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Kinetics and Mechanism of Acetylene Reduction with Europium Amalgam Catalyzed by Isolated Active Center of Nitrogenase

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Abstract—The reaction kinetics of C_2H_2 reduction with europium amalgam (Eu/Hg) catalyzed by nitrogenase active center separated from the enzyme, the molybdenum–iron–sulfur cluster [$MoFe_7S_9$ · homocitrate] (FeMoco), was studied. The dependence of the rates of ethylene and ethane formation on the concentrations of catalyst, substrate, protonating agent, and amalgam was studied. The stereospecificity of the reaction was studied by Fourier transform IR spectroscopy. It was found that the reaction occurred at the amalgam surface via the adsorption of the compound [FeMoco · PhSH]. Upon reduction, this compound can simultaneously coordinate several substrate molecules to activate them for the subsequent reactions. A study of the IR spectra of the gas phase of the reaction demonstrated that *cis*-1,2-dideuterioethylene is the main product of C_2D_2 reduction. Taking into account this fact and the dependence of the reaction rate on the concentration of a protonating agent, we concluded that substrate molecules bound to the cofactor underwent protonation by intramolecular hydrogen transfer from the iron or sulfur atoms of FeMoco to coordinated C_2H_2.

INTRODUCTION

The enzyme nitrogenase is a catalytic binary system that contains the Fe protein and the MoFe protein [1]. In addition to the physiologically important conversion of molecular nitrogen into ammonia, nitrogenase catalyzes the ATP-dependent reduction of other simple molecules with multiple bonds, including C_2H_2 [2], and the reduction of H_3O^+ to hydrogen. The Fe protein is a specific reductant of the MoFe protein. The coordination and reduction of substrates occur at the cluster [$MoFe_7S_9$ · homocitrate], which is known as the FeMo cofactor (FeMoco), arranged within the α-subunit of the MoFe protein. Although the structures of the nitrogenase protein components and the constitution of the included metal clusters are well known [3], the chemical mechanism of substrate conversion into products remains unknown. The FeMoco cluster, which is the main part of the enzyme active center, exhibits a number of special features, in particular, a unique constitution, which has no analogues among the numerous synthetic FeS and MoFeS clusters. It can be separated from the protein in the form of quaternary ammonium salts in various organic solvents without cluster structure degradation [4, 5]. In this case, it mainly retains its spectroscopic and redox properties [6, 7]. Outside the protein, it exhibits a high stability to acids and chelating agents [7], which are usually destructive to synthetic FeS and MoFeS clusters. Studying the chemical properties of this cluster under nonenzymatic conditions, in particular, its behavior as a catalyst in reactions characteristic of this cluster within the nitrogenase, is of undoubted interest because it provides information on the mechanism of FeMoco catalysis. This information can be useful for understanding the chemistry of substrate conversion at the active center of the enzyme.

Previously [8, 9], we found that the cofactor separated from the nitrogenase MoFe protein in dimethylformamide (DMF) (where it is stable in the absence of oxygen for a long time) can catalyze the reduction of C_2H_2 to ethylene and ethane at high rates upon the delivery of electrons and protons to the cofactor. These reaction rates are lower than the rates observed in nitrogenase under the optimum conditions of catalysis by a factor of only 3 to 4 [1].

In this paper, we report on a study of the steady-state kinetics of catalytic acetylene reduction in the presence of FeMoco separated from the protein as a catalyst under nonprotein conditions.

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), mercury R0, and bromine (Reakhim); tetra-*N*-butylammonium hexafluorophosphate

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and disodium adenosine triphosphate (ATP) (Aldrich); 2,2'-dipyridyl of analytical grade (Reanal); deuterium oxide D_2O(VO Izotop); sodium hydroxide (Chemapol); and pure argon.

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

Pure 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.

Dideuterioacetylene was synthesized by the reaction of calcium carbide with deuterium oxide. The prepared C_2D_2 was purified of water and oxygen impurities as follows: the gas frozen in liquid nitrogen was evacuated to a residual pressure of 6×10^{-3} torr; next, it was evaporated in an alcohol bath (–80°C); the frozen gas was repeatedly evacuated in an alcohol bath (–110°C) followed by its evaporation into a preevacuated cylinder in an alcohol bath (–30°C).

Tetrabutylammonium dithionite was synthesized according to the published procedure [5]. Sodium hypobromite was synthesized by the reaction of sodium hydroxide with bromine according to the procedure described in [10].

The Fe protein and MoFe protein were isolated from *Azotobacter vinelandii* nitrogenase by R.I. Gvozdev and L.A. Syrtsova with coworkers according to the procedure described in [11].

The FeMoco-deficient MoFe protein from the mutant strain *Klebsiella pneumoniae Kp 5058* was prepared by C.A. Gormal (John Innes Centre, the United Kingdom) according to the procedure in [12].

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

The amalgam of europium was prepared and its potential was measured according to the previously described procedure [9]. Dilute europium amalgam (0–1.1 M) was prepared by adding mercury to a certain amount of concentrated amalgam (1.1 M). Thereafter, the concentrations of the resulting amalgams were established by titrimetric analysis. The prepared amalgams were stored in an argon atmosphere.

All manipulations with oxygen-sensitive substances (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, dideuterioacetylene, and argon) and the absence of oxygen impurities in them were controlled by mass spectrometry.

Preparation of FeMoco

FeMoco was isolated from the MoFe protein of *Azotobacter vinelandii* nitrogenase (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 [5]. 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 procedure in [5]. 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 a range of 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 Kp 5058* MoFe toward acetylene. The assay was performed according to the published procedure [12, 13]. A sample of the desalted cofactor was incubated with a crude extract of *Kp 5058* 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 with acetylene was stopped by the addition of TCA, and the amount of formed ethylene was measured as described below. The specific activity of FeMoco samples used in this study was 200 ± 20 nmol C_2H_2 min^{-1} (nmol Mo)$^{-1}$.

The quality of FeMoco after its participation in catalytic reactions outside the protein was checked in a similar manner. To reconstruct *Kp 5058*, 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 reconstruction. This test demonstrated that the FeMoco cluster did not

decompose in DMF in the presence of a reducing agent such as europium amalgam (E = –1.4 V with reference to a normal hydrogen electrode (NHE)) and thiophenol with a concentration of up to 0.2 M. 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.

Study of the Catalytic Activity of Isolated FeMoco

The experiments were performed in a specially designed thermostated flat-bottomed glass vessel [14] equipped with a magnetic stirrer for operations with metal amalgams. The vessel was evacuated and filled with argon; next, 0.5 ml of Eu(Hg) was introduced into a side tumbler tube in an argon flow, and 4.0–4.3 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: acetylene–argon in various ratios (dideuterioacetylene in studies on reaction stereospecificity). In the course of an experiment, the gas phase was forcedly mixed in the circulation unit, and the liquid phase was stirred with a magnetic stirrer under the conditions when the amalgam was maximally disintegrated. When studying the dependence of the reaction rate on the FeMoco concentration, the cofactor content of the reaction mixture was varied over a range of $(0–8) \times 10^{-8}$ mol at a constant acetylene pressure of 0.22 atm and a constant volume of the liquid phase. When studying the dependence of the reaction rate on the concentrations of thiophenol and europium amalgam, the concentration of PhSH in the reaction mixture was varied over a range of 0–0.36 M at a constant acetylene pressure of 0.2 atm. When studying the possibility of the homogeneous reduction of FeMoco, the required amount of solid cobaltocene was placed in the side tumbler tube.

The course of the reaction was monitored by sampling the 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 value was calculated as the average of two or three values obtained in replicate experiments. Usually, the difference between reaction rates in compared experiments was no higher than 10%. The initial steady-state rates (w) that we use here are taken as the amounts of reaction products formed in a unit time per mole of Mo at the initial linear portions of the kinetic curves of reaction product formation.

Analytical Procedures

The molybdenum content of FeMoco samples was determined by atomic absorption spectrometry using a 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 the 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 90°C; argon was the carrier gas; and a flame-ionization detector was used. Samples for analysis were taken at regular intervals directly from the circulating gas mixture into an evacuated sampling loop, from which the sample was transferred to the chromatograph with the carrier gas. To determine the amount of hydrogen formed, gaseous reaction products unfrozen in liquid nitrogen were collected with the use of a Toepler pump. Hydrogen was determined by chromatography with the use of a column packed with molecular sieves 5 Å and a thermal-conductivity detector; argon was the carrier gas.

The IR absorption spectra were measured with a FTIR 1600 Perkin-Elmer spectrometer in a range of 4000–400 cm^{-1} with a resolution of 2 cm^{-1}. A hermetically sealed evacuated KBr cell for gaseous samples was used in the spectrometric measurements. The test samples were the gaseous products of the catalytic reduction of C_2D_2 collected with the use of a Toepler pump from the reaction vessel cooled in an alcohol bath (–93°C).

RESULTS AND DISCUSSION

Generally, the test reaction can be represented by the following equations:

$$2C_2H_2 + 3Eu(Hg) + 6PhSH$$

$$\xrightarrow[\text{DMF, 21°C}]{\text{FeMoco}} C_2H_4 + C_2H_6 + 3Eu^{2+} + 6PhS^-(+Hg),$$

$$Eu(Hg) + 2PhSH \longrightarrow Eu^{2+}(+Hg) + 2PhS^- + H_2.$$

In fact, C_2H_2 is reduced to ethylene and ethane with the use of electrons from europium amalgam and protons from thiophenol. The reaction is catalytic: neither ethylene nor ethane was formed in the absence of the cofactor, although europium amalgam actively reduces thiophenol to hydrogen and leads to H–D exchange in the reaction of C_2D_2 + PhSH with the formation of C_2DH and C_2H_2 (see below).

When using a standard procedure for the extraction of FeMoco from the protein in the presence of dithion-

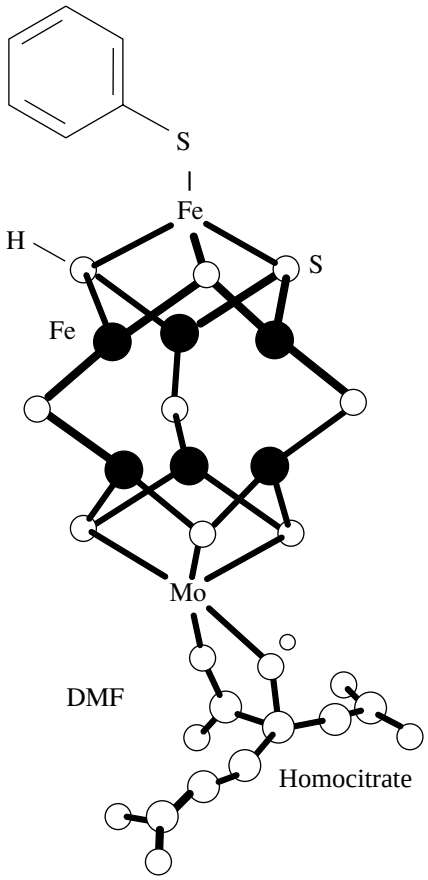


Fig. 1. Assumed structure of $FeMoco_{s\text{-}r}$ separated from the enzyme in DMF in the presence of thiophenol according to published data [3, 17–19].

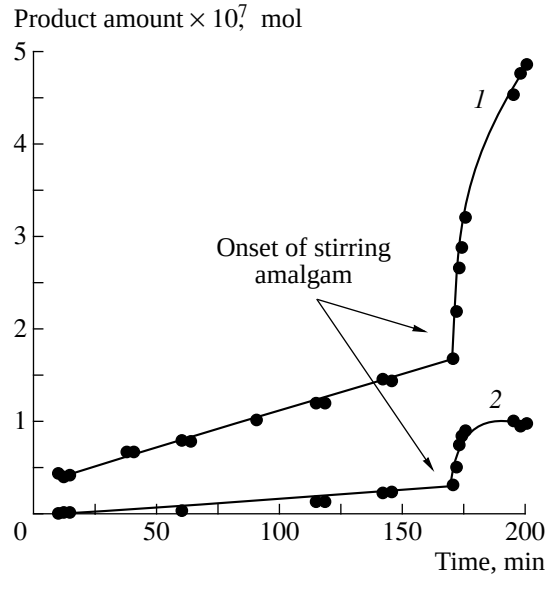


Fig. 2. Effect of amalgam surface area on the formation of (*1*) ethylene and (*2*) ethane upon C_2H_2 reduction with europium amalgam. [FeMoco] = 1.5×10^{-5} M.

ite, the isolated cofactor occurs in a so-called semireduced (s-r) state [7]. In this state, the cofactor does not bind acetylene [7, 15]; consequently, it cannot catalyze its reduction. The first step of the reaction is thiophenol binding to the $FeMoco_{s\text{-}r}$ cluster [16, 17] before the addition of a reducing agent (Eu(Hg)). According to published data [3, 17–19], it is most likely that the species shown in Fig. 1 is formed. Next, this compound is reduced at the surface of an amalgam electrode; thereafter, it becomes capable of coordinating C_2H_2. Because the reduced form of FeMoco is formed at the amalgam surface, the rate of reaction with the participation of the reduced cofactor is proportional to the surface area: $w \sim kSC_s$, where S is the contact surface area of a given amalgam amount with a solution and C_s is the surface concentration of FeMoco.

Figure 2 illustrates the effect of the surface area of an amalgam electrode on the rate of reaction. In the time interval 0–175 min, only the gas phase in the reactor was stirred, and the gas phase was chromatographically analyzed for ethylene and ethane at regular intervals. It can be seen that ethylene and methane were slowly accumulated. Then, at the point in time indicated with arrows in Fig. 2, the stirring of the liquid phase was turned on. So the amalgam pool was smashed into small drops. As a result, the surface of the "electrode" at which the catalytic cluster is reduced considerably increased; in turn, this considerably increased the rate of accumulation of the reduction products. In this case, the reaction mixture (both the amalgam and the solution over it) should also be stirred to eliminate the effect of diffusion (mass transfer) on the reaction kinetics. The rotational speed of the electromagnetic stirrer was chosen so that the rate of reduction product formation reached a maximal and reproducible value (for a given amount of amalgam) and was independent of a further increase in the intensity of stirring.

Ethylene and ethane, the products of C_2H_2 reduction in this system, simultaneously accumulated with time (Fig. 3). Ethylene was found to be a very poor substrate; it underwent only stoichiometric reduction to ethane under the specified reaction conditions [9]. The europium amalgam and thiophenol were consumed not only for the catalytic reduction of C_2H_2, but also for the H_2 formation. The accumulation of the reduction products was almost completed after the reaction had occurred for approximately 5 min. We found that this was simply due to the complete consumption of thiophenol present in the system. The degree of europium amalgam conversion for the same time was only ~10%. In this case, 0.36×10^{-5} mol of C_2H_4, 0.09×10^{-5} mol of C_2H_6, and 1.7×10^{-5} mol of H_2 were formed; that is, the source of H^+ present in the system (PhSH) was consumed as follows: ~25% H^+ for the formation of ethylene and ethane (in a ratio of 2 : 1) and 75% for the release of hydrogen. In this case, it was found that almost all the hydrogen released was formed in a noncatalytic reaction, because the same amount of

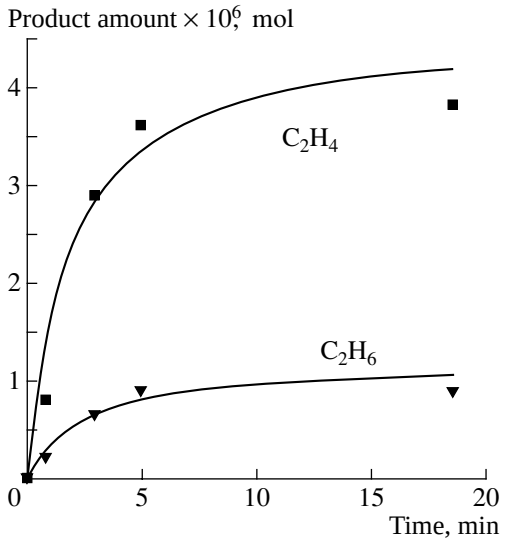


Fig. 3. Kinetic curves of formation of the products of C_2H_2 reduction with europium amalgam catalyzed by FeMoco. [FeMoco] = 0.6×10^{-5} M; Eu(Hg) (1.1 M) 0.5 ml.

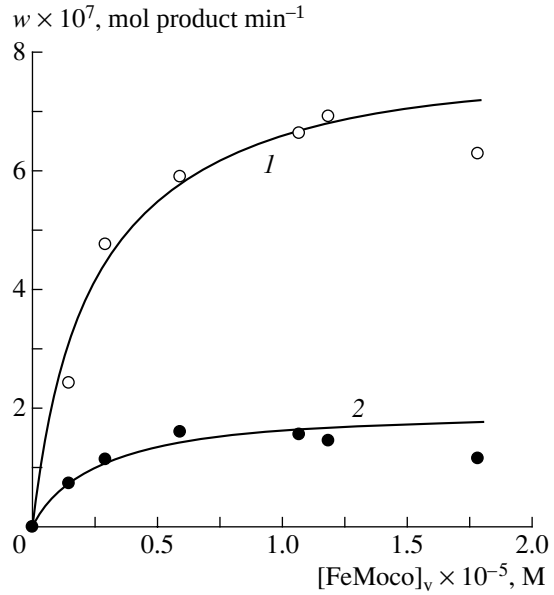


Fig. 4. Rates of formation of (*1*) ethylene and (*2*) ethane as functions of the bulk concentration of $[FeMoco]_v$ in the reaction of acetylene reduction with europium amalgam. Points and curves indicate experimental data and data calculated by Eq. (1), respectively. Eu(Hg) (1.1 M) 0.5 ml.

hydrogen was released with europium amalgam in the absence of FeMoco for the same time. Therefore, in the presence of C_2H_2, the cofactor adsorbed on the amalgam surface forwards all the reducing equivalents to the reduction of the substrate rather than catalyzes the release of H_2. Without acetylene, thiophenol present in the reaction medium was almost quantitatively reduced to hydrogen in the presence of FeMoco.

Effect of Catalyst Concentration

It is likely that all reactions leading to the formation of reduction products occur without the release of the catalyst–substrate complex $[FeMoco_{red}(H)(PhS)(C_2H_2)]$ into the bulk of the solution. In this case, the dependence of the rates of ethylene and ethane formation on the bulk concentration of FeMoco is described by the following equation, which is similar to the equation of an adsorption isotherm showing the change in the surface concentration of a catalyst as its bulk concentration increases: $w = k[FeMoco]_s$,

$$\text{where} \qquad [FeMoco]_s = \frac{k_{ads}[FeMoco]_v}{1 + k_{ads}[FeMoco]_v}.$$

Here, $[FeMoco]_s$ and $[FeMoco]_v$ are the surface and bulk concentrations of the catalyst, respectively, and k_{ads} is the adsorption coefficient. Figure 4 demonstrates the rates of C_2H_4 and C_2H_6 formation as functions of cofactor concentration in the reaction solution. At low catalyst concentrations (from 0 to 1.0×10^{-5} M FeMoco), the product formation rates were proportional to the concentration of FeMoco. A further increase in the catalyst concentration had almost no effect on the rate of reaction. As the catalyst concentration in solution was increased, an apparent zero-order reaction was observed at $[FeMoco] \geq 1.2 \times 10^{-5}$ M. It is likely that this concentration corresponds to the complete surface coverage of the given amalgam amount with the cofactor at a constant rate of stirring. We found that the formation rates of C_2H_4 and C_2H_6 as functions of FeMoco concentration are described by the equations (solid lines in Fig. 4)

$$w_{C_2H_4} = \frac{8.2[FeMoco]_v}{0.25 + [FeMoco]_v}$$

$$\text{and} \qquad w_{C_2H_6} = \frac{2[FeMoco]_v}{0.25 + [FeMoco]_v}. \qquad (1)$$

The values of k_{ads} that appear in the expressions for the rates of product formation as functions of catalyst concentration are equal. This fact suggests that both of the products are formed with the participation of the same species $[FeMoco_{red}(H)(PhS)(C_2H_2)]$ adsorbed on the amalgam surface.

A quasi-heterogeneous reaction type was also observed for the nitrogenase enzyme: in the reconstruction of the specific activity of the FeMoco-deficient apoenzyme toward the reduction of C_2H_2, the enzyme activity as a function of the amount of the extracted cofactor exhibits a similar dependence as a saturation curve. Initially, the higher the concentration of FeMoco, the higher the reaction rate. Then, the surface capacity (the number of binding sites for the cofactor in a given amount of the apoenzyme) becomes filled, and the

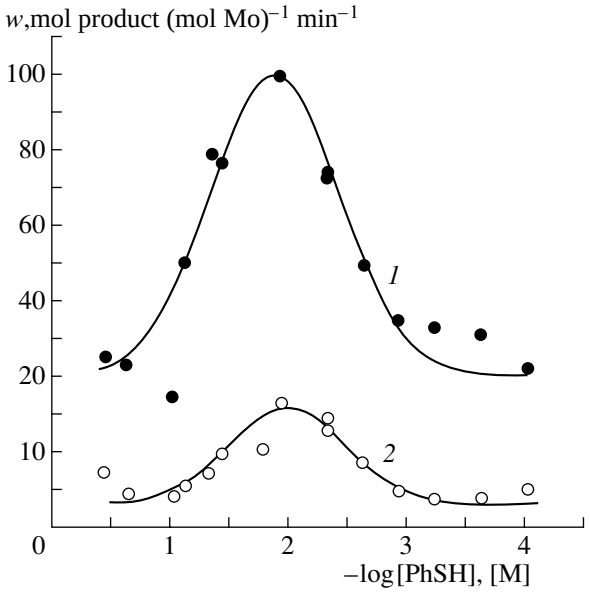


Fig. 5. Rates of (*1*) ethylene and (*2*) ethane formation in the reaction of acetylene reduction with europium amalgam as functions of thiophenol concentration on semilogarithmic coordinates. [FeMoco] = 0.6×10^{-5} M; Eu(Hg) (0.37 M) 0.5 ml.

reaction rate remains unchanged as the amount of the cofactor is increased [4, 7].

Dependence on the Concentration of Thiophenol

Figure 5 demonstrates the dependence of the initial steady-state rates of ethylene and ethane formation on thiophenol concentration in solution. This dependence is expressed as an extremum function, and the maximum rates of formation of both products correspond to a concentration of 10^{-2} M PhSH in DMF. The nonzero rates of ethylene and ethane formation observed at [PhSH] = 0 were explained by the presence of trace water in the reaction medium; water can also be a source of protons in this reaction. It is likely that water was introduced into the reaction mixture with a cofactor solution in DMF. We did not study the dependence of the reaction rate on the concentration of water. However, water specially added to a concentration of ~10^{-2} M did not change the reaction rate; that is, impurity water was present in saturating concentrations.

The reaction rate noticeably decreased as [PhSH] was increased above 1×10^{-2} M. At [PhSH] = 0.1 M, the rate became equal to the rate of C_2H_4 or C_2H_6 formation in the absence of PhSH when the reaction occurs because of water impurity. Several processes occurring in the system in the presence of thiophenol can be responsible for this effect. Usually, the reduction of H^+ to hydrogen is a predominant reaction in systems containing H^+ (an acid) and a reducing agent. The formation of hydrogen by the reduction of H^+ with amalgams under conditions similar to those used in our experiments (a nonaqueous medium and phenol as an acid) was a first-order reaction with respect to the acid [20, 21], and an increase in [PhSH] increased the rate of this reaction. The reaction rates of both ethylene and ethane formation should decrease because of the rapid removal of the reactant H^+ from the reaction medium. Ethane formation suffered mainly because a greater amount of H^+ is required for the formation of this product. Two additional factors associated with an increase in the concentration of PhSH act in the same direction, that is, toward a decrease in the formation rate of protonation products. Not only the coordinated substrate but also the atoms of the metal (metals) and sulfur in reduced FeMoco undergo protonation under the action of thiophenol. In this case, it is likely that "excessive" protonation deteriorates the catalytic reaction because it can impair, first, the protonation of coordinated acetylene (by the impairment of intramolecular hydrogen transfer from a metal to a substrate) and, second, the conditions of the primary binding of C_2H_2 to the reduced cluster [19].

Reaction Stereospecificity

To study the binding of C_2H_2 to the cluster and the protonation mechanism of coordinated acetylene, the reaction stereospecificity of ethylene formation from fully deuterated acetylene (C_2D_2) by its protonation with PhSH was examined by Fourier transform IR spectroscopy.

Figure 6 demonstrates the IR spectrum of gaseous reaction products in a range of 1000–800 cm^{-1}. It can be seen that *cis*-1,2-dideuterioethylene (ν = 843 cm^{-1}; A = 30.0 [22], where A is the absolute IR band intensity) is the main component of the resulting ethylenes. Its concentration in the mixture was 76%; the concentrations of *trans*-1,2-$C_2H_2D_2$ (ν = 988 cm^{-1}; A = 16.6) and monodeuterioethylene C_2H_3D (ν = 943 cm^{-1}; A = 16.0) were 11 and 13%, respectively. Spectrum *2* in Fig. 6 demonstrates an analogous range of the IR spectrum of a gas phase of the reaction performed under identical conditions, although without the cofactor. It can be seen that europium amalgam in the absence of the catalyst did not reduce the substrate (an analogous result was obtained by chromatographic analysis of the gaseous products of the reaction without a catalyst). In this case, europium amalgam catalyzes H–D exchange between acetylene and thiophenol. This can be easily seen when comparing the spectra of initial acetylene and acetylene after the reaction Eu(Hg) + C_2D_2 + PhSH (see Fig. 7 and the table).

The presence of FeMoco had almost no effect on this reaction; that is, the cofactor does not catalyze additional H–D exchange in acetylene coordinated to the cofactor. The only result of its presence in the system was the appearance of reduction products in a considerable amount, and the main product was *cis*-dideuterioethylene. It is likely that monodeuterioethylene C_2H_3D was formed by the protonation of the isotopically substituted acetylene C_2HD, which was present in

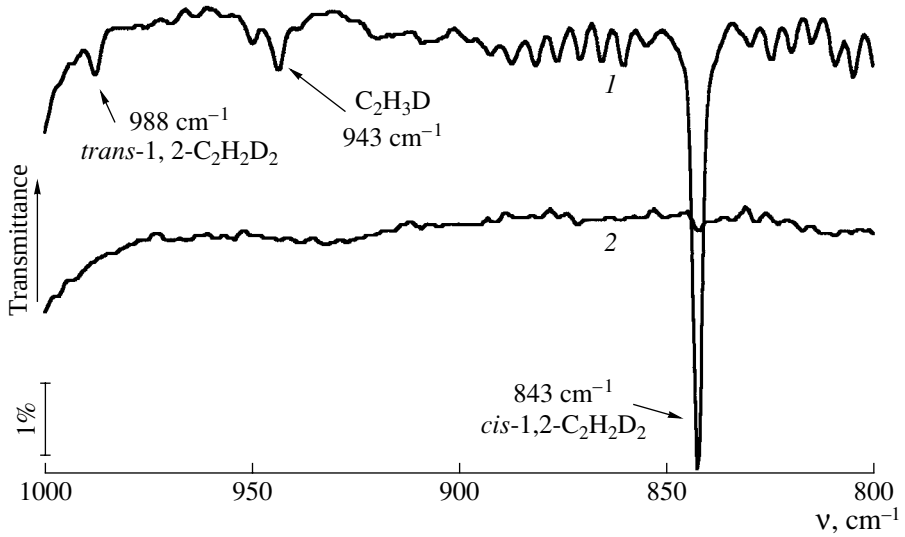


Fig. 6. IR spectra of the gaseous products of C_2D_2 reduction with europium amalgam (*1*) in the presence of the FeMoco catalyst and (*2*) without the catalyst.

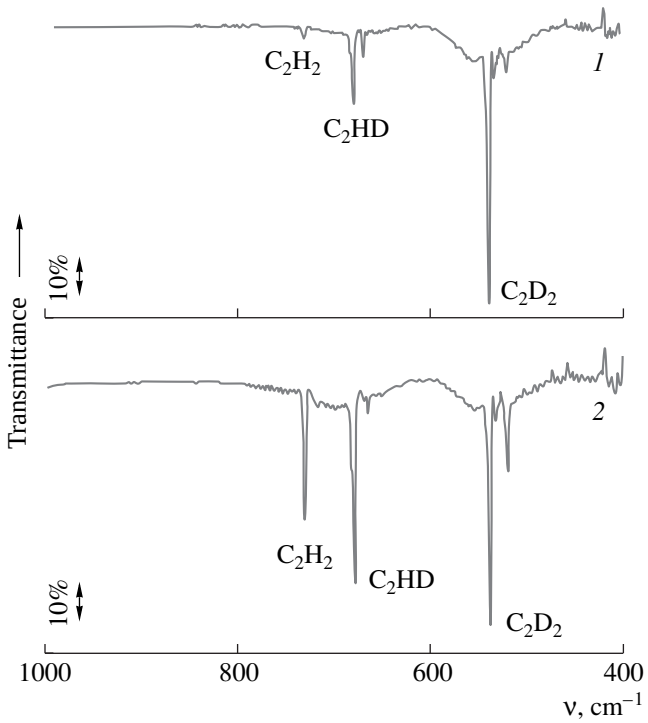


Fig. 7. IR spectra of (*1*) initial deuterated acetylene and (*2*) the acetylene after contact with Eu(Hg) and PhSH in DMF.

a considerable amount in the initial deuterated acetylene (see the table). For the reduction of C_2D_2 with both native and mutant nitrogenases, no exchange takes place between acetylene and a solvent. The absence of exchange was demonstrated using ^{1}H ENDOR (electron nuclear double resonance) spectroscopy for the C_2D_2/H_2O and C_2H_2/D_2O pairs [23, 24].

It was found [23, 25] that the replacement of amino acids near FeMoco within the protein considerably affected the stereochemistry of H^+ binding during the reduction of C_2D_2. Classical molybdenum-containing nitrogenase produces 96% *cis*-product [2, 23]. In mutant nitrogenases with some amino acid replacement near FeMoco [23, 25], *cis*-1,2-$C_2D_2H_2$ remained the main product; however, *trans*-1,2-dideuterioethylene

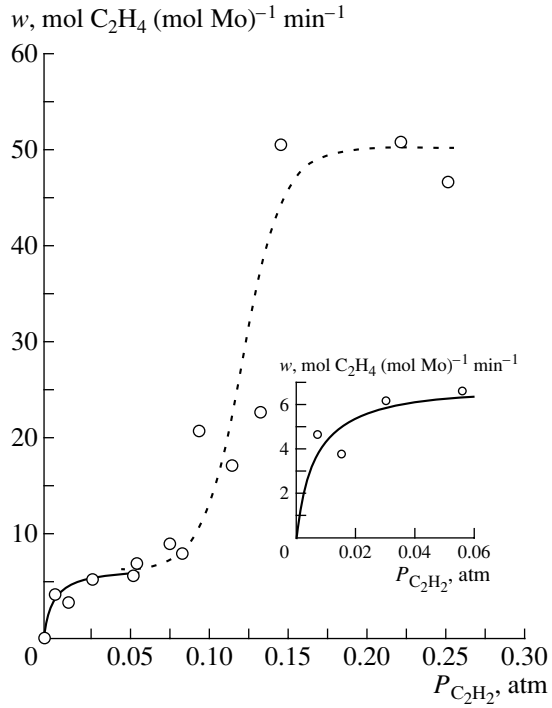


Fig. 8. Rate of ethylene formation in the reaction of C_2H_2 reduction with europium amalgam catalyzed by FeMoco as a function of substrate pressure (insert: a portion of this function at $P_{C_2H_2}$ = 0–0.06 atm). [FeMoco] = 0.6×10^{-5} M; Eu(Hg) (1.1 M) 0.5 ml.

appeared in considerable amounts (from 5 to 35% in different mutants). In this case, no correlation was found between the amount of the *trans*-product formed and the Michaelis constant K_M of acetylene reduction or the presence or absence of C_2H_6 in the reduction products [25]. As found in the studies [26, 27] of transition metal complexes with acetylene, the stereoselectivity of product formation depends on the mechanism of protonation; that is, it depends on which site (metal or substrate) of the metal complex containing C_2H_2 is first subjected to a proton attack. In the case of the direct protonation of coordinated C_2H_2 from the solution, the resulting intermediate *trans*-vinyl intermediate forms *trans*-ethylene upon subsequent protonation. If the metal center is initially protonated, the subsequent intramolecular migration of hydrogen gives the *cis*-vinyl derivative, which is converted into *cis*-1,2-dideuterioethylene upon protonaton. A real path of protonation in each particular case depends on the nature of ligands and on electron density distribution on the reduced cluster with the coordinated substrate. It is likely that slight changes in the ligand environment of the active center in mutant enzymes (these changes occur due to some amino acid replacement near the cofactor) and much more significant changes in our systems (where the cluster is simply placed in a medium of DMF + PhSH) are responsible for a change in the reaction stereospecificity. The observed results illustrate the well-known fact that both enzymatic catalysis and metal complex catalysis are highly selective and specific: insignificant changes in the coordination sphere of a catalytic complex are often responsible for considerable changes in the nature of the products.

Based on the dependence of the rate of acetylene reduction on thiophenol concentration and on the stereospecificity of reaction, we can conclude that the major portion of ethylene is formed via intramolecular hydrogen transfer to the substrate from preprotonated iron or sulfur atoms of the cofactor.

Dependence on Substrate Concentration

Figure 8 demonstrates the dependence of the rate of ethylene formation on the substrate pressure for the reduction of acetylene with europium amalgam in the presence of FeMoco. In an acetylene pressure range of 0–0.06 atm (from 0 to 45 torr), the dependence of the formation rates of both C_2H_4 and C_2H_6 on substrate concentration are described by a hyperbolic function. This function was linearized on the Lineweaver–Burk coordinates, and the values of K_M = 0.006 atm C_2H_2 for the formation of both ethane and ethylene were found from this linearization. Thus, at low substrate concentrations, one center that produces ethylene and ethane in ~1 : 1 ratio is active toward acetylene on the cofactor reduced with europium amalgam. The saturation of this center with acetylene induced the activity of other centers of the cluster toward the substrate. In a pressure range from 0.06 to ~0.29 atm, an S-shaped curve was observed for the reaction rate plotted against substrate

Isotopic composition (%) of deuterated acetylene before and after reaction in systems with and without the catalyst

System	C_2D_2 539 cm^{-1}, A = 38.8	C_2HD 678 cm^{-1}, A = 42.0	C_2H_2 729 cm^{-1}, A = 81.0
Initial acetylene	79	19	2
Eu(Hg) + PhSH + acetylene	47	39	14
Eu(Hg) + PhSH + FeMoco + acetylene	46	38	16

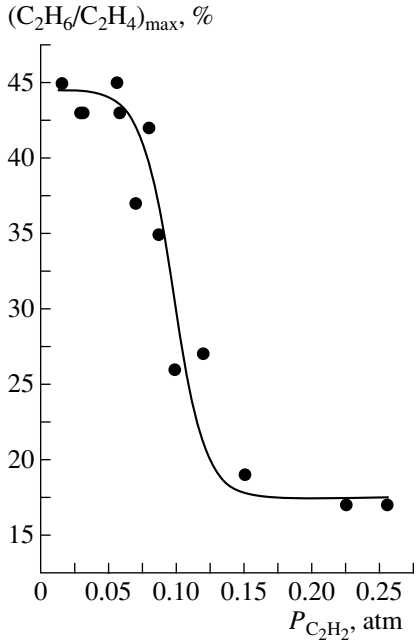


Fig. 9. Maximum C_2H_6/C_2H_4 molar ratio in the reaction products of C_2H_2 reduction with europium amalgam catalyzed by FeMoco as a function of $P_{C_2H_2}$. [FeMoco] = 0.6×10^{-5} M; Eu(Hg) (1.1 M) 0.5 ml.

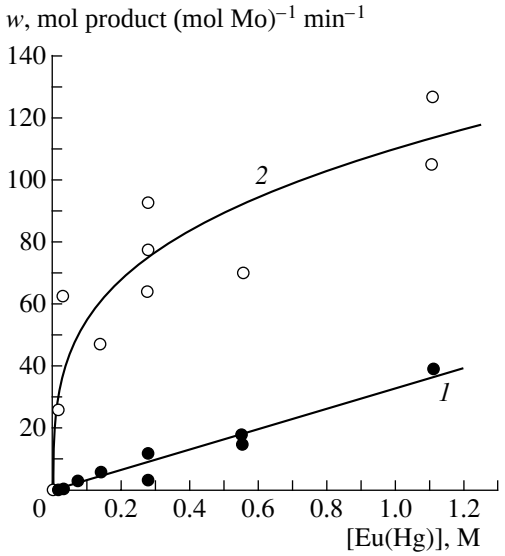


Fig. 10. Rates of (*1*) ethane and (*2*) ethylene formation in the reaction of C_2H_2 reduction as functions of europium amalgam concentration. [FeMoco] = 1.5×10^{-5} M.

concentration. This fact indicates that several acetylene molecules were simultaneously coordinated to a catalytic cluster [28]. The linearization of the S-shaped curve on Hill coordinates resulted in the coefficient $n = 1.6$, which is indicative of the existence of substrate-induced cooperativity between at least two centers active toward acetylene.

Figure 9 demonstrates the relative yield of ethane as a function of substrate concentration ($p_{C_2H_2}$). It can be seen that in a C_2H_2 pressure range of 0–0.07 atm (from 0 to 50 torr), the relative yield of ethane is independent of the pressure of acetylene. As noted above, at these substrate pressures, one center that gives both ethane and ethylene is active toward acetylene on the reduced FeMoco cluster. In this case, the independence of the formation rates of these products from the pressure of C_2H_2 suggests a dissociative mechanism of decomposition of the catalyst–substrate complex. The formation of C_2H_4 and C_2H_6 occurs without the displacement of a coordinated reduced ligand that corresponds to a reaction product by acetylene. The fraction of ethane considerably decreased with increasing pressure of C_2H_2. This is due to the fact that centers with a lower affinity to acetylene give a relatively lower amount of ethane than the most active center with $K_M = 0.006$ atm C_2H_2. In this case, it is likely that the products are formed by an associative mechanism: acetylene displaces the reduced coordinated intermediate that produces ethane and ethylene.

Experimental data are indicative of the presence of several centers of substrate and inhibitor coordination on both FeMoco as a constituent of the MoFe protein and extracted FeMoco [15, 29, 30]. For FeMoco in the protein, Shen *et al.* [29] found that two C_2H_2 molecules and a CO molecule can be bound simultaneously, whereas Lee *et al.* [30] demonstrated that two CO molecules can be bound. Only isocyanides [31], azide [15], and cyanide [32] can be bound to isolated $FeMoco_{s-r}$. It was found that each molecule of FeMoco binds two CN^- ions [33]. We were the first to experimentally demonstrate the existence of simultaneously operating interdependent active centers on the FeMoco cluster reduced outside the protein. It is likely that such a behavior is typical of this catalyst.

Dependence on the Concentration of Europium in Amalgam

Figure 10 demonstrates the experimentally measured rates of ethylene and ethane formation as functions of the concentration of europium amalgam. It can be seen that the rates of reduction of both ethane and ethylene increased with an increase in the metal concentration in the amalgam. However, the behaviors of these functions are different. The rate of C_2H_6 formation linearly increased with [Eu(Hg)], whereas the rate of ethylene formation is proportional to the amalgam concentration to the power of 0.3. Because, as can be seen, the formation rate of ethane increases more rapidly than that of ethylene with increasing metal concentration in the amalgam, the relative ethane content of the products should increase with increasing [Eu(Hg)], as we observed experimentally (Fig. 11).

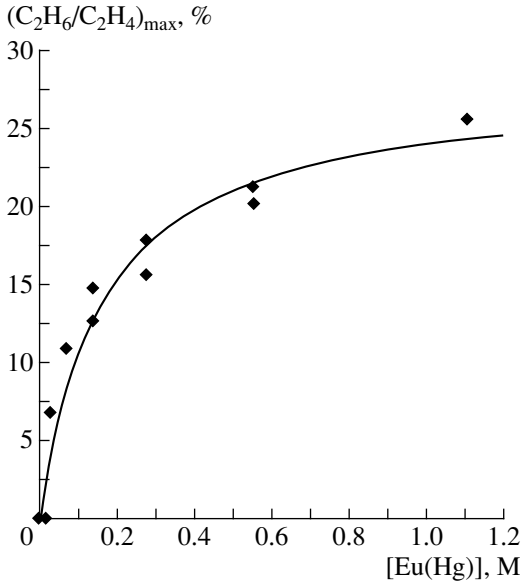


Fig. 11. Maximum C_2H_6/C_2H_4 molar ratio in the reaction products of C_2H_2 reduction with europium amalgam catalyzed by FeMoco as a function of Eu(Hg) concentration. [FeMoco] = 1.5×10^{-5} M.

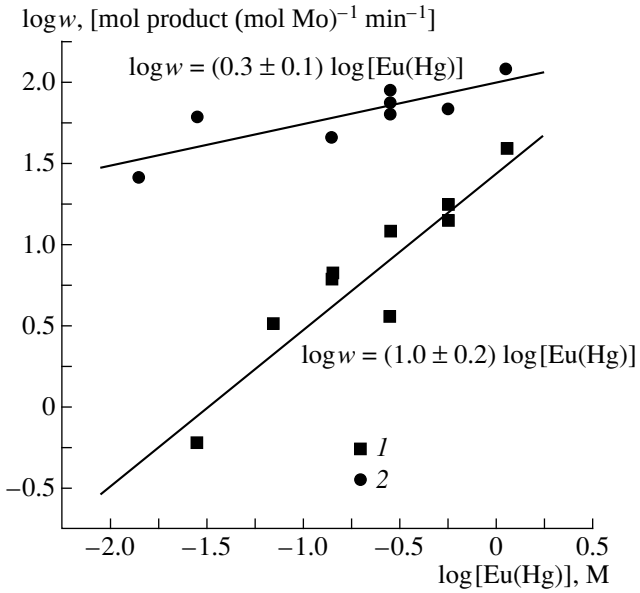


Fig. 12. Rates of (*1*) ethane and (*2*) ethylene formation in the reaction of C_2H_2 reduction as functions of europium amalgam concentration on logarithmic coordinates. [FeMoco] = 1.5×10^{-5} M.

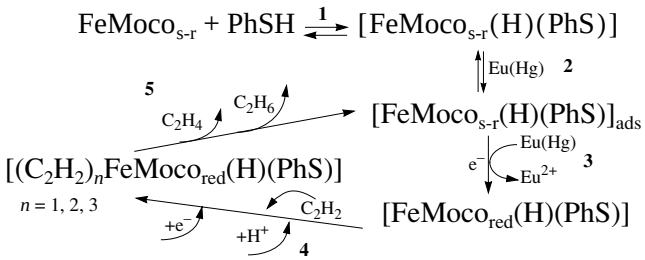
As mentioned above, the rate of reaction with the participation of amalgam depends on the contact surface area between amalgam and solution, and this surface area *S* is a multiplier in the expression for the reaction rate. In principle, europium amalgams with different concentrations could have different surface areas, all other factors being the same. In general, the specific surface area of amalgam depends on the surface tension and the composition of the surrounding solution [34, 35]. The surface tension of the liquid amalgam depends on the metal concentration in the amalgam so that even very small metal quantity added to mercury with the formation of a dilute amalgam with a concentration lower than 10^{-3} M dramatically decrease the surface tension of mercury (by a factor of ~2 to 3). A further increase in the metal concentration in mercury had almost no effect on the surface tension of the amalgam over the entire range of concentrations up to solid amalgams [34]. Therefore, we believed that, at the same rate of stirring (which provides maximum disintegration of the amalgam) and at the same composition of the surrounding solution (DMF, PhSH, FeMoco, Bu_4NBr, and $(Bu_4N)_2S_2O_4$), equal amounts of amalgams with different europium concentrations form equal surface areas, so the rate equation includes a constant coefficient equal to this surface area. It is likely that thiophenol and quaternary ammonium salts, which are surface-active compounds, exert a stabilizing effect by preventing amalgam droplets from coalescing.

In neutral or weakly acidic aqueous solutions and nonaqueous media, the rate of amalgam decomposition due to hydrogen release obeys the equation $w \sim [M(Hg)]^{1/2}\,[H^+]$, as was first found by Brønsted and Kane [36] as early as 1931 and repeatedly supported in more recent publications. This is true of alkaline and alkaline earth metal amalgams, as well as europium, samarium, and terbium amalgams, the formation of which is accompanied by considerable heat effects and which have relatively stable chemical compounds between the metals and mercury with variable composition. If amalgam is consumed in the reduction of an organic compound along with hydrogen release, the type of experimental dependence of the rate of this reaction on amalgam concentration is determined by one-electron or two-electron transfer as a rate-limiting step in the reduction reaction.[1] It was found [20 and references therein] that, if one-electron transfer is a rate-limiting step of a chemical reaction, the rate of formation of the reduced product is proportional to the amalgam concentration to the power of 1/2. We observed for the ethylene formation rate that $w_{C_2H_4} \sim [Eu(Hg)]^{0.3}$. In the case when the addition of two electrons to a molecule to be reduced is a rate-limiting step, the reaction rate is proportional to the amalgam concentration to the first power. For the formation of ethane, we observed the direct proportionality of the reaction rate to the concentration of europium amalgam $w_{C_2H_6} \sim [Eu(Hg)]$ (Fig. 12).

[1] Note that this is true of cases in which amalgam and solution are intensely stirred so that neither metal diffusion in the amalgam nor the diffusion of a reduced substance to the amalgam–solution interface limit the process, and the act of reduction (electron transfer) is a limiting step.

CONCLUSIONS

The above data on the steady-state reaction kinetics of acetylene reduction by europium amalgam catalyzed by the FeMoco, the nitrogenase active center separated from the enzyme allowed us to propose the following reaction scheme:



Reaction step **1** is thiophenol binding to the FeMoco cluster in a semireduced state with the formation of the compound [FeMoco$_{s\text{-}r}$(H)(PhS)], which can undergo adsorption on the surface of an amalgam electrode (step **2**) and reduction with the amalgam (step **3**). Next, it can coordinate C_2H_2 (from one to three molecules simultaneously depending on the pressure of acetylene). Coordinated HC≡CH is reduced and protonated at the subsequent step. Initially, metal or sulfur atoms of the cofactor undergo protonation; then, ethylene and ethane are formed by intramolecular hydrogen transfer to the substrate. We believe that the catalyst is not desorbed in the course of a catalytic cycle because, under steady-state conditions, the rates of release of C_2H_4 and C_2H_6 as functions of catalyst concentration are similar in shape to an adsorption isotherm; that is, they depend on the surface concentration of the catalyst (Fig. 4). Note that adsorption is ultimately reversible because, after the termination of the reaction by the cessation of stirring, the amalgam is aggregated in a large drop, and the catalyst goes into solution. In this case, the catalyst structure remains unchanged, as shown by the reconstruction of the defect MoFe protein by the FeMoco samples.

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