while studying the inhibiting effect of CO, with a CO–acetylene–argon mixture. In experiments on the relationship between the rate of reaction and the concentration of FeMoco, the cofactor content of the reaction mixture was varied within the range $(0–8) \times 10^{-8}$ mol at a constant acetylene pressure of 0.22 atm and a constant volume of the liquid phase. The experiments were performed at 21°C.

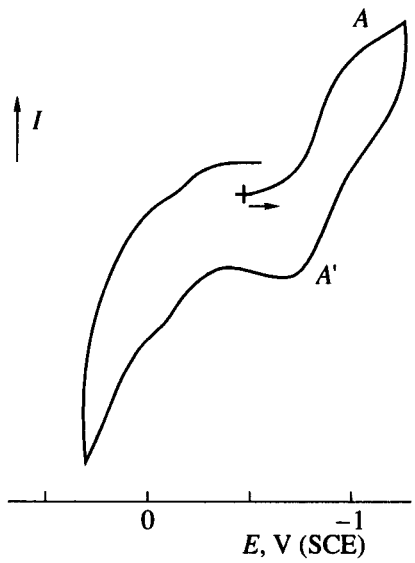

Fig. 1. Cyclic voltammogram of the FeMo cofactor in a DMF/0.05 M $[Bu_4N][PF_6]$ medium on a glassy carbon electrode (Tokaii, Japan) at a potential sweep rate of 200 mV/s.

Analytical Procedures

The molybdenum content of FeMoco samples was determined by either atomic absorption spectrometry on a Carl Zeiss AAS1 spectrometer with a Perkin-Elmer HGA 74 graphite furnace or colorimetry using the dithiol method [17]. The iron content was determined by spectrophotometry using a Fe^{3+} complex with the CNS^- ion. Absorption spectra were recorded on a Hewlett Packard 8451A Diode Array Spectrophotometer.

Gaseous reaction products were analyzed by gas chromatography. Ethylene, ethane, and methane were determined on a Biokhrom chromatograph using a column with activated alumina (Al_2O_3 fraction of 0.25–0.5 mm); the column temperature was 80°C; argon was a carrier gas; and a flame-ionization detector was used. Samples for analyses were taken directly from the circulating gas mixture into an evacuated calibrated sample loop, from which the sample was transferred to the detector with a carrier-gas flow. To determine the amount of hydrogen formed, gaseous products that were not frozen in liquid nitrogen were collected using a Töpler pump; hydrogen was determined by chromatography using a column packed with molecular sieves 5 Å; a thermal-conductivity detector was used; argon was a carrier gas.

RESULTS

I. Electrochemical Properties of FeMoco in DMF

It is known [8] that the formal potential of the redox transition FeMoco(s-r) ⟶ FeMoco(red) in NMF (ε = 182.4) is –1 V (with respect to NHE), whereas the redox transition M-center(s-r) ⟶ M-center(red) inside protein, where the polarity of the medium is considerably lower ($\varepsilon \approx 5$–10), takes place at a less negative potential of –0.47 V (with respect to NHE) [9]. Here, FeMoco(s-r) and FeMoco(red) denote the cofactor in dithionite-reduced and reduced states, respectively, and M-center(s-r) and M-center(red) are the above states of the cofactor inside a protein globule, respectively.

In nonaqueous media, the formal potential of a redox pair may strongly depend on the polarity and solvation ability of the solvent [18]. We used DMF (ε = 37) as a solvent for extracting the cofactor from protein and for studying its reactions with substrates. To find amalgams that exhibit reduction potentials sufficient for transforming FeMoco into a reduced (substrate-bound) state, we examined the electrochemical behavior of FeMoco in DMF by cyclic voltammetry.

Figure 1 demonstrates the cyclic voltammogram of a FeMoco solution in DMF. It can be seen that, in the examined range of potentials, a quasi-reversible redox pair of peaks *A*/*A*' is observed. As well as Schultz *et al.* [8a], we attributed this pair to the transition FeMoco(s-r)/FeMoco(red). The formal redox potential of this pair $E^0 = (E^A + E^{A'})/2$, where E^A and $E^{A'}$ are the potentials of peaks *A* and *A*', respectively, was found equal to –0.97 (with respect to SCE) or –0.73 V (with respect to NHE).

II. FeMoco-Catalyzed Acetylene Reduction with Zn and Eu Amalgams

Recently [10–12], we found that using zinc, europium, and sodium amalgams as electron donors (in aprotic solvents with thiophenol as a proton source), the FeMo cofactor of nitrogenase is an efficient catalyst for acetylene reduction, and the yield of products increases in the order Zn(Hg) < Eu(Hg) < Na(Hg), increasing with the cathode (negative) potential of the reducing agent.

Figure 2 shows the kinetic curves of ethylene and ethane buildup in the reduction of acetylene by zinc and europium amalgams. It can be seen that the reaction rate, as well as the amount of products, considerably increases with an increase in the cathode potential of the reducing agent. On going from Zn(Hg) (E = –0.84 V with respect to NHE) to Eu(Hg) (E = –1.4 V with respect to NHE) (the potentials were obtained by direct experimental measurements), the rate increased by more than an order of magnitude (from 3–5 to 40–50 mol of C_2H_4 (mol of Mo)$^{-1}$ min^{-1} for Zn(Hg) and Eu(Hg), respectively). The ethane fraction of products also increased considerably (from less than 1% in the case of Zn(Hg) to 25% for Eu(Hg)).

Without a catalyst (or in the presence of FeMoco preoxidized by atmospheric oxygen), acetylene is not reduced on either europium amalgam or zinc amalgam. Oxygen irreversibly destroys the cofactor. At temperatures above 40°C, the rates of acetylene reduction on both Zn amalgam and Eu amalgam dramatically decrease; a probable reason is the thermal decomposi-

tion of the catalytic cluster. Ethylene is a poor substrate in these systems: at a C_2H_4 pressure higher than 0.26 atm, only the stoichiometric reduction to C_2H_6 was observed for a sufficiently long time. The rate of product accumulation depends on the reduction potential, stirring intensity of the amalgam (in fact, the "electrode" surface area), and a solvent (using NMF instead of DMF, the reaction rates were noticeably lower in the case of Zn(Hg); this fact is consistent with the potentials of FeMoco transition into a state active toward substrates in these solvents).

It is well known that nitrogenase in the absence of substrates catalyzes the reduction of H^+ to hydrogen. We found that the reduction of H^+ (PhSH) to hydrogen on both zinc amalgam and europium amalgam catalyzed by the cofactor was also observed in chemical systems with the participation of FeMoco. The rate of this reaction also increased significantly with an increase in the cathode potential of the reducing agent. The reaction occurred at an amalgam surface, because the amount of H_2 released in a certain time depended heavily on the intensity of amalgam stirring.

The currently available literature data on the composition and spectral properties of nitrogenase cofactors from different bacterial sources indicate that the enzymes in various microorganisms contain identical cofactors [3, 9]. It was of interest to test to what extent the catalytic behavior of nitrogenase cofactors extracted from the protein matrix from various types of microorganisms is similar under nonprotein conditions. We performed a comparative study of the behaviors of the following two cofactors: the cofactor obtained from the MoFe protein of *Azotobacter vinelandii* and a sample of FeMoco from the MoFe protein of *Klebsiella pneumoniae* provided by our British colleagues. Figure 3 demonstrates the kinetic curves of acetylene reduction into ethylene and ethane by europium amalgam catalyzed by FeMoco. It can be seen that under identical conditions (solvent, temperature, amalgam amount, stirring intensity, and FeMoco amount), the cofactors behaved almost identically. These cofactor samples also exhibited identical specific activities in experiments on the reactivation of defect FeMo protein of *Klebsiella pneumoniae Kp 5850*.

The inhibition of acetylene reduction with time (Figs. 2 and 3) can be explained by several causes, namely: the gradual decomposition of the catalyst, thiophenol consumption, or a drop in the reduction potential of amalgam in the course of reaction. The relative contributions from these causes are not equal for zinc and europium amalgams. To understand how a decrease in the rate in the course of reaction is associated with the consumption of thiophenol, we measured the yields of hydrogen for both of the systems in the time it takes for the reaction rate to fall noticeably; this time was ~1 h or 5 min for Zn(Hg) or Eu(Hg), respectively. We found that in the latter case, more than 50% thiophenol was consumed in 5 min in the reaction of

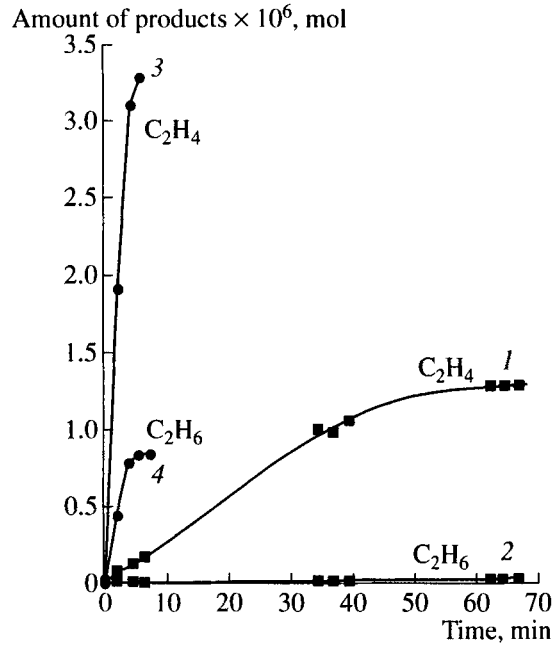

Fig. 2. Kinetics of the reduction of acetylene to (*1*, *3*) C_2H_4 and (*2*, *4*) C_2H_6 under exposure to (*1*, *2*) zinc amalgam or (*3*, *4*) europium amalgam. Catalyst: FeMoco from MoFe protein of *Azotobacter vinelandii*. [Mo] = 0.53×10^{-5} M; [PhSH] = 0.012 M.

hydrogen release; it is likely that this is the main reason for the decrease of acetylene reduction. In the case of zinc amalgam, the thiophenol concentration decreased by only 10–15% in an hour because of the reaction of hydrogen release. It is likely that in this case, the main reason for a decrease in the reaction rate with time is a drop in the absolute value of the amalgam potential in the course of reaction as a result of a decrease in the concentration of zinc in the amalgam. Because the reduction potential of zinc amalgam is close to the critical minimum required for FeMoco conversion into the substrate-bound state, even a small decrease in the potential (in magnitude) in the course of reaction can result in that the potential of the reducing agent is inadequate to transform FeMoco into the state active toward C_2H_2 reduction, and the catalytic reaction terminates.

Dependence of the reaction rate on [FeMoco]

Figure 4 demonstrates the initial steady-state rate[2] of C_2H_4 formation (w) as a function of cofactor concentration for europium amalgam as a reducing agent. At low catalyst concentrations (from 0 to 1×10^{-5} M FeMoco), the rate of product buildup is proportional to the FeMoco concentration. Then, an increase in the cata-

[2] Here, the initial steady-state rate is a rate at the initial linear portion of a kinetic curve under steady-state conditions with respect to the intermediate catalyst–substrate complex, which is converted into the product (C_2H_4 and (or) C_2H_6) with regeneration of the active center (Michaelis scheme) [19].

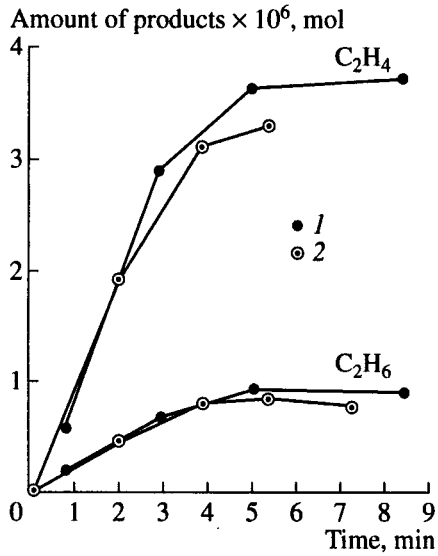

Fig. 3. Comparison between the catalytic behaviors of nitrogenase cofactors from different bacterial sources: (*1*) FeMoco from nitrogenase of *Klebsiella pneumoniae*, [Mo] = 0.6×10^{-5} M; (2) FeMoco from nitrogenase of *Azotobacter vinelandii*, [Mo] = 0.53×10^{-5} M. Eu(Hg); [PhSH] = 0.012 M.

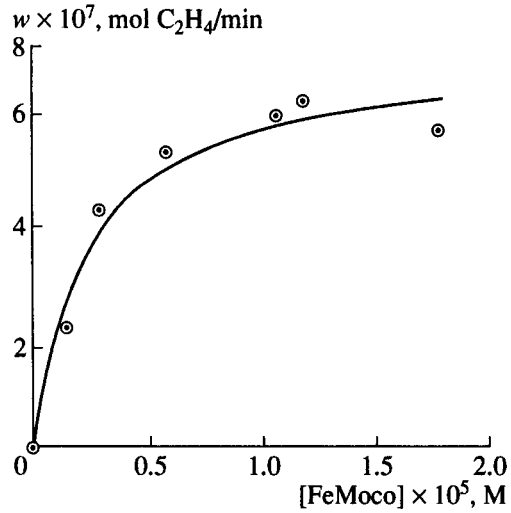

Fig. 4. Initial steady-state rate of acetylene reduction by europium amalgam as a function of cofactor concentration. Eu(Hg); [PhSH] = 0.012 M.

lyst concentration no longer affects the reaction rate. A change from the first-order reaction with respect to FeMoco to an apparent zero order with an increase in the concentration of the catalyst in solution takes place at [FeMoco] ≥ 1.2×10^{-5} M. This can be explained by a heterogeneous character of the reaction (the cofactor reduction to the substrate-bound state occurs after the adsorption on the amalgam surface). The curve reflects an increase in the surface concentration with an increase in the volume concentration of the catalyst, and the portion of a constant rate corresponds to the complete coverage of the amalgam surface at a constant intensity of stirring.

Dependence of the reaction rate on the substrate concentration

Zinc amalgam. Figure 5 demonstrates the initial steady-state rate[2] of cofactor-catalyzed acetylene reduction into ethylene on a per-mole basis in terms of Mo ($w^{sp}_{C_2H_4}$) as a function of the partial pressure of C_2H_2 in the case of zinc amalgam used as a reducing agent. The relationship is adequately described by the Michaelis equation. The Michaelis constant K_M = 0.045 atm was found by the linearization of the curve in the reciprocal coordinates (Lineweaver–Burk plot). At $P_{C_2H_2} \geq$ 0.29 atm, the reaction rate no longer depends on acetylene pressure. At pressures close to this value ($P_{C_2H_2} >$ 0.13 atm), ethylene is almost the only product (less than 1% ethane is formed). However, at low acetylene pressures (0.007–0.013 atm), the formation of noticeable amounts of ethane is observed (up to 10% of the total products).

Europium amalgam. Figures 6 and 7 demonstrate the specific initial steady-state rates of formation of ethylene and ethane, respectively, as functions of substrate concentration in the reduction of acetylene by europium amalgam in the presence of FeMoco. In the acetylene pressure range from 0 to 0.06 atm, the dependence of the rate of either C_2H_4 or C_2H_6 buildup on substrate concentration is described by a hyperbolic function and can be linearized in the Lineweaver–Burk coordinates. The value of K_M = 0.006 atm for the formation of both ethane and ethylene was found from this plot. Thus, at low substrate concentrations, one site of the cofactor reduced with europium amalgam exhibits activity in acetylene reduction; this site gives ethylene and ethane in the ratio ≈4 : 1 as products. The saturation of this site with acetylene induced the activity of other sites of the cluster in this reaction. In a pressure range from 0.06 to ~0.29 atm, the reaction rate plotted as a function of the substrate concentration exhibits an *S*-shaped curve; this fact is indicative of the simultaneous coordination of several acetylene molecules to a catalytic cluster [19]. The coefficient n = 1.6 was found by the linearization of the *S*-shaped curve in the Hill coordinates (Figs. 6b and 7b). The value of this coefficient suggests substrate-induced cooperativity between at least two sites that are active toward acetylene reduction.

Acetone reduction. In some kinetic experiments that use zinc amalgam as a reducing agent, we observed irreproducible rates of acetylene reduction and the formation of methane in considerable amounts in addition to ethylene. We found that acetone was responsible for irreproducibility and served as a source of methane. Acetone was used at a stage of FeMoco extraction from protein and remained in the final preparations in extremely low uncontrollable amounts. The formation of methane was not observed in the case of FeMoco isolated from protein without using acetone. Acetone

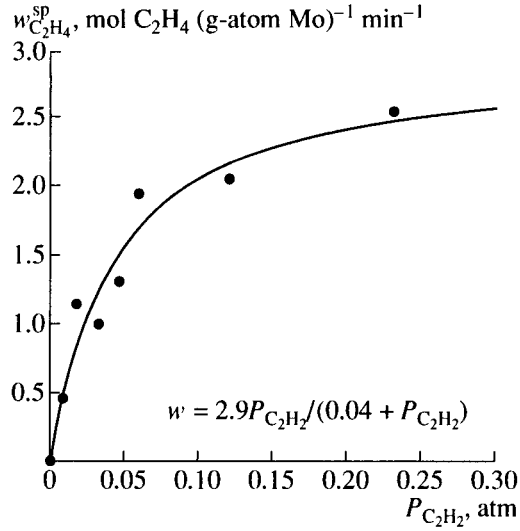

Fig. 5. $w^{sp}_{C_2H_4}$ as a function of substrate concentration for the system with Zn(Hg) as a reducing agent. [PhSH] = 0.012 M.

additives to the reaction medium almost completely inhibited the reaction with acetylene, and the amount of methane formed increased dramatically. If pure argon was used as a gas phase instead of a mixture of C_2H_2 with Ar, the cofactor-catalyzed catalytic reduction of acetone with zinc amalgam to form methane was observed (Fig. 8). Because ethanol was detected in the liquid phase after completion of the reaction, it is likely that the following reaction occurred:

$$H_3C-\overset{\overset{O}{\|}}{C}-CH_3 \xrightarrow[4H^+(PhSH)]{4e^-(Zn(Hg))} CH_4 + CH_3CH_2OH,$$

Carbon monoxide and acetylene were found to inhibit this reaction. In the system with a europium amalgam as a reducing agent, the catalytic reduction of acetone to methane did not occur, and acetone did not inhibit the reduction of acetylene.

Inhibition of FeMoco-catalyzed acetylene reduction by carbon monoxide

It is well known that CO inhibits (both *in vivo* and *in vitro*) the reduction of all nitrogenase substrates, with the exception of H^+. It was of interest to reveal the features of carbon monoxide inhibition of the reduction of acetylene by nonprotein systems with the participation of FeMoco. Early experiments demonstrated that in this case CO also exerts a strong inhibiting effect, as well as in enzymatic systems, but only if it is present in the system in the course of the catalytic reaction. The preexposure of a cofactor solution to a carbon monoxide atmosphere without a reducing agent has no effect on the subsequent reactions with acetylene. This fact indicates that CO is not bound to FeMoco in the dithionite-reduced state. An analogous result was obtained earlier [20]. The reaction was inhibited to different extents in the presence of zinc and europium amalgams: the stronger the reducing agent, the lower the carbon monoxide pressure that exhibits an inhibiting effect. In the presence of zinc amalgam, the inhibition constant is $K_i = 0.05$ atm. In this case, we experimentally found that the inhibition is reversible: after the removal of CO by evacuating the gas phase over the reaction mixture frozen in liquid nitrogen and the admission of a new portion of acetylene, the catalytic reduction of C_2H_2 continued. The presence of CO at 0.09 atm decreased by half the amount of hydrogen released on a zinc amalgam with the cofactor used as a catalyst. In the presence of the stronger reducing agent

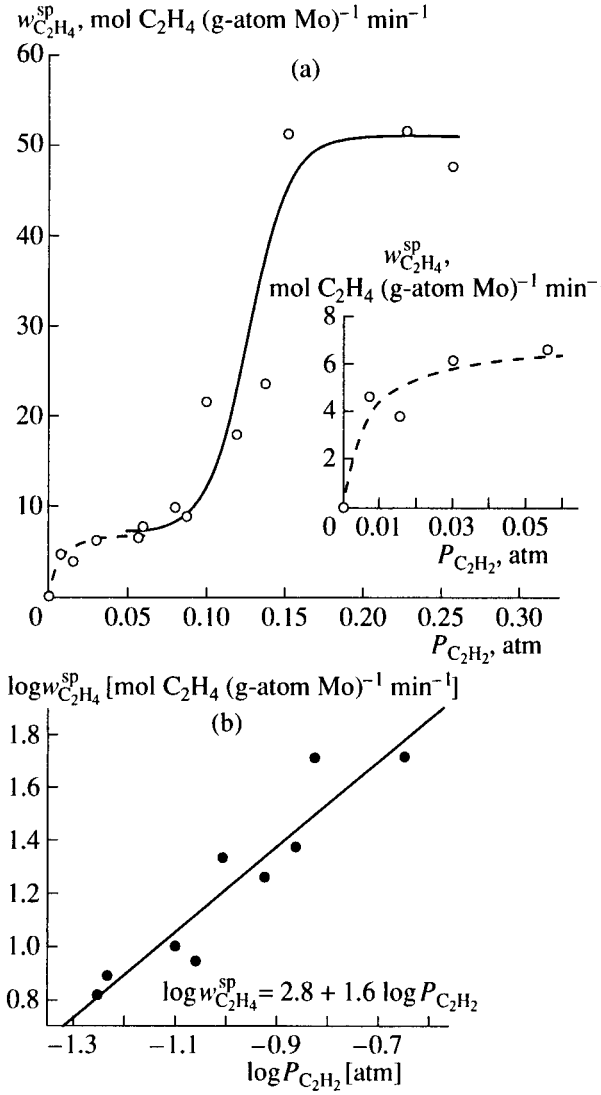

Fig. 6. $w^{sp}_{C_2H_4}$ as a function of substrate concentration for the system with Eu(Hg) as a reducing agent; (a) a portion of the plot for $P_{C_2H_2}$ = 0–0.06 atm; (b) linearization of the *S*-shaped portion of the curve ($P_{C_2H_2}$ = 0.06–0.29 atm) on the Hill coordinates. [FeMoco] = 3×10^{-6} M; [PhSH] = 0.010 M.

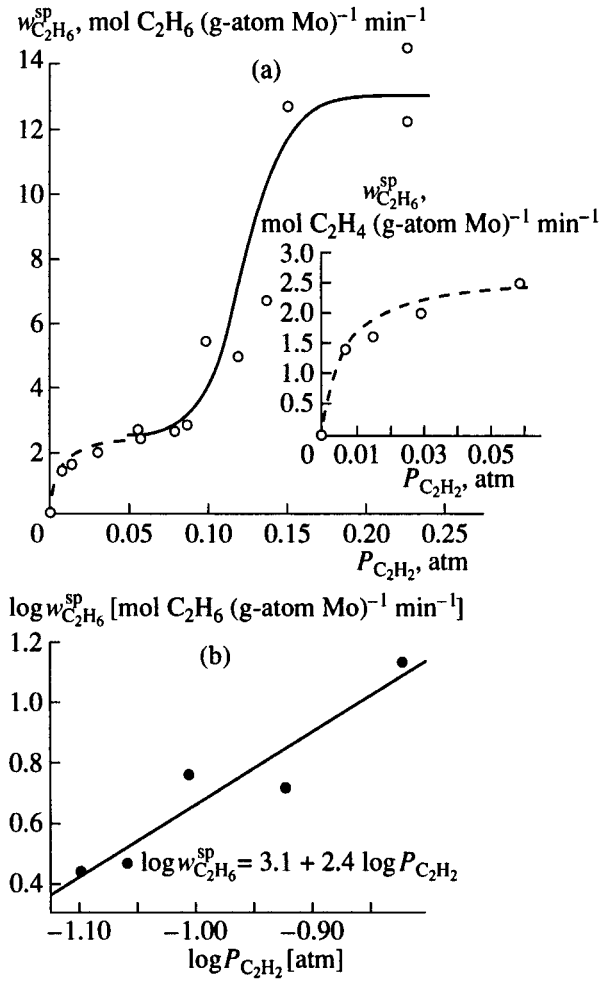

Fig. 7. $w^{sp}_{C_2H_6}$ as a function of substrate concentration (all conditions are analogous to those specified in Fig. 6).

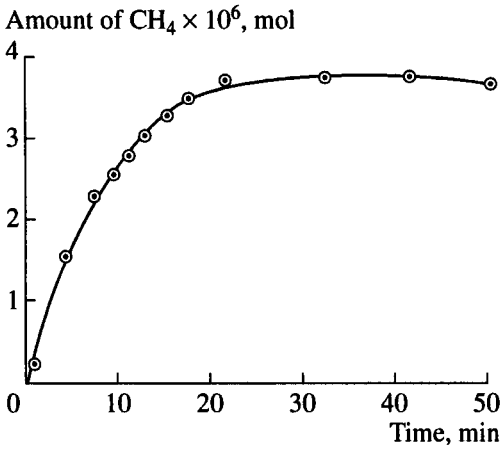

Fig. 8. Methane formation in the reduction of acetone on zinc amalgam. Catalyst: FeMoco, [Mo] = 0.55×10^{-5} M; 3.6 ml of DMF; 0.4 ml of CH_3COCH_3; [PhSH] = 0.010 M.

Eu(Hg), CO exerts a considerable inhibiting effect at pressures lower than those in the case of Zn(Hg) by an order of magnitude; the inhibition of ethylene formation was much more pronounced than that of ethane formation. Thus, the relative fraction of ethane in the products increased from 25 to 60% in the reduction of C_2H_2 in the presence of small amounts of CO (0.007–0.05 atm). The inhibition constants found from the rates of product formation as functions of CO pressure (Fig. 9) were 0.004 and 0.009 atm for ethylene and ethane, respectively. These data also confirm the presence of different acetylene-binding sites in a catalytic cluster, which exhibit different responses to the inhibiting action of CO.

DISCUSSION

Electrochemical Properties of FeMoco

It is well known that the M-center in the MoFe protein of nitrogenase can occur at least in the following three oxidation states: dithionite-reduced or semireduced (s-r), oxidized (ox), and reduced (red). The s-r state of the M-center results from protein purification in the presence of an excess of dithionite according to a standard procedure. This M (s-r)-center can be reversibly oxidized or reduced to M (ox)- or M (red)-centers, respectively. The M (red)-center results from the reduction of M (s-r) at a potential of –0.465 V (NHE); the number of electrons transferred in this process is unknown [8, 9]. The M (red) state is the substrate-reducing state of the center inside protein. This state is accomplished only under the conditions of a nitrogenase reaction, i.e., in the presence of the complete nitrogenase system (ATP, Fe protein, MoFe protein, Mg^{2+}, creatine phosphate, creatine kinase, and a reducing agent).

An electrochemical study of the cofactor extracted from protein in an NMF solution by cyclic voltammetry demonstrated that it underwent quasi-reversible reduction at a formal potential of –1.1 V (NHE) [8a]. Schultz *et al.* [8a] assigned this transition to the pair FeMoco(s-r)/FeMoco(red).

The electrochemical behavior of FeMoco in DMF is consistent with the published results [8a]; the only difference is that the reduction of FeMoco in DMF takes place at a more positive potential (–0.73 V (NHE)). This is not surprising taking into account a considerable difference in the donor properties and solvating ability of NMF and DMF. The cofactor extracted from protein is always specifically solvated by the solvent used for the extraction. Thus, the cofactor containing strong donor molecules of NMF as ligands is more difficult to reduce; that is, it is reduced at a more negative potential.

Acetylene Reduction

Let us consider how our data on the acetylene reduction by the nonenzymatic reducing system with the participation of FeMoco as a catalyst compare with the information known for various protein systems. After Dilworth's discovery [21] that C_2H_2 is a nitrogenase

substrate, this reaction became a common test for nitrogenase activity. Acetylene is reduced by all nitrogenases to different degrees. An exception is the nitrogenase of *Streptomyces thermoautotrophicus* discovered recently, which has a different active center [22]. Table 1 summarizes data on the specific activity toward acetylene hydrogenation and on the composition of reduction products for classical FeMoco-containing nitrogenase, for alternative nitrogenases containing analogous clusters with Fe or V as heteroatoms as active centers, and for hybrid systems obtained by incorporating FeMoco extracted from classical nitrogenase into the protein of alternative nitrogenases. It is likely that these hybrid systems are the most similar to our nonenzymatic systems formed by placing FeMoco extracted from protein in another reducing medium. Classical Mo-containing nitrogenase exhibits a maximum specific activity toward C_2H_2, which is as high as 250 mol of C_2H_4 (mol of Mo)$^{-1}$ min^{-1} [23]. Alternative and hybrid nitrogenases and nonprotein systems with the participation of FeMoco exhibit a somewhat lower activity (Table 1). For a long time, nitrogenase was believed to be capable of reducing C_2H_2 to ethylene only. This was even a test for the similarity of model systems to nitrogenase. Now data on the reaction rates and sets of products are accumulated for various nitrogenases containing almost identical cofactors (a very high degree of similarity between metal clusters—active centers of alternative nitrogenases—was found in spectroscopic and biochemical experiments (see the review [25] and references therein)). These data indicate that the reaction conditions, in particular protonation conditions, are responsible for the appearance of ethane in the products of C_2H_2 reduction. The formation of ethane is typical of alternative nitrogenases [28]; classical nitrogenase also gives ethane at elevated temperatures (50°C) [29]. The single replacement of amino acids near FeMoco in an FeMo protein results in mutants that produce a considerable percentage of ethane in the reduction of C_2H_2 [30]. The molar fraction of ethane increases with an increase in the "electron flux" (the number of electrons transferred to the cofactor), i.e., for enzymatic systems, with an increase in the Fe protein/MoFe protein ratio (because Fe protein is a source of electrons in the reduction of the cofactor in MoFe protein) and, for our model systems, with an increase in the cathode potential of the reducing agent. Note that the following is also typical of reactions with the participation of FeMoco: (a) the rate of reduction depends on an electron flux (the more "accessible" the electrons are, the higher the activity is) and (b) the C_2H_6/C_2H_4 ratio depends on pH (the more "accessible" the protons are, the higher the relative fraction of ethane formed is [25]).

It is likely that the occurrence of simultaneously working interdependent active centers on the reduced FeMoco cluster, which we observed in the experiments, is also typical of the performance of this catalyst. A number of experimental data indicate that several

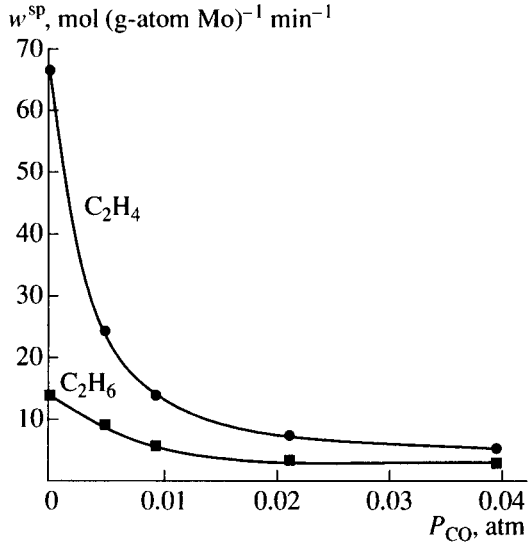

Fig. 9. Dependence of w^{sp} for ethylene and ethane on the amount of CO added. [Mo] = 0.6×10^{-5} M; Eu(Hg); [PhSH] = 0.012 M.

substrate and (or) inhibitor molecules can simultaneously be coordinated to the MoFe protein of nitrogenase. For example, it was found that reduced FeMoco can simultaneously coordinate two CO molecules [31]. Because the inhibition of substrate reduction by carbon monoxide is noncompetitive, this suggests the simultaneous coordination of CO and a substrate at the reduced form of the FeMoco cluster.

Chen *et al.* [32] observed an *S*-shaped relation between the rate of ethylene formation and acetylene concentration in the presence of very small amounts of CO on the mutant MoFe protein *DJ788* (in which the amino acid arginine α-277 near the cofactor is replaced with histidine). This relation was explained by the inhibitor-induced cooperativity between two C_2H_2-coordinating sites (the Hill coefficient is equal to 1.6), so that a total of three substrate and inhibitor coordination sites on the reduced FeMoco cluster were occupied simultaneously.

Thus, a comparison of the specific activity of FeMoco and its behavior as a catalyst in different reducing media of both protein and nonprotein origin demonstrates that this behavior primarily depends on the composition and structure of the catalyst and secondarily, on the type of reducing agent. In this case, critical parameters are the redox potential of the reducing agent (it should be less negative than the potential of the FeMoco transition into the substrate-bound state), the reaction medium (it should not decompose the FeMoco cluster structure in none of the redox states passed by the catalyst in the course of a catalytic cycle), and the acidity of the protonating agent (it should be sufficient for the protonation of the coordinated substrate, but not damaging for the integrity of the FeMoco cluster framework).

Table 1. Reduction of C_2H_2 by different nitrogenases and nonenzymatic systems with the participation of the active center of classical nitrogenase as a catalyst

System	Activity*, mol of C_2H_2 (g-atom M)$^{-1}$ min^{-1}	$[C_2H_6]$, %	References
FeMoco-containing nitrogenase (*A. vinelandii*)	150–250	0	[23]
FeVco nitrogenase	10–27	1–77.5	[23–25]
FeFeco nitrogenase	4–8	5.5 (pH 6.5) 1.6 (pH 7.8) 0 (pH >8.5)	[26a]
FeMoco in VFe protein	10	28	[27]
FeMoco in FeFe protein	–	60	[26a and 26b]
FeMoco + Zn(Hg) + PhSH	2–3	<1.0	This work
FeMoco + Eu(Hg) + PhSH	40–50	25	This work

* Calculated on a per-mole basis in terms of Mo, V, or Fe for Mo-, V-, or Fe-containing systems, respectively.

Inhibition by Carbon Monoxide

Christie *et al.* [33] unambiguously demonstrated that the inhibiting effect of CO in nitrogenase systems is associated with the fact that CO forms a complex with a reduced form of FeMoco in the course of a catalytic cycle. Depending on the pressure of CO, either one molecule is coordinated by forming a bridge between two neighboring iron atoms, or two molecules are terminally coordinated to two neighboring iron atoms [31]. Because the inhibition is noncompetitive, the absence of a reaction between the center and substrates in the presence of CO can be explained by the fact that the coordination of carbon monoxide to the cluster dramatically affects the potential sites of substrate coordination. The inhibition was found to be reversible.

It was believed that the inhibiting effects were identical for all nitrogenases. Presently, many inhibition modes are known, depending on the type of nitrogenase and reaction conditions. An extraordinary phenomenon was described by Cameron *et al.* [34], who were the first to find that, in the presence of V-nitrogenase, carbon monoxide accelerates rather than inhibits acetylene reduction at very low pressures (Table 2). The inhibition constant varies over a wide range and depends on both the type of nitrogenase (classical, alternative, mutant, or hybrid) and the accessibility of electrons (the degree of saturation with respect to the reducing agent, which demonstrates to what extent the number of electrons transferred from the reducing agent approaches the number required for the complete reduction of the cofactor). It was found for a wide variety of nitrogenases that the CO inhibition efficiency increases with an increase in the electron flux (or with an increase in the Fe protein/MoFe protein ratio); this fact indicates that different redox states of FeMoco react differently with CO [34].

Earlier, it was believed that CO does not inhibit the reduction of H^+ by nitrogenase. This is only true of classical nitrogenase, where the protein environment is arranged optimally for electron and proton transfer to the FeMoco cluster. However, even an insignificant distortion of this optimality, for example, by single replacement of amino acids close to the cofactor, results in noticeable differences in the behavior of the formed mutants toward CO; in particular, the inhibition of hydrogen release takes effect under exposure to CO. Thus, it was found that a disturbance in the system of hydrogen bonds around homocitrate, for example, by the replacement with citrate [36] or by replacing amino acids that participate in the formation of hydrogen bonds [37], renders the reduction of protons sensitive to inhibition by CO. For classical nitrogenase, such an effect was observed at high pH (7.5–9), when amino acids close to FeMoco were deprotonated [38].

Schneide *et al.* [26b] studied an alternative Fe-containing nitrogenase and observed unusual behavior toward CO, which bears a strong resemblance to the behavior of our systems. They also obtained different inhibition constants for C_2H_4 and C_2H_6. At relatively high pressures of CO added, the ethane fraction of the reduction products was as high as 80%. Schneide *et al.* [26b] explained this phenomenon by the occurrence of two sites of C_2H_2 coordination in the catalytic FeFeco cluster; these sites exhibit different responses to the additional coordination of an inhibitor molecule to the cluster.

Thus, various protein systems with the participation of FeMoco (FeVco or FeFeco) as a catalyst exhibited all of the special features of inhibition observed in this study, namely: the interaction of CO with only the

Table 2. Constants of CO-induced inhibition of the reduction of C_2H_2 by various nitrogenases and a nonenzymatic system with the participation of the active center of classical nitrogenase as a catalyst

System	K_i, atm	Note	References
FeMoco + Eu(Hg) + PhSH	0.004 (C_2H_4) 0.009 (C_2H_6)	At $P_{CO} > 0.04$ atm, the C_2H_6 fraction increased from ≈20 to 60%	This work
FeMoco-containing nitrogenase (*A. vinelandii*)	0.0003	–	[32, 35]
FeVco nitrogenase	0.03	–	[23, 25]
FeFeco nitrogenase	0.009 (C_2H_4) 0.15 (C_2H_6)	At $P_{CO} > 0.13$ atm, the C_2H_6 fraction increased up to 80%	[26a]
FeMoco in VFe protein	0.03	At $P_{CO} \approx 0.001$–0.007 atm, the formation of products was stimulated	[34]

reduced state of FeMoco, the dependence of the inhibition constant on the potential of the reducing agent, the reversibility of inhibition, the partial inhibition of cofactor-catalyzed reduction of H^+ to hydrogen, and the occurrence of several interdependent sites of substrate and inhibitor coordination. The above features primarily characterize this type of clusters.

Acetone Reduction

It is well known that nitrogenase can reduce a number of molecules having multiple C≡C-, C≡N-, N≡N-, or CO_2 bonds [39]. Recently, this range was extended by compounds with C=O and C=S bonds (CO_2, COS, and CS_2) [40]. Thus, it is not surprising that FeMoco catalyzes the reduction of acetone by zinc amalgam. It is likely that the range of substrates for FeMoco in a nonprotein environment can be extended further, because the limitations on the substrate size are removed in this case (these limitations are associated with particular parameters of a channel for the approach of a substrate to the active center deep inside the protein). Moreover, it is likely that not all potentially active sites in FeMoco embedded in protein are accessible to substrates, and FeMoco outside protein can be more reactive than that inside a protein globule.

The fact that acetone is reduced on Zn(Hg) rather than Eu(Hg) at a high rate is not surprising either, if the chemical nature of the reacting species is taken into consideration. It is likely that reducing agents of different strengths produce different reduced states of FeMoco. These states differ from each other in the number and character of substrate and inhibitor coordination sites. Under exposure to zinc amalgam, the FeMoco cluster is reduced to a state at which it can coordinate C_2H_2 and CO. In this case, it is not too strongly reduced and can be a σ-acceptor and coordinate donor molecules such as acetone. It is likely that, in this case, the nature of coordination sites is similar to that in a labile coordinatively unsaturated Ru(II) complex reported in [41]. It was found that this complex can reversibly bond to various molecules different in the donor–acceptor properties (N_2, H_2O, acetone, CO, CH_3CN, and RC≡CH) primarily by σ-donation from the ligand to the metal. Using europium amalgam as a reducing agent, we obtained a more reduced state of the cofactor. The cluster in this state reduces acetylene at a much higher rate. In the case of Eu(Hg), as judged from the dependence of the reaction rate on substrate concentration and from the type of inhibition of this process by carbon monoxide, the number of coordination and reduction sites is greater than that in the case of Zn(Hg), and the nature of these sites is different (different reduction products are formed, and the CO inhibition character is different). In this case, acetone is not a substrate (and does not inhibit acetylene reduction) because the conditions of its possible coordination are not met. The cluster lost the property to serve as a σ-acceptor (it is too strongly reduced for that), and the π-acceptor properties of acetone are insufficient for the coordination due to the π-donor properties of the metal (here, no more than 2 mol of CH_4 per mole of FeMoco was formed; it is likely that acetone that was initially present in the solvation sphere of FeMoco was immediately reduced, whereas the other acetone molecules present in the solution cannot coordinate to the strongly reduced form of FeMoco). Carbon monoxide as a good π-acceptor can readily bind to a strongly reduced metal center, and the inhibition by this compound is much more pronounced.

CONCLUSION

A comparison of the catalytic behavior of FeMoco in protein and purely chemical systems toward acetylene reduction and inhibition of this process by carbon monoxide demonstrates that both the reduction and the inhibition are quite similar in various systems with the participation of FeMoco. Thus, our approach shows promise as a tool for obtaining new information on the chemical mechanism of substrate reduction at the active center of nitrogenase and for understanding the role of the protein environment and its contribution to

the capability of the enzyme to reduce such a difficult-to-reduce substrate as molecular nitrogen under mild conditions using comparatively weak reducing agents.

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