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Effect of the Acidity and Chemical Nature of the Protonating Agent on the Rate of Acetylene Reduction Catalyzed by the Nitrogenase Active Site Isolated from the Enzyme

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Abstract—The effect of the acidity (pK_a) of the source of protons on the rate and selectivity of acetylene reduction has been investigated in order to elucidate the mechanism of protonation of substrate molecules coordinated to the reduced FeMoco cluster. A number of compounds whose pK_a in DMF varies between 6 and 20 have been examined as protonating agents. The rate of the reaction is almost independent of the acidity of the proton donor in a wide pK_a range. This can be explained in terms of the specific features of substrate protonation catalyzed by iron–sulfur clusters. Active protonating agents in the system are those which react with the catalyst to form hydrogen-bonded association species or those which are ligands reversibly binding to the cluster and are capable of donating protons, likely with simultaneous electron transfer.

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The enzyme nitrogenase, which occurs in nitrogen-fixing bacteria, catalyzes N_2 reduction to NH_3. The latter, unlike molecular nitrogen, can be utilized by living organisms in the synthesis of proteins, nucleic acids, and other essential biomolecules.

Although there have been great advances in the study of nitrogenase in recent years [1, 2], it is still unknown how this enzyme functions at the molecular level. The active site of the enzyme contains an iron–molybdenum cofactor (FeMoco). This cofactor is an octanuclear cluster, whose composition is [(C)$MoFe_7S_9$ homocitrate] [3, 4]. Its function is to coordinate and reduce (via successive transfer of a number of electrons and protons) various substrates whose molecules are small and have multiple bonds, primarily N_2. The molecular mechanism of the processes taking place on FeMoco is unknown.

Kinetic studies of the nitrogenase reaction in vitro have provided little information concerning substrate conversions on the active site, because these conversions are not the limiting ones for the overall enzymatic process and, therefore, have no effect on its kinetics. In order to investigate the transformations occurring at the active site, it is necessary to find some simplified model systems that could imitate separate aspects of the enzymatic reaction and could be experimentally studied.

The FeMoco cluster has a number of specific features. In particular, it possesses a unique structure that has no analogues among the numerous synthetic FeS and MoFeS clusters. It can be isolated from the protein as a quaternary ammonium base salt dissolved in an organic solvent without breaking its cluster structure [3–7], and the isolated cofactor retains many of its initial spectral and redox properties [8–11]. Outside the protein, it is extraordinarily resistant to acids and chelating agents as compared to related compounds [11], whereas these chemicals are usually destructive for synthetic FeS and MoFeS clusters.

In order to elucidate the function of the FeMoco cluster itself and to see how the protein particle and the amino acids nearest to FeMoco contribute to nitrogen reduction by comparatively weak reductants under mild conditions, we studied the catalytic activity of the free FeMoco cluster in nitrogenase substrate reduction reactions in a nonprotein medium [12, 17]. The cofactor was isolated from the MoFe protein of nitrogenase of the *Azotobacter vinelandii* microorganism. The integrity of the cluster framework after its isolation from the protein and after catalytic reactions involving FeMoco in nonprotein systems was estimated as the ability of FeMoco to reproduce the enzymatic activity of the cofactor-deficient MoFe protein of *Klebsiella pneumoniae* Kp5058. We demonstrated [12–14] that the iron–molybdenum cofactor is an active reduction catalyst for a variety of nitrogenase substrates in a dimethylformamide (DMF) medium containing a Zn or Eu amalgam as the reductant and thiophenol as the proton donor. To gain insight into the mechanism of the catalytic action of FeMoco in nonenzymatic systems, we carried out a detailed kinetic study of the FeMoco-catalyzed reduction of nitrogenase substrates [11] and of the reaction of FeMoco with nitrogenase inhibitors. Based on the

results of these studies, we made some inferences as to the interactions of the substrates and inhibitors with FeMoco separated from the protein [16]. A comparison between the catalytic behavior of isolated FeMoco under conditions of acetylene reduction inhibited by carbon(II) oxide and molecular nitrogen and the behavior of the same cofactor inside the protein demonstrated that the process involving the whole enzyme and the process involving the isolated cofactor are very similar in their basic regularities, even to the extent of being characterized by equal kinetic constants. We were the first to establish that FeMoco can efficiently coordinate with molecular nitrogen outside the protein environment as well [16], and we did not detect any nitrogen reduction products under the conditions of this reaction. This might be due to the insufficient basicity of the coordinated N_2 molecule or to the inappropriate acidity and/or chemical nature of protonating agent. The substrate protonation mechanism in FeMoco-involving systems is known neither for nitrogenase in vitro nor for biomimetic systems involving the cofactor. In order to see whether the FeMoco cluster separated from the protein catalyze nitrogen reduction in a purely chemical system and to elucidate the mechanism of substrate protonation during reduction, we studied how acetylene reduction catalyzed by FeMoco outside the protein depends on the nature of the protonating agent.

Here, we report the steady-state kinetics of cofactor-catalyzed acetylene reduction in a nonprotein environment in the presence of proton donors varying in acidity and chemical composition.

EXPERIMENTAL

Chemicals and Preparations

The protonating agents—thiophenol (special-purity grade), citric acid (special-purity grade), 2-methylimidazole (Fluka), pentafluorothiophenol (Sigma), imidazole (99%, Aldrich), oxalic acid (reagent grade), orthophosphoric acid (reagent grade), stearic acid (reagent grade), 2-mercaptobenzimidazole (reagent grade) (Reakhim), benzoic acid (Veb Jenapharm)—were used as received.

Pyrocatechol (Reakhim) was purified by vacuum sublimation in a water bath.

Preparations of the Fe and MoFe proteins of *Azotobacter vinelandii* nitrogenase were obtained by R.I. Gvozdev, L.A. Syrtsova, and their colleagues via an earlier published procedure [18]. The cofactor-deficient MoFe protein from the mutant strain *Klebsiella pneumoniae* Kp5058 was kindly provided by B.E. Smith (John Innes Centre, Great Britain). The reagents and their solutions to be used in the isolation and study of the FeMo cofactor were purified from traces of oxygen and water by standard methods and were stored in an argon atmosphere.

The iron–molybdenum cofactor was isolated from the MoFe protein of *Azotobacter vinelandii* nitrogenase using earlier described procedures [15, 16]. According to the analysis of FeMoco for molybdenum and iron (see below), the FeMoco yield was 70–85%. The capacity of isolated FeMoco for reproducing the catalytic activity of the cofactor-deficient MoFe protein of *Klebsiella pneumoniae* Kp5058 in acetylene reduction was estimated using a proven procedure [19–21]. Samples of FeMoco in DMF and solutions of the Fe and MoFe proteins were stored frozen in liquid nitrogen.

The solvent was DMF (reagent grade, Reaknim). It was predried and was then vacuum-distilled at 15 Torr from molecular sieve 4 Å. Aqueous solutions were prepared using triply distilled water.

Preparation of a zinc amalgam and measurement of its potential were described in an earlier publication [13].

All manipulations with oxidizer-sensitive substances were performed under strictly anaerobic conditions using Schlenk techniques. All aqueous buffer solutions contained 5×10^{-3} mol/L of sodium dithionite. Organic solvents contained $(2–5) \times 10^{-3}$ mol/L of tetrabutylammonium dithionite. The presence of sodium dithionite was checked using the benzyl viologen indicator.

Experimental Study of the Catalytic Activity of FeMoco outside the Protein

Experiments were carried out in a special-purpose temperature-controlled glass reactor with a flat bottom and a magnetic stirrer [21]. This reactor allowed use of metal amalgams. The reactor was vacuumized and filled with argon. Thereafter, 0.7 mL of Zn(Hg) was placed in an overturnable vessel and 4.0–4.3 mL of a solution of FeMoCo ($(1–2) \times 10^{-5}$ mol/L, $(4–7) \times 10^{-8}$ mol) and the necessary amount of a proton donor solution in DMF were placed in the reactor. The reaction mixture was frozen with liquid nitrogen, and the reactor was connected to a circulation unit and was then vacuumized. Next, the liquid phase was thawed and was warmed to 21°C and the reactor was filled with an acetylene (nitrogen) + argon mixture. During the run, the gas phase was stirred using the circulation unit and the liquid phase was stirred with the magnetic stirrer. The stirrer speed was adjusted so as to maximize the breakup of the amalgam.

The reaction was monitored by sampling, at certain intervals, the gas phase from the reactor for chromatographic analysis (see below) and by plotting the amount of reaction product as a function of the reaction time. The rate of the reaction was calculated from the slope of the initial portion of the product accumulation curve. The value of the reaction rate was refined by averaging the results of two or three replica experiments, whose spread typically did not exceed 10%. The initial steady-state reaction rate (w) was determined as the amount of product forming per unit time

in the initial, linear portion of the product accumulation curve.

Analytical Procedures

The molybdenum content of FeMoco samples was determined by atomic absorption spectroscopy on a Carl Zeiss AAS1 spectrometer with a PerkinElmer HGA 74 graphite furnace.

Iron in the cofactor was oxidized to Fe^{3+} by heating the cofactor with dilute nitric acid (1 : 10) and was then quantified spectrophotometrically as the complex between the Fe^{3+} and CNS^- ions by measuring the absorbance of an ethanolic solution of the complex at a wavelength of 500 nm. Absorption spectra were recorded on a Hewlett Packard 8451A Diode Array spectrophotometer.

The concentration of hydrazine was determined spectrophotometrically via converting it into the colored compound dihydrazone (whose extinction coefficient at λ_{max} is $\varepsilon = 7 \times 10^4$ mol^{-1} cm^{-1}) by reacting it with *para*-dimethylaminobenzaldehyde in an acid medium [22].

The ammonia concentration was measured by the modified indophenol method [23]. For ammonia determination in the experiments involving labeled nitrogen $^{15}N_2$, 2.3 mL of the solution resulting from the nitrogen fixation reaction and 5×10^{-5} mol of $^{15}NH_4Cl$ were placed in one leg of a Rittenberg tube and 2 mL of a saturated sodium hypobromite solution was placed in the other one. After the tube was thoroughly vacuumized, the solutions were combined and the gas phase was analyzed mass-spectrometrically.

The acetylene reduction products (ethylene and ethane) were quantified on a Biokhrom chromatograph (2 m × 5 mm stainless steel column packed with activated alumina (0.25–0.5 mm size fraction), oven temperature of 90°C, argon as the carrier gas, flame-ionization detector). The circulating gas mixture was sampled at regular intervals into an evacuated sample loop with a calibrated volume, and the sample was then transported by the carrier gas to the detector chamber.

RESULTS AND DISCUSSION

A study of the electrode reduction potential effect on the catalytic reduction of C_2H_2 in the presence of FeMoco [17] demonstrated that the smaller the absolute value of the potential, the higher the significance of the acidity of the protonating agent. For this reason, systematic experiments on the effect of pK_a on the reaction were performed using zinc amalgam ($E = -0.84$ V versus the standard hydrogen electrode) as the reductant. It was expected that the effect of the acidity of the proton donor would be stronger in this case.

Twelve compounds were examined as potential proton donors for the formation of C_2H_4 or C_2H_6 from C_2H_2. Their structures and their pK_a values in H_2O [24] and in DMF [25] are listed in the table. The following requirements were imposed upon these compounds. (1) The compound should be soluble in DMF. (2) The pK_a value of the compound in DMF should be larger than 5, because the cluster decomposes irreversibly at $pK_a \le 5$ [26]. Choice of the chemical nature of the compound was dictated by the structure of the ligand environment of the cofactor in the protein in the in vivo and in vitro reactions (thiophenol, imidazole, their analogues, water, orthophosphoric acid) and by the necessity of considering different classes of compounds (carboxylic acids).

As was demonstrated in an earlier study [27], thiophenol interacts with the cofactor isolated from the enzyme, coordinating to the iron atom Fe1.[1] Next is pentafluorothiophenol, a thiophenol analogue whose SH group has a higher acidity owing to the strong electron-withdrawing effect of the substituents in the benzene ring. Conversely, phenol is a weaker acid than thiophenol.

According to X-ray crystallographic data for a MoFe protein single crystal [3, 4], the imidazole ring of histidine is a ligand at the molybdenum atom in the cofactor of the protein. 2-Mercaptobenzimidazole as a protonating agent can apparently coordinate to the cofactor in two ways. However, owing to the higher acidity of its SH group, it likely interacts with the cofactor at the Fe1 atom.

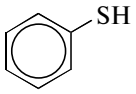
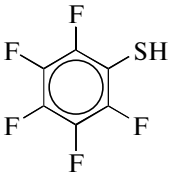
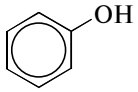
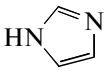
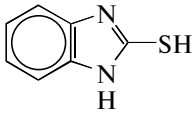
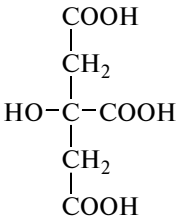
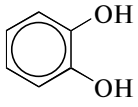
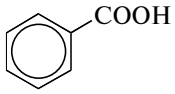
Citric and oxalic acids and pyrocatechol were included in the list of compounds to be examined for the reason that, on the one hand, they possess chelating properties and can thus coordinate to the cofactor to replace solvent molecules and, on the other hand, they can vacate a coordination site to bind to a zinc ion as the substrate is approaching. Since the rate of the reduction reaction depends considerably on the surface area of the amalgam [15], it is obvious that adding a compound that has both proton-donating and surface-active properties is favorable for the system to stay in the finely dispersed state. These requirements are met by stearic acid. Because of lack of information concerning the capacity of monocarboxylic acids for binding to the cofactor, it was interesting to study the behavior of an aromatic acid, specifically, benzoic acid. The class of inorganic acids was represented by orthophosphoric acid.

Although water has a small pK_a value in DMF, it was also considered as a potential protonating agent, for it is among the participants of the nitrogenase reaction in vitro. In addition, since the DMF used as the solvent in this study contained 0.05 mol/L of water, it was necessary to allow for a competing reaction taking place.

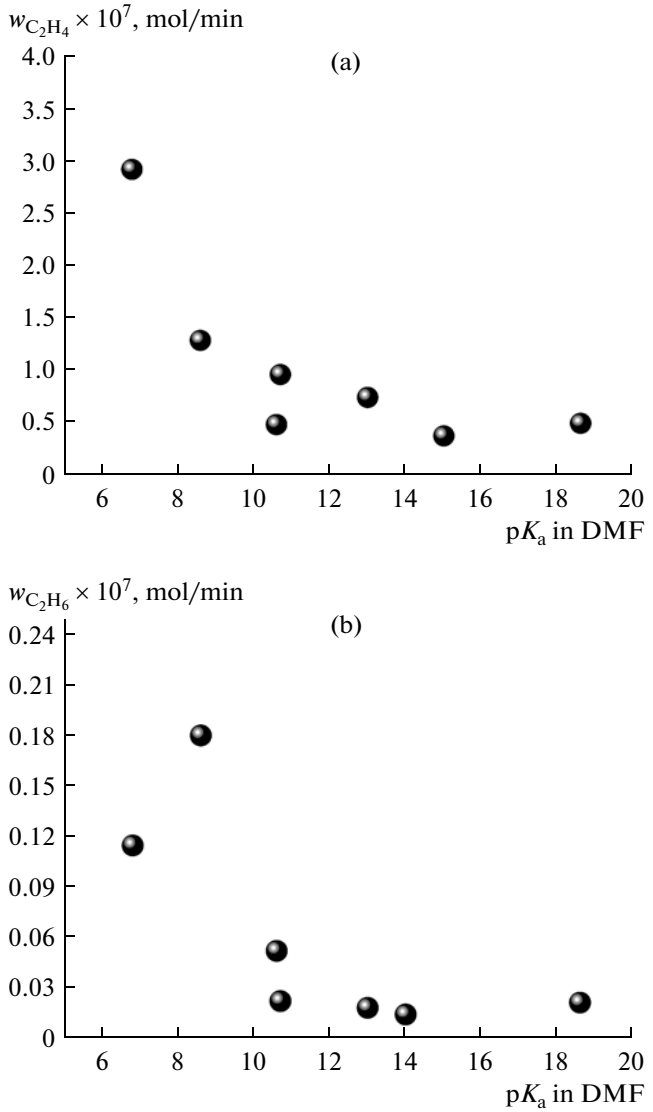
Not all of the compounds appearing in the table are characterized by a precise pK_a value. The key factors

[1] Here, the atoms are numbered in the same way as in the X-ray crystallographic analysis of the MoFe protein of *Azotobacter vinelandii* (ProteinDataBank accessing code 1M1N(Av1)) [3, 4].

pK_a data for protonating agents

Entry	Compound		pK_a	
	molecular formula	name	in H_2O	in DMF
1		thiophenol	6.6	10.7
2		pentafluorothiophenol	2.7	6.8
3		phenol	9.95	15.4
4		imidazole	14.5	18.6
5		2-mercaprobenzimidazole	~8	~13
6		citric acid	3.13 4.76 6.40	10.6 13.3 15.7
7		oxalic acid	1.2 4.2	8.6 16.6
8		pyrocatechol	9.2	~15
9	$C_{18}H_{38}COOH$	stearic acid	4.9	~13
10		benzoic acid	4.2	12.3
11	H_3PO_4	orthophosphoric acid	2.12 7.21 12.32	~10 ~15 ~20
12	H_2O	water	15.7	~32

Note: The sign ~ is placed before the pK_a values for compounds in DMF estimated by extrapolation of pK_a data for DMSO.



(a) Ethylene and (b) ethane formation rates as a function of the pK_a of the protonating agent. Reaction conditions: [FeMoco] = 1×10^{-5} mol/L, [HA] = 1×10^{-2} mol/L, 0.7 mL Zn(Hg) at a concentration of 4.27 mol/L, DMF as the solvent (4 mL), C_2H_2 pressure of 100 Torr, 21°C.

determining the acidic properties of a solute are the proton-donating power (acidity), proton-accepting power (basicity), and dielectric constant of the solvent [28]. The solvent that is most similar to DMF in terms of these parameters is DMSO. The pK_a values of some compounds in this solvent are known from the literature [25, 29]. For this reason, we considered the pK_a values of the protonating agents in these two solvents. In the table, the sign ~ is placed before the pK_a values for compounds in DMF obtained by extrapolation of pK_a data for DMSO. A precise pK_a value for 2-mercaptobenzimidazole is known for none of the above solvents. The value given in the table was calculated by comparing pK_a data for related compounds in different solvents.

For each of the above [H^+A^-] compounds, we measured the C_2H_4 and C_2H_6 accumulation rates in the FeMoco–Zn/Hg–C_2H_2–[H^+A^-] system in DMF. The C_2H_2 reduction products in this system—ethylene and ethane—accumulate simultaneously [15]. The reaction is catalytic: neither ethylene nor ethane form in the absence of the cofactor. The figure presents the plots of the observed ethylene and ethane formation rates as a function of the pK_a of the proton donor used in acetylene reduction. Clearly, as the acidity of the proton donor is changed in the wide range from 8 to 19 and as its chemical nature is varied, the accumulation rates of the reduction products, particularly that of ethylene, change only slightly.

The ethane formation rate passes through a maximum, whose position corresponds to oxalic acid. However, under our experimental conditions oxalic acid itself can undergo reduction to yield ethane; that is, it can be both a source of protons and a substrate. Therefore, the observed effect (increased ethane accumulation rate) can be attributed in part to the conversion of oxalic acid.[2]

Five of the 12 compounds examined—water, orthophosphoric acid, monocarboxylic (benzoic and stearic) acids, and phenol—turned out to be inactive. In the case of the monocarboxylic acids, whose pK_a in DMF is about 13, the C_2H_4 formation rate was very low and C_2H_6 did not form at all. With water as the proton donor, neither ethylene nor ethane formed at then reduction potential of zinc amalgam. It is likely that water, a very weak H^+ donor in DMF, cannot protonate FeMoco, so its reduction to the substrate-binding state takes place at more negative potentials. This assumption is corroborated by the observed dependence of the reaction rate on the preset potential of the mercury electrode. It was demonstrated that, in the presence of water, ethylene as an acetylene reduction product appears at an electrode potential lower than –0.95 V versus the standard hydrogen electrode. It is interesting that ethane did not form in this experiment up to a potential of –1.75 V.

As was noted above, the mechanism of substrate protonation on the cofactor in the protein is unknown. In principle, the substrate in a [catalyst–substrate] complex can be protonated directly by the medium or via the intermediate protonation of catalyst atoms followed by proton transfer to the substrate. There are empirical criteria for assigning a mechanism to one type or the other [30]. If the proton binds directly to the atom that is the "destination" of protonation, the

[2] Earlier, when choosing operating conditions for the cofactor isolated from the protein, we carried out experiments on acetylene reduction in the [FeMoco–PhSH] (**1**) and [FeMoco–PhSH–$(COOH)_2$] (**2**) systems at component concentrations of [FeMoco] = 6×10^{-6} mol/L, [PhSH] = 6×10^{-3} mol/L, and [$(COOH)_2$ = 5×10^{-3} mol/L, a C_2H_2 pressure of 200 Torr, and a temperature of 30°C. In system **2**, the ethane yield in 15 min was 3.1 mol per mole of FeMoco, 6 times higher than in system **1** (0.49 mol of C_2H_6 per mole of FeMoco).

dependence of the reaction rate on the acid concentration will be described by a first-order equation. The addition of protons to a substrate having multiple bonds will result in the formation of a *trans* product. In catalysis by metal complexes, which takes place via indirect proton transfer to the substrate, it is necessary that the catalytic cluster form an association species with the proton donor as well, producing a [catalyst–acid–substrate] ternary ensemble. As a consequence, the reaction rate will depend on the structure of the acid. Thus, if the reaction rate is described a complicated equation, including the case in which the reaction rate as a function of proton concentration is not a first-order equation, the mechanism of protonation in the system is considered to be indirect. In this case, the reaction rate depends on the nature of the protonating agent and the reduction reaction yields a *cis* product [30].

The totality of data for reactions involving FeMoco isolated from the protein suggests that the protonation of the acetylene molecule coordinated to the reduced cofactor occurs intramolecularly in the [FeMoco–C_2H_2–proton donor] complex. Earlier measurements of the acetylene reduction rate as a function of the thiophenol concentration [14] and an earlier study of the stereospecificity of the reaction [15] confirm that the greater part of ethylene results from intramolecular H transfer from protonated iron or sulfur atoms of the cofactor to the substrate.

Pham and Burgess [31], who experimentally investigated the medium acidity effect on substrate reduction, discovered that, in the presence of nitrogenase in vitro, the plot of the activity of the system versus the pH of the medium is dome-shaped. An analysis of this plot demonstrates that, in a catalytic cycle, a group with pK_a ~6.3 undergoes deprotonation and a group with pK_a ~9.0 undergoes protonation. It was hypothesized that these groups are located near the cluster and are involved in regulated proton transfer to the cluster and, eventually, to the substrate.

The results of theoretical studies of the mechanism of protonation in nitrogenase [32, 33] and experimental data on the protonation of Fe–S clusters [26] indicated that, out of the possible mediating atoms (metal and sulfur), the sulfur atoms are the most likely mediators in proton transfer by the cofactor. In substrate protonation, proton transfer from amino acid residues or water molecules to the μS bridges of FeMoco yields sulfhydryl groups SH. The issue of the place at which the cofactor in the protein undergoes primary and subsequent protonations is extensively discussed in the literature [1, 32–34]. The three central μ2S groups are more basic than the μ3S groups of the cubane moieties constituting the cofactor structure [35]; nevertheless, according to X-ray structure determination data for the nitrogenase complex [3, 4] and quantum chemical calculations [32, 33], the proton-conducting channel in the protein leads to the 4Fe moiety of the cofactor, which includes Fe2, Fe3, Fe6, Fe7, and μ3S groups. The proton is transferred from the μS groups to the substrate, which is likely coordinated to the same 4Fe moiety [36–38].

Investigation of all reactions taking place on the cofactor isolated from the protein has demonstrated that they are very similar to the reactions occurring in nitrogenase in vitro [15, 16, 39]. In view of this, we think that, for these two case, it is quite allowable to compare medium acidity effects, specifically protonating agent acidity effects. Under the assumption that substrate reduction in the systems involving the isolated cofactor proceeds via the same protonation mechanism as in the case of nitrogenase in vitro, it is clear why the reaction rate depends weakly on the acidity of the protonating agent in the pK_a 8–19 range. It is essential that the threshold acidity of the source of protons be sufficient for the protonation of the bridging sulfur atom followed by hydrogen transfer to the substrate. The lower limit of the acidity is determined by the pK_a^1 value of the cofactor, which was calculated to be 7.8 [26].

In order to determine the rate-determining step of acetylene reduction catalyzed by the cofactor outside the protein, we studied the dependence of the rate of this reaction on the preset potential of the external electron donor and on temperature [17]. It turned out that the rate of the reaction may be determined by different steps, depending on the acidity of the protonating agent. When the protonating agent is thiophenol or water, whose acidities fall in the range in which the reaction rate is independent of the nature of the protonating agent, the rate is determined by the electrochemical step, specifically, electron transfer from the cathode to the catalytic cluster. It is, therefore, natural that the reaction rate depends only weakly on the acidity of the protonating agent. In this case, the apparent activation energy of the formation of ethane, which is the product of the four-electron reduction of C_2H_2, is 1.5 times lower than the apparent activation energy of ethylene formation and is about 13 kcal/mol. With pentafluorothiophenol, a more acidic protonating agent, the observed product formation rates are higher than in protonation with the other compound examined (figure) and the rate of the overall process is limited by the chemical step, specifically, intramolecular rearrangement in the [catalyst–substrate–proton donor] complex. In this case, the apparent activation energies of ethylene and ethane formation are equal and are 32 kcal/mol, much higher than are observed for less acidic proton donors.

Thus, the results of this study of the protonating agent acidity effect on the rate of acetylene reduction catalyzed by FeMoco outside the protein and the results of earlier studies dealing with the dependence of the acetylene reduction rate on the thiophenol concentration [14] and with the stereospecificity of the reaction [15] demonstrate that the protonation of the acetylene molecule coordinated to the reduced cofac-

tor takes place in the [FeMoco–C_2H_2–proton donor ensemble] and the greater part of the ethylene results from intramolecular proton transfer from the pre-protonated iron or sulfur atoms of the cofactor to the substrate.

In the systems involving FeMoco separated from the protein, protonating activity is shown by compounds that can interact with the catalyst to yield hydrogen-bonded association species or can serve as ligands reversibly binding to the cluster and donating protons to the cluster, the latter process being likely to occur simultaneously with electron transfer.

Protonation with none of the proton donors was accompanied by N_2 reduction on the cofactor outside the protein, even though intensive acetylene reduction took place under the same conditions and nitrogen inhibited this process. Therefore, the only unquestionable inference from this study is that FeMoco outside the protein can be reduced to the state in which it ensures the reversible coordination of the N_2 molecule and catalyzes acetylene reduction. The rate and regularities of this reaction are very similar to those of the reaction catalyzed by FeMoco inside the protein globule.

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