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Mutual Effects of Substrates and Inhibitors in Reactions Catalyzed by Isolated Iron–Molybdenum Cofactor of Nitrogenase[1]

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Abstract—The inhibiting effects of CO and N_2 on the ability of the nitrogenase iron–molybdenum cofactor (FeMoco) to catalyze acetylene reduction outside the protein were studied to obtain data on the mechanism of substrate reduction at the active center of the enzyme nitrogenase. It was found that CO and N_2 reacted with FeMoco that was separated from the enzyme and reduced by zinc amalgam (E = –0.84 V relative to a normal hydrogen electrode (NHE)) (I) or europium amalgam (E = –1.4 V relative to NHE) (II). In system I, CO reversibly inhibited the reaction of acetylene reduction to ethylene with K_i = 0.05 atm CO. In system II, CO inhibited the formation of the two products of C_2H_2 reduction in different manners: the mixed-type or competitive inhibition was found for ethylene formation with K_i = 0.003 atm CO and the incomplete competitive inhibition was found for ethane formation with K_i = 0.006 atm CO. The fraction of C_2H_6 in the reaction products was greater than 50% at a CO pressure of 0.05 atm because of the stronger inhibiting effect of CO on the formation of C_2H_4. The change in the product specificity of acetylene-reduction centers under influence of CO was explained by some stabilization of the intermediate complex [FeMoco · C_2H_2] upon the simultaneous coordination of CO to the catalytic cluster. Because of this, the fraction value of ethane as a multielectron reduction product increased. The experimental results suggest that several active sites at the FeMoco cluster reduced outside the protein can be simultaneously occupied by substrates and (or) inhibitors. The inhibition of both ethane and ethylene formation by molecular nitrogen in system II is competitive with K_i = 0.5 atm N_2 for either product. That is, N_2 and C_2H_2 as ligands compete for the same coordination site at the reduced FeMoco cluster. The inhibiting effects of CO and N_2 on the catalytic behaviors of both isolated FeMoco and that in the enzyme were compared.

INTRODUCTION

The enzyme nitrogenase catalyzes the reduction of molecular nitrogen to ammonia at room temperature and atmospheric pressure. This process of converting atmospheric nitrogen into a biologically assimilable form provides a basis for the biosynthesis of proteins and nucleic acids. The enzyme contains two protein components: the Fe protein and the MoFe protein. In addition to both of the proteins, the presence of a reducing agent, for example, sodium dithionite; ATP; and an ATP-regenerating system is required to perform an *in vitro* nitrogenase reaction. The reaction is carried out in an aqueous buffer solution (pH ~7) in the absence of atmospheric oxygen. Under these conditions, nitrogenase catalyzes the reduction of not only N_2 but also a number of C-, O-, and N-containing small molecules with multiple bonds [1–3]. Up to now, it was unambiguously established [4–6] that the active center of the enzyme, that is, the center of substrate coordination and conversion, is the polynuclear complex [$MoFe_7S_9$ · homocitrate]—the so-called iron–molybdenum cofactor (FeMoco), which is a constituent of the MoFe protein [7–10].

One of the most important unresolved problems in nitrogenase catalysis is the mechanism of substrate conversion at the active center of the enzyme. To solve this problem, we used the following approach [11]: we studied reactions that occur at the active center of the enzyme using the cofactor separated from the protein matrix as a catalyst in the chemical nonenzymatic system. This approach allowed us to understand what kind of the reactions FeMoco cluster can catalyze without the protein environment. The nitrogenase reactions that can be performed on the cofactor outside the protein and the rate laws of these reactions can provide data on the chemical nature of the process and on the necessity of amino acids adjacent to the cofactor at particular steps of the reaction.

Previously [11, 12], we found that FeMoco separated from the nitrogenase MoFe protein is an active catalyst of the reaction of acetylene reduction by zinc amalgam or europium amalgam in aprotic solvents with the use of thiophenol as a source of protons. The integrity of the FeMoco cluster framework before and

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after the catalytic process was established [13]. A comparison of the reaction kinetics of acetylene reduction catalyzed by extracted FeMoco with analogous data for an *in vitro* nitrogenase system demonstrated that these different systems with the participation of FeMoco as a catalyst exhibited very similar apparent reaction rates, set of products, and chemical behavior. The possibility of N_2 reduction with the participation of FeMoco separated from the protein remains unclear. We failed to detect the products of N_2 reduction under the conditions of active C_2H_2 reduction. It is well known that the formation of ammonia from molecular nitrogen is a complex multistep process. However, it must begin with the reduction of FeMoco and the coordination of the N_2 molecule to the reduced cofactor. To recognize whether this first step, which is absolutely necessary, occurs under the conditions of our experiments, we examined the effect of nitrogen on the kinetics of FeMoco-catalyzed acetylene reduction. If N_2 can at least be coordinated to the reduced cluster, it has to affect the kinetics of C_2H_2 reduction, for example, by inhibiting this reaction.

The inhibitor analysis method is widely used in studies of enzyme catalysis in general and nitrogenase catalysis in particular because it provides useful information on both the mechanism of action of an enzyme as a whole and substrate transformations at the active center. A great body of information on the effects of various inhibitors on nitrogenase has been accumulated. In particular, on this basis, assumptions can be made about the mechanisms of electron and proton transfer in the protein and the sites and modes of substrate coordination to FeMoco. A study of the interrelations between various substrates and inhibitors on FeMoco separated from the protein can provide additional important information on the chemistry of the active center of nitrogenase, on its redox states, and on the properties of cluster coordination sites. From this standpoint, it is very interesting to study the inhibiting effect of CO, which is isoelectronic to nitrogen, the main substrate of nitrogenase. Carbon monoxide is not reduced by the enzyme; however, it inhibits the reduction of almost all nitrogenase substrates.

In this paper, we report on a study of the inhibiting effects of molecular nitrogen and CO on the ability of FeMoco to catalyze acetylene reduction outside the protein. We also compare the effects of these inhibitors on the catalytic behaviors of FeMoco outside the protein and as a constituent of the enzyme.

EXPERIMENTAL

The following chemicals were used in this study without additional purification: 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, mercury R0, and bromine (Reakhim); tetra-*N*-butylammonium hexafluorophosphate and adenosine triphosphate (ATP), disodium salt (Aldrich); 2,2'-dipyridyl of analytical grade (Reanal); $^{15}N_2$ (VO Izotop); sodium hydroxide (Chemapol); and high-purity nitrogen and pure argon.

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

Pure dimethylformamide (DMF) (Reakhim) and *N*-methylformamide (NMF) (Fluka) were used as solvents. They were dried and distilled in a vacuum (15 torr) over molecular sieves 4 Å and then degassed by evacuation at reduced temperature. After evacuation, the solvents were stored in an argon atmosphere.

The *Tris*-HCl (pH 7.4) and HEPES (pH 7.5) buffer solutions were prepared using triply distilled water.

Acetylene of 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 (–100°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 formiate (Reakhim) with concentrated sulfuric acid.

Tetrabutylammonium dithionite was synthesized according to the published procedure [14].

Sodium hypobromite was synthesized by the reaction of sodium hydroxide with bromine according to the procedure described in [15].

The Fe protein and MoFe protein from *Azotobacter vinelandii* nitrogenase were isolated by R.I. Gvozdev and L.A. Syrtsova with coworkers according to the procedure described in [16].

The FeMoco-deficient MoFe protein from the mutant strain *Klebsiella pneumoniae Kp5058* was prepared by C.A. Gormal (John Innes Centre, the United Kingdom) according to the published procedure [13].

The samples of FeMoco in different solvents and the solutions of the Fe and MoFe proteins were kept frozen in liquid nitrogen.

The amalgams of europium and zinc were prepared and their potentials were measured according to the previously described procedures [12].

All manipulations with substances sensitive to oxidation (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 with the use of a benzylviologen indicator.

The purity of all gases used in this study (acetylene, argon, nitrogen, and carbon monoxide) and the absence of oxygen impurities in them were controlled by mass spectrometry.

Preparation of FeMoco

FeMoco was isolated from the MoFe protein of nitrogenase from *Azotobacter vinelandii* (the concentration of a protein solution was 40–70 mg/ml in 0.25 M NaCl–25 mM *Tris*-HCl) according to the procedure described in [14, 17]. The cofactor was extracted from the DMF-denatured MoFe protein bound to a DEAE Sepharose anion-exchange support with a Bu_4NBr solution in DMF.

The desalting of cofactor samples was performed according to the published procedure [14, 17]. A concentrated FeMoco solution was passed through a column packed with Sephadex LH-20 in DMF, and the elution was performed with an excess of the solvent.

Analysis of FeMoco

Based on the determination of molybdenum and iron in FeMoco samples (see below), the yield of FeMoco varied from 70 to 85%. The [Fe]/[Mo] molar ratio varied within the range 7–10.

The quality of FeMoco after the extraction (the retention of the cluster framework and the presence of homocitrate in its composition) was checked by the biological activity of FeMoco, that is, by its ability to reconstruct the catalytic activity of the FeMoco-deficient MoFe protein *Klebsiella pneumoniae Kp5058* toward acetylene. The assay was performed according to the published procedure [13, 18]. A sample of the desalted cofactor was incubated with a crude extract of *Kp5058* in a 50 mM *Tris* buffer solution (protein concentration of 10 mg/ml). Next, the resulting reconstructed MoFe protein was added to a reaction mixture containing the Fe protein of *A. vinelandii*, ATP, $MgCl_2$, creatine phosphate, creatine kinase in a HEPES buffer solution, and acetylene. After 15 min, the reaction was stopped by the addition of TCA, and the amount of formed ethylene was measured as described below. The specific activity of the FeMoco samples used in this study was 200 ± 20 nmol C_2H_2 (nmol Mo)$^{-1}$ min^{-1}.

The quality of the FeMoco after its participation in catalytic reactions outside the protein was checked in a similar manner. To reconstruct *Kp5058*, the samples of a reaction mixture containing FeMoco were used without additional purification. The presence of thiophenol and europium or zinc compounds in the mixtures caused no interference with the reconstruction. The specific activity of these FeMoco samples was found to be almost equal to the activity of the cofactor after its extraction from the protein.

Experiments on the Inhibition of the Catalytic Activity of FeMoco outside the Protein

The experiments were performed in a specially designed thermostatted flat-bottomed glass vessel [19] equipped with a magnetic stirrer for operations with metal amalgams. The vessel was evacuated and filled with argon; next, 0.7 ml of Zn(Hg) (system **I**) or 0.5 ml of Eu(Hg) (system **II**) was introduced into a side tumbler vessel in an argon flow, and 4–4.3 ml of a FeMoco solution in DMF with a concentration of $(1–2) \times 10^{-5}$ M ($(4–7) \times 10^{-8}$ mol) and 0.5 ml of a 0.1 M (5×10^{-5} mol) thiophenol solution in DMF were added to the main vessel (in the reactor, [PhSH] = 0.012 M). The reaction mixture was frozen with liquid nitrogen; then, the reaction vessel was connected to a circulation unit and evacuated. After thawing the liquid phase and heating it to 21°C, the vessel was filled with the required gas mixture (CO (or N_2)–acetylene–argon in various ratios). In the course of the experiment, the gas phase was forcedly mixed in the circulation unit, and the liquid phase was stirred with a magnetic stirrer choosing the conditions under which the amalgam was maximally disintegrated.

The course of the reaction was monitored by sampling a gas phase from the reaction vessel at regular intervals for chromatographic analysis (see below). As a result, kinetic curves were plotted as the time dependence of the amount of reaction products. Next, the reaction rates were calculated from the initial portions of the kinetic curves. Each reaction rate was found as the average of two or three values obtained in replicate experiments. The experimental errors in the reaction rates were usually no higher than 10%.

*Determination of the Activity of System **II** toward the Reduction of Molecular Nitrogen*

To determine the activity toward the reduction of molecular nitrogen, the reaction was performed in an atmosphere of either N_2 (and the reaction mixture was analyzed for hydrazine and ammonia as described below after terminating the reaction by the addition of an acid) or $^{15}N_2$ (see below). The reaction vessel was filled as described above. In the latter case, the incorporation of $^{15}N_2$ into ammonia was analyzed using mass spectrometry. Ammonium sulfate was added as a carrier to the reaction mixture before the analysis. Ammonia for analysis was separated with the use of a modification of the Conway diffusion method. Next, the resulting ammonia was converted into gaseous nitrogen by oxidation with sodium hypobromite in a Rittenberg apparatus. The gas phase unfrozen in liquid nitrogen was collected with the use of a Toepler pump. An excess of ^{15}N was determined by mass spectrometry [20].

Analytical Procedures

The molybdenum content in the FeMoco samples was determined by atomic absorption spectrometry

using Carl Zeiss AAS1 spectrometer with a Perkin-Elmer HGA 74 graphite furnace.

The iron content was determined by spectrophotometry as a Fe^{3+} complex with CNS^- ion. The absorbance of solutions in ethanol was measured at 500 nm. Iron contained in the cofactor samples was oxidized to the Fe^{3+} state by heating with dilute nitric acid (1 : 10). The 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 with a Biokhrom chromatograph using a column with activated alumina (Al_2O_3 fraction of 0.25–0.50 mm); the column temperature was 80°C; argon was the carrier gas; and a flame-ionization detector was used. Samples for analysis were taken directly from the circulating gas mixture into an evacuated sample loop, from which the sample was transferred to the detector with a carrier gas.

The determination of hydrazine was performed by spectrophotometry according to a procedure [21] based on the reaction of N_2H_4 with *para*-dimethylaminobenzaldhyde. The absorbance of solutions was measured at 458 nm. The absorption spectra were recorded on a Hewlett-Packard 8451A diode array spectrophotometer.

Ammonia was determined by spectrophotometry with the use of the indophenol method [22].

Methanol was determined by chromatography on a Hewlett-Packard 5880A gas chromatograph with a DB-WAX column.

RESULTS

Inhibition of Cofactor-Catalyzed Acetylene Reduction by Carbon Monoxide

Previously [12], we found that CO inhibited the reduction of acetylene with zinc and europium amalgams catalyzed by FeMoco outside the protein in a DMF–thiophenol medium. As well as in nitrogenase [23], CO reacted with only the reduced cofactor ($FeMoco_{red}$). The preincubation of a solution of the cofactor in a dithionite-reduced state ($FeMoco_{s-r}$) in a CO atmosphere in the absence of a reducing agent had no effect on the rate of the subsequent reaction with acetylene.

With the use of zinc amalgam (E = –0.84 V with reference to a normal hydrogen electrode (NHE)) (**I**) as a reducing agent, CO reversibly inhibited the reaction of acetylene reduction to ethylene with K_i = 0.05 atm CO. The inhibition constant was obtained by the treatment of experimental data on the inverse partial inhibition[2]—the inverse pressure of CO coordinates in the approximation of complete noncompetitive inhibition [24].

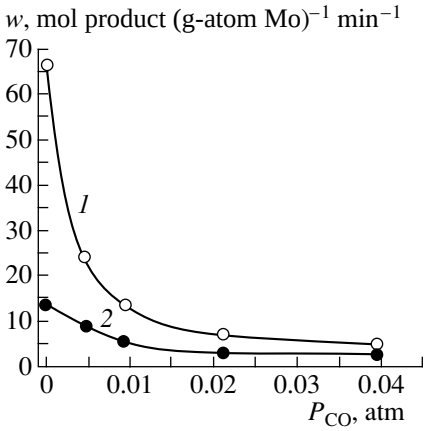


Fig. 1. Initial steady-state rates of formation of (*1*) ethylene and (*2*) ethane as functions of CO pressure. [Mo] = 0.6×10^{-5} M; Eu(Hg); $P_{C_2H_2}$ = 0.07 atm.

The reversibility of CO inhibition in this system was experimentally demonstrated as follows: after freezing the liquid phase, removing CO from the gas phase of the reactor by evacuation, and adding a new substrate portion, the rate of catalytic acetylene reduction increased again almost up to the values observed in the absence of the inhibitor.

With the use of a stronger reducing agent, europium amalgam (E = –1.4 V with reference to NHE) (**II**), the inhibiting effect of CO appeared at much lower pressures of the inhibitor. As found previously [12], unlike system **I**, the reduction of acetylene in system **II** in the presence of FeMoco as a catalyst resulted in the formation of two products: ethane and ethylene (the products of four-electron and two-electron acetylene reduction, respectively). The types of CO inhibition of the formation of these products were found to be different. Figure 1 demonstrates the initial steady-state rates[3] of ethylene and ethane formation as functions of CO pressure. It can be seen that the rates of formation of both reaction products decreased as the pressure of CO was increased. At the same inhibitor pressure, the rate of ethylene formation more strongly decreased than that of ethane formation. Figure 2 clearly illustrates this effect: the ratio between ethane and ethylene in reaction products increased with CO pressure in the system (the maximum ratio between the amounts of formed C_2H_6 and C_2H_4 changed from 22 to 52% as the pressure of CO was increased from 0 to 0.05 atm).

Figure 3 illustrates the treatment of the initial steady-state rate of ethylene formation as a function of CO pressure on the Dixon coordinates. It can be seen that the straight lines related to two different substrate concentrations on the Dixon coordinates intersect in the fourth quadrant close to the axis of abscissas (at a neg-

[2] The partial inhibition is taken to mean the quantity $i = 1 - w_{CO}/w$, where w_{CO} and w are the initial steady-state rates of ethylene formation in the presence and in the absence of CO, respectively [24].

[3] The initial steady-state rate is taken to mean the amount of a reaction product formed in unit time per mole of Mo at the initial linear portion of a kinetic curve of the buildup of the reaction product.

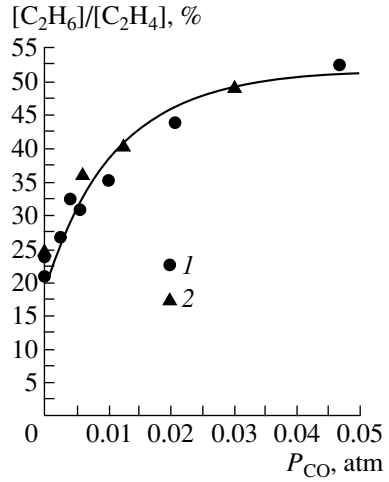


Fig. 2. Maximum molar ratio between ethane and ethylene in the reaction products of FeMoco-catalyzed acetylene reduction with europium amalgam as a function of inhibitor (CO) pressure. [Mo] = 0.6×10^{-5} M; Eu(Hg); $P_{C_2H_2}$ = (*1*) 0.09 or (*2*) 0.2 atm.

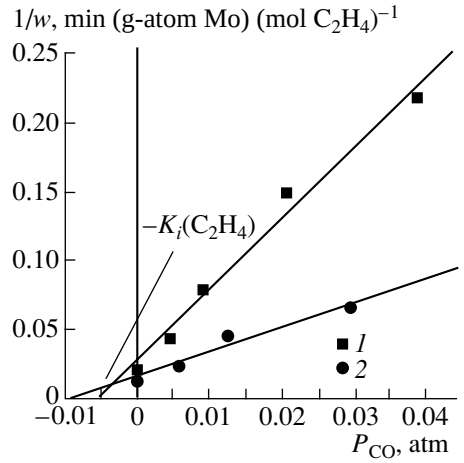


Fig. 3. Inhibition of FeMoco-catalyzed acetylene reduction with europium amalgam to ethylene by CO (on the Dixon coordinates). [Mo] = 0.6×10^{-5} M; Eu(Hg); $P_{C_2H_2}$ = (*1*) 0.07 or (*2*) 0.2 atm.

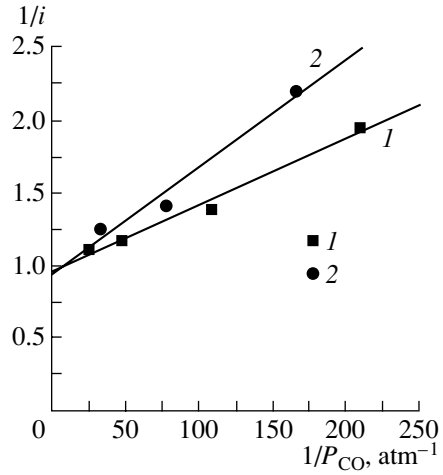


Fig. 4. Partial inhibition (*i*) of FeMoco-catalyzed ethylene formation from acetylene by CO as a function of inhibitor pressure. [Mo] = 0.6×10^{-5} M; Eu(Hg); $P_{C_2H_2}$ = (*1*) 0.07 or (*2*) 0.2 atm.

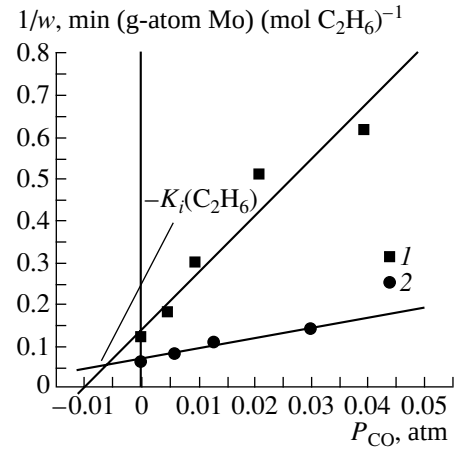


Fig. 5. Inhibition of FeMoco-catalyzed acetylene reduction with europium amalgam to ethane by CO (on the Dixon coordinates). [Mo] = 0.6×10^{-5} M; Eu(Hg); $P_{C_2H_2}$ = (*1*) 0.07 or (*2*) 0.2 atm.

ative value of $P_{C_2H_2}$). The type of the inhibition of ethylene formation by CO cannot be unambiguously determined from the shape of this plot; additional information is required for assigning the inhibition to a competitive or noncompetitive type [24]. To reveal the type of inhibition, we treated the data on the inverse partial inhibition–inverse CO pressure coordinates [24]. As can be seen in Fig. 4, the plots of the partial inhibition as a function of CO pressure on the above coordinates are linear at different substrate concentrations, and they intersect the axis of 1/*i* at a point with the ordinate equal to unity. The slopes of the two straight lines are similar (the partial inhibition depends on substrate pressure only slightly). This shape of the plot indicates that the inhibition of ethylene formation by CO can be ascribed to either mixed or competitive types. Based on the plots in Figs. 3 and 4 and taking into account the behavior of CO as a ligand (see below), we believe that the inhibition of ethylene formation on FeMoco by CO is most likely of a mixed type: CO affects both the substrate binding to the cofactor and the rate of decomposition of the catalyst–substrate complex with the formation of products. The inhibition constant K_i = 0.003 atm CO for

the inhibition of ethylene formation by CO was determined from the plots in Fig. 3.

Figures 5 and 6 illustrate the inhibition of ethane formation by CO. The experimental data presented in Fig. 5 on the Dixon coordinates indicate that straight lines related to two different substrate concentrations intersect in the fourth quadrant, and the intersection point is equidistant from both of the Cartesian axes at the chosen scale. In this case, the experimental points corresponding to a lower substrate concentration in the given coordinate system are arranged so that a convex curve can be drawn through them (Fig. 5). These two facts suggest that the inhibition of ethane formation by CO is of a competitive or incompletely competitive type. That is, the inhibitor partially (in the case of incomplete inhibition) prevents the binding of the substrate to the cofactor, and it has no effect on the rate of conversion of the cofactor–substrate complex into products. The inhibition constant $K_i = 0.006$ atm CO for the inhibition of ethane formation by CO was determined from the plots in Fig. 5. The dependence of the partial inhibition of ethane formation on the pressure of CO (Fig. 6) provides support for the hypothesis that inhibition is incomplete and competitive. The curves for two substrate concentrations do not coincide with each other (a curve for a lower concentration lies lower), and the points of intersection of these curves with the axis of ordinates lie above unity; such a behavior is typical of incomplete inhibition. Moreover, it can be seen that these functions are nonlinear. This nonlinearity can be explained by several causes: First, this can be a consequence of the interaction of several sites of substrate and inhibitor coordination to FeMoco with different affinities to CO and C_2H_2 (see, for example, [25, 26]). Second, the nonlinearity of these kinetic functions can be due to the simultaneous binding of two (or more) CO molecules to the cofactor, which is also possible under these conditions [27–29]. Moreover, a slight deviation of the observed functions from a purely competitive type might be a consequence of the fact that CO is not only an inhibitor but also a substrate in the test system. However, thorough attempts to detect CO reduction products (methane and methanol) demonstrated that FeMoco outside the protein did not catalyze the reduction of CO. As well as in nitrogenase, CO is only an inhibitor rather than a substrate.

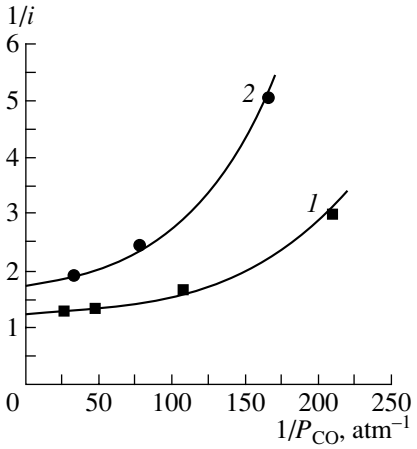


Fig. 6. Partial inhibition (i) of FeMoco-catalyzed ethane formation from acetylene by CO as a function of inhibitor pressure. [Mo] = 0.6×10^{-5} M; Eu(Hg); $P_{C_2H_2}$ = (*1*) 0.07 or (*2*) 0.2 atm.

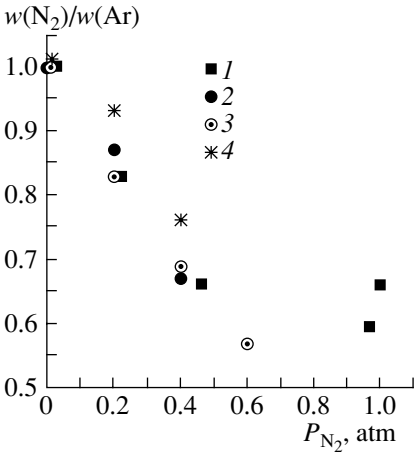


Fig. 7. Acetylene reduction to ethylene inhibited by molecular nitrogen in various systems with the participation of FeMoco: (*1*) system **II** with the participation of FeMoco outside the protein, (*2*) *Azotobacter vinelandii* nitrogenase [43], (*3*) *Azotobacter vinelandii* nitrogenase [37], and (*4*) α*Gln195* mutant *A. vinelandii* nitrogenase [37]. $w(N_2)$ and w(Ar) are the reaction rates of C_2H_2 reduction in the systems ($N_2 + C_2H_2$ + Ar) and (C_2H_2 + Ar), respectively; [FeMoco] = 0.7×10^{-5} M; Eu(Hg); $P_{C_2H_2}$ = 0.0145 atm.

Inhibition of Cofactor-Catalyzed Acetylene Reduction by Molecular Nitrogen

Previously [30], we found that molecular nitrogen inhibits the reduction of acetylene catalyzed by the cofactor outside the protein in system **II**. Figure 7 demonstrates the relative decrease of the initial reaction rate of ethylene formation in system **II** in the presence of nitrogen in the gas phase. A maximum rate of reaction was observed in the absence of nitrogen from the gas phase, and the rate was ~60% of the maximum value at a pressure of N_2 equal to 0.50–0.65 atm (400–500 torr). Because acetylene is a very good nitrogenase substrate with a small Michaelis constant K_M, nitrogen can compete with it only at low unsaturating pressures of C_2H_2. At an acetylene pressure higher than 0.026 atm (20 torr), the inhibiting effect of N_2 was eliminated; the inhibition is reversible.

As mentioned above, the catalytic reduction of acetylene in system **II** resulted in the formation of two products: ethylene and ethane. Figures 8 and 9 show the rates of formation of ethylene and ethane, respectively,

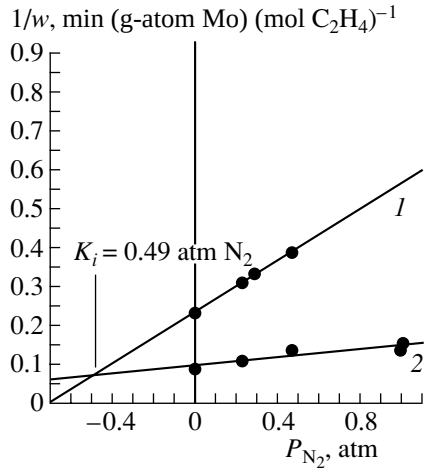


Fig. 8. Inhibition of FeMoco-catalyzed acetylene reduction with europium amalgam to ethylene by molecular nitrogen (on the Dixon coordinates). [Mo] = 0.7×10^{-5} M; Eu(Hg); $P_{C_2H_2}$ = (*1*) 0.0033 or (*2*) 0.0146 atm.

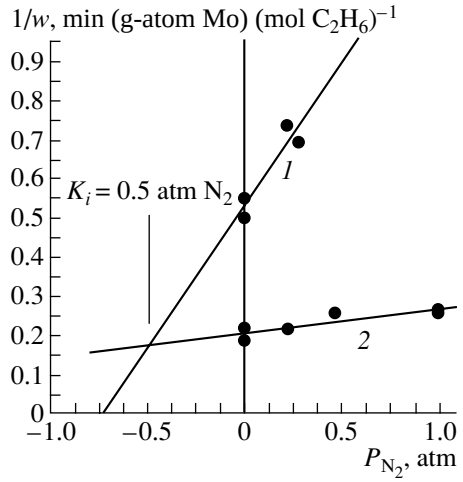


Fig. 9. Inhibition of FeMoco-catalyzed acetylene reduction with europium amalgam to ethane by molecular nitrogen (on the Dixon coordinates). [Mo] = 0.7×10^{-5} M; Eu(Hg); $P_{C_2H_2}$ = (*1*) 0.0033 or (*2*) 0.0145 atm.

as functions of the pressure of the N_2 inhibitor on the Dixon coordinates. It can be seen that in both cases the inhibition was competitive: the experimental results are linearized on the Dixon coordinates and the straight lines related to different substrate (acetylene) concentrations intersect in the fourth quadrant (at negative values of P_{N_2}). The presentation of these data as the dependence of the partial inhibition on the pressure of the inhibitor (analogously to those for inhibition by CO; not shown in figures) provides support for the hypothesis on the competitive character of inhibition by nitrogen. The inhibition constants for the inhibition of the formation of both products by nitrogen were calculated from the functions plotted in Figs. 8 and 9 (as the abscissas of the intersection points of straight lines for two substrate pressures). They were found to be almost the same and equal to 0.49 and 0.50 atm N_2 for the formation of ethylene and ethane, respectively.

We attempted to detect nitrogen reduction products—ammonia or hydrazine—in system **II** with the use of the ^{15}N isotope. We found by thorough experiments that nitrogen reduction catalyzed by FeMoco outside the protein, even stoichiometric, did not occur under conditions when C_2H_2 reduction was inhibited by nitrogen. We also attempted to detect the reduction products of N_2 in system **II** by terminating the catalytic reaction by the addition of an acid (as described by Thorneley *et al.* [31]). We detected neither hydrazine nor ammonia.

DISCUSSION

Inhibition of FeMoco-Catalyzed Acetylene Reduction by Carbon Monoxide

It is well known that carbon monoxide inhibits the reduction of all substrates of classical (Mo-containing) nitrogenase, except for H^+. It was found [27, 28, 32, 33] that the inhibiting effect is due to the fact that CO is bound to FeMoco reduced in the course of a catalytic cycle. One or two carbon monoxide molecules can be bound depending on the pressure of CO. A study of the chemical properties of FeMoco outside the protein demonstrated that the cluster in a dithionite-reduced state ($FeMoco_{s-r}$) in an NMF or DMF solution does not react with CO [12, 26]. The interaction was only observed with either electrochemically reduced FeMoco [32] (as was observed by measuring the IR spectra of carbonyl complexes ($FeMoco_{red}$) in NMF) or FeMoco outside the protein reduced by zinc or europium amalgams (as follows from the strong inhibition of cofactor-catalyzed acetylene reduction by CO) [12].

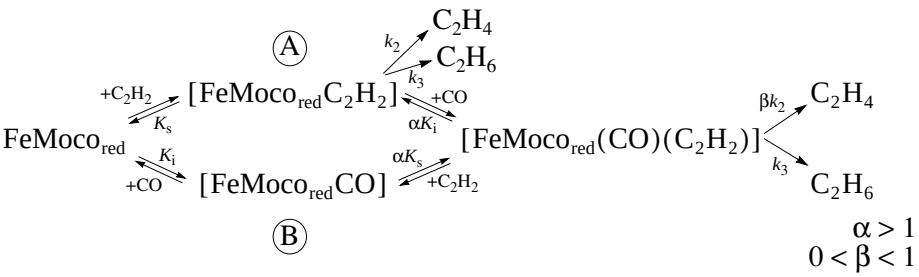
The CO inhibition constant of acetylene reduction with a stronger reducing agent—europium amalgam (system **II**)—is much lower than the K_i of C_2H_2 reduction with zinc amalgam (system **I**). This can be explained by the possibility of attaining a more deeply reduced state of the FeMoco cluster under the action of europium amalgam, and this state is favorable for binding a ligand such as CO. It was also found that K_i for enzyme systems depends on the degree of reduction of the MoFe protein: the CO inhibition constant increases with decreasing electron flow (with decreasing Fe protein : MoFe protein ratio) [34].

At present, it is almost universally recognized that CO is a noncompetitive inhibitor of the reduction of nitrogenase substrates. Little information is available about the competitive CO inhibition of N_2 reduction and on the occurrence of the signs of inhibition of both types [35]. This is true of the classical Mo-containing nitrogenase. At the same time, it is well known that even very small changes in the protein environment of the FeMoco cluster, especially the replacement of

amino acid residues nearest to FeMoco, which can form hydrogen bonds with the metal framework and thereby affect the electronic properties of the cluster and its orientation within the protein, often produce considerable changes in the catalytic behavior of the resulting mutant nitrogenase. In particular, they lead to the decrease of the enzyme activity toward the reduction of substrates, and to the change in the substrate specificity and product specificity [25, 36–38]. For example, an *Azotobacter vinelandii* mutant nitrogenase is known [38] in which the glycine residue αGly-69 in the MoFe protein from the nearest environment of FeMoco is replaced with serine (Ser). Only this replacement would suffice to change the type of inhibition: in the αSer69 mutant, CO competitively inhibits the reduction of acetylene, nitrogen, azide, and N_2O, whereas Christiansen *et al.* [38] confirmed noncompetitive inhibition for the native nitrogenase.

In our approach, we completely removed a protein environment from the FeMoco cluster by replacing all bonds with the amino acid residues of a protein matrix by bonds with the molecules of an amide solvent (NMF or DMF). In principle, this is an extremely rough case as compared with mutants where only some bonds between the cluster and the protein are changed. The complete destruction of the protein environment seems to result in catastrophic consequences for FeMoco as a catalyst. Nevertheless, a comparison between the catalytic behaviors of isolated FeMoco and FeMoco as a constituent of the MoFe protein of the enzyme system [12, 39] indicates that numerous characteristic properties of this metal cluster as a catalyst are also reproduced under nonenzymatic conditions. Thus, these properties primarily depend on the structure and composition of the cluster and secondarily, on the protein environment, the reaction medium, and the type of reducing agent. It is likely that a comparative study of the catalytic behavior of the isolated FeMoco could be more correctly performed with mutant nitrogenases, in which the protein environments of the cofactor are changed, or with hybrid nitrogenase systems like FeMoco in the VFe protein [40]. We found for FeMoco outside the protein in system **II** that the inhibition of ethylene formation by CO can be assigned to a mixed type, whereas that of ethane formation can be assigned to a competitive type. Thus, the type of inhibition by CO in this system is the same as in the above *Azotobacter vinelandii* αSer69 mutant nitrogenase [38], in which CO competitively inhibits the reduction of acetylene and other substrates.

Now the presence of more than one (two or three) substrate and inhibitor coordination sites on FeMoco as a constituent of the MoFe protein is commonly accepted [25, 26, etc.]. Our previous data [12] and this work allowed us to state that this property is also retained for the isolated FeMoco. At least two or, more probably, three interdependent sites of acetylene coordination and reduction occur on FeMoco reduced with europium amalgam, and one of them is highly active with K_M = 0.006 atm C_2H_2. As the pressure of C_2H_2 was increased, the saturation of this site with the substrate induced the activity of one or two additional sites, and the apparent K_M became equal to 0.08 atm C_2H_2 [12]. All of these three sites reduce acetylene with the formation of ethylene and ethane, although the fraction of ethane in the reaction products slightly decreased with increasing acetylene concentration: sites with a lower affinity to acetylene give a relatively smaller amount of ethane. Thus, the differences between the types of inhibition of two products of acetylene reduction by carbon monoxide observed in our system **II** and between the corresponding inhibition constants cannot be explained by the fact that one of the sites gives predominantly (or only) ethane and the other gives ethylene, as was assumed, for example, in alternative "iron" nitrogenase [41]. The addition of a "strong" ligand such as CO to the reduced FeMoco cluster will affect acetylene coordination and conversion by this cluster. First, CO, which is an isoelectronic analog of C_2H_2, can simply compete with it for a coordination site. Second, even CO bound at another site on the cluster can significantly affect the electronic properties of a C_2H_2 binding site toward impairing its coordination ability. Kinetically, both of these effects manifest themselves as competitive inhibition, which were observed upon the formation of both products in the presence of CO. In this case, competitive or incompletely competitive inhibition was observed for ethane (i.e., CO hardly inhibits the formation of ethane at all at the step of decomposition of the catalyst–substrate complex), whereas the inhibition of ethylene formation was of a mixed type (i.e., CO affects both acetylene coordination and decomposition of the intermediate complex with the formation of ethylene). Scheme 1 demonstrates in the simplest form the catalytic conversion of acetylene into ethylene and ethane (using K_S in place of K_M, we believe that all FeMoco complexes occur in equilibrium with their components [24]).



Scheme 1.

Because $K_i \le K_s$, the reaction in the presence of CO proceeded via pathway B to a considerable extent. In this case, the acetylene-containing intermediate complexes [$FeMoco_{red}$ C_2H_2] and [$FeMoco_{red}$ (C_2H_2)(CO)] were formed in smaller amounts than in the absence of CO ($\alpha > 1$). Because of this, the amounts of both ethylene and ethane in the reaction products decreased. The catalytic complex [$FeMoco_{red}(C_2H_2)(CO)$] should be more stable toward degradation and longer lived, because CO as a strong π-acceptor ligand stabilized the reduced state of the cluster. This stabilization allowed acetylene to occur in the coordination sphere of the cofactor for a longer time without converting into ethylene and enable it to be reduced to ethane. This is most likely the reason for the increase of the ethane fraction in the reaction products with increasing pressure of CO. Moreover, we assume a noncompetitive contribution of inhibition by CO in the case of ethylene formation ($0 < \beta < 1$), which also decreased the fraction of C_2H_4 in the reaction products.

Thus, we can state that CO impairs the binding of acetylene to the cofactor and affects the product specificity. This manifests itself in different values of K_i and in different characters of inhibition of the formation of the two products.

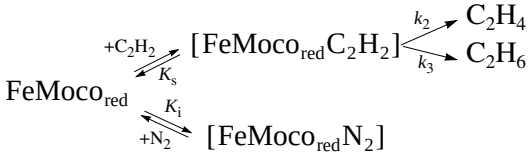
We cannot obtain information on the separate effects of CO on each of the sites of acetylene reduction from the available experimental data. The inhibition by CO was studied at substrate pressures when all of the three acetylene reduction sites were active, so that only the total effect can be observed.

We did not perform special studies to determine the quantity and the coordination mode of CO molecules that were bound to the isolated FeMoco in our systems. However, taking into account data on the conditions of CO coordination in nitrogenase *in vitro* [27–29, 33, 42], we can conclude that either only one CO molecule or two molecules can be bound under conditions of our experiments. For example, at a CO pressure of 0.05 atm, the concentration of CO in solution was higher than the concentration of FeMoco by one order of magnitude (CO : FeMoco = 10 : 1). According to published data [27, 28], at this ratio, FeMoco bound two or more CO molecules. Data obtained by Davis *et al.* [29] also suggest the binding of more than one CO molecule at inhibitor pressures higher than 0.01 atm. However, more recently [33, 42], a switch from one to two molecules coordinated was observed only at CO pressures higher than 0.08 atm. It is of importance that the substrate reduction is inhibited by CO through the binding of the first molecule of CO, which takes place even at CO : FeMoco = 1 : 1 [27, 28].

Inhibition of FeMoco-Catalyzed Acetylene Reduction by Molecular Nitrogen

Rivera-Ortiz and Burris [43] were the first to describe the competitive inhibition of acetylene reduction by dinitrogen for *in vitro* nitrogenase samples from *Azotobacter vinelandii*. Rivera-Ortiz and Burris [43] found the average inhibition constant to be equal to 0.4 atm N_2 (the values of K_i from 0.25 to 0.6 atm were obtained in different experiments) and demonstrated that the inhibition is competitive. More recently, Newton and coauthors [37] studied a similar effect of the inhibition of acetylene reduction by nitrogen with highly purified *Azotobacter vinelandii* nitrogenase samples. They found that $K_M = 0.005$ atm C_2H_2 and the inhibition constant of C_2H_2 reduction is 0.219 atm N_2 and confirmed that the inhibition is competitive. Figure 7 illustrates the published data [37, 43] on the inhibition of acetylene reduction with different enzyme systems by nitrogen and our results obtained in system **II**. It can be seen that the quantitative inhibition effects of the C_2H_2 reduction reaction in the presence of N_2 were almost equal in all of the above systems of both protein and nonprotein nature.

Previously [12], we found that, at acetylene pressures at which the inhibition effect was observed, only one site of acetylene coordination and reduction on the reduced FeMoco outside the protein was active. Both of the reaction products (ethane and ethylene) were formed at this site, and $K_M = 0.006$ atm C_2H_2 for both of the products. Under these conditions, nitrogen acted as a competitive inhibitor of ethane and ethylene formation, and the inhibition constants were equal. This fact indicates that the inhibiting effect of nitrogen is due to its coordination to the same site on the cofactor at which acetylene is bound. Competing for the site on FeMoco, N_2 interferes with the coordination of acetylene by reversibly binding a portion of the catalyst; however, it has no effect on the subsequent conversion of the substrate into the products (Scheme 2). As in *in vitro* nitrogenase systems, the inhibition by nitrogen was eliminated at high pressures of the substrate.



Scheme 2.

The fact that the inhibiting effect of nitrogen was observed is of paramount importance because the possibility of nitrogen reacting with the cofactor reduced outside the protein was first demonstrated. To this point, it was unclear whether the cofactor separated from the enzyme in an organic solvent could reach a state (in terms of the degree of reduction and the conformation of the catalyst) when it becomes capable of reacting with molecular nitrogen.

In enzyme systems, the coordination of the nitrogen molecule to the cofactor is accompanied by its reduction to ammonia. In nonprotein systems with the participation of FeMoco separated from the enzyme, the resulting nitrogen complex [$FeMoco_{red}N_2$] does not

undergo protonation. A further study can provide the answer to the question of whether this is because the redox potential of FeMoco required for the reduction of coordinated nitrogen is not attained in system **II** or the conditions of protonation are nonoptimal.

The behavior of our nonprotein test system with the participation of FeMoco is most similar to the behavior of the $\alpha 195^{Asn}$ mutant nitrogenase [36], in which the amino acid histidine in the 195-position of the α-subunit of the MoFe protein is replaced by asparagine. The $\alpha 195^{Asn}$ nitrogenase actively reduces acetylene to ethylene and ethane; K_M = 0.01 atm C_2H_2. Nitrogen reversibly and competitively inhibits the reduction of C_2H_2: 1 atm of N_2 decreased the activity toward acetylene by approximately one-third. Nitrogen can undergo only complexation; its reduction to ammonia and hydrazine was not observed at all. It is likely that an analogous situation took place in our case: the extraction of FeMoco from a protein matrix resulted in a loss in the ability to catalyze N_2 reduction, whereas the ability to reduce acetylene and protons and to coordinate the nitrogen molecule in the presence of a sufficiently strong reducing agent remained almost unaffected.

CONCLUSION

Three sites on the FeMoco cluster reduced by europium amalgam in DMF are active toward substrates and inhibitors (C_2H_2, N_2, and CO). At the most active of these sites, C_2H_2 can undergo coordination and reduction with K_M = 0.006 atm, and N_2 and (or) CO can undergo coordination without reduction. Sites 2 and 3 exhibit much lower activity (K_M of acetylene is higher than K_M for site 1 by more than one order of magnitude), and they can bind C_2H_2 and (or) CO.

The parameters of the inhibition of acetylene reduction catalyzed by FeMoco separated from the protein in DMF by molecular nitrogen have almost the same values as those in enzyme systems (in both native nitrogenase [35, 37] and mutants [36, 37]). Thus, we can conclude that the FeMoco cluster is a sorption site of the active center of the enzyme (it forms a complex of the substrate with the enzyme and is responsible for the specificity of the catalyst). The FeMoco cluster with the surrounding amino acids should be considered as a catalytic site (where the redistribution of electron densities and the transfer of groups in the chemical act of catalysis take place) or, in other words, an active center of the enzyme. The amino acids stabilize the intermediates of a catalytic cycle and participate in the transfer of protons of a required (optimum) acidity to coordinated nitrogen.

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