Nitrogenase Reactivity: Effects of pH on Substrate Reduction and CO Inhibition†

David N. Pham and Barbara K. Burgess*

Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717

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ABSTRACT: Molybdenum nitrogenase is composed of two separately purified proteins designated the iron protein (Fe protein) and the molybdenum–iron protein (MoFe protein), with the latter containing the substrate reduction site which is a metal cluster designated the iron–molybdenum cofactor (FeMo cofactor). In addition to its physiological substrates H^+ and N_2, nitrogenase reduces a number of nonphysiological substrates (e.g. C_2H_2 and N_3^-) and interacts with a number of similar molecules (e.g. CH_3NC and CO) that serve as specific inhibitors. Despite their great diversity, all substrates are reduced by multiples of two electrons and require equivalent numbers of electrons and protons. Although the electron donor to a substrate is believed to be FeMo cofactor, the nature of the proton donor is unknown and might be different for different substrates. Here we report a three-component buffer assay system that eliminates variables of buffer type, ionic strength, and ATP and reductant availability and that is compatible with the nitrogenase system in the pH range 5.0–9.8. Preincubated studies and studies of the effects of pH on H_2 evolution under Ar, H_2 evolution under N_2, H_2 evolution under CO, and C_2H_2 reduction show that there is a group with a pK of ca. 6.3 that must be deprotonated for substrate reduction to occur and that there is a group with a pK of ca. 9.0 that must be protonated for substrate reduction to occur. The pK of the 9.0 group is shifted 0.5 pH unit in the acid direction by both CO and C_2H_2 but not by N_2, while the pK of the 6.3 group is shifted 0.4 pH unit in the acid direction by C_2H_2 but not by CO or N_2. Thus, CO appears to inhibit H_2 evolution by native nitrogenase at high pH. These substrate effects strongly suggest that the groups being titrated are at or near the active, FeMo cofactor, site of the enzyme.

The enzyme nitrogenase catalyzes the six-electron reduction of atmospheric N_2 to ammonia, a critical step in the global nitrogen cycle. Molybdenum nitrogenase is composed of two separately purified proteins called the iron protein (Fe protein) and the molybdenum–iron protein (MoFe protein), both of which are absolutely required for substrate reduction (Yates, 1992; Burgess, 1993; see also references therein). The Fe protein is a dimer composed of identical ca. 30 000 M_r subunits that are bridged by a single [4Fe-4S] cluster. It has two binding sites for MgATP. During nitrogenase turnover, the Fe protein serves as a specific one-electron donor for the much more complex MoFe protein. This ca. 240 000 M_r protein has an $\alpha_2\beta_2$ subunit pattern and contains two different types of metal centers designated the P-clusters and the iron–molybdenum cofactor (FeMo cofactor) centers (Smith & Eady, 1992; Burgess, 1990; Newton, 1992; see also references therein).

The direction of electron flow through nitrogenase has been extensively studied, and the steps involved were recently reviewed (Yates, 1992; Burgess, 1993; Thorneley & Lowe, 1985). *In vitro* experiments show that the Fe protein is first reduced by one electron using $SO_2^{\bullet-}$ as the electron donor. The Fe protein, with two molecules of MgATP bound, then forms a complex with the MoFe protein. Within that complex, a single electron is then transferred to the MoFe protein, a reaction that is coupled to the hydrolysis of two molecules of MgATP. The complex then dissociates in what is usually the rate-limiting step in nitrogenase turnover (Thorneley & Lowe, 1985). Substrates are believed to be ultimately reduced at the FeMo cofactor site of the reduced MoFe protein (Smith & Eady, 1992; Burgess, 1990; Newton, 1992). Because no nitrogenase substrate is completely reduced by only one electron, the enzyme must go through more than one cycle of electron transfer between the Fe protein and the MoFe protein before products can appear.

The physiological substrates for nitrogenase are N_2 and protons from H_3O^+, with the latter being reduced by two electrons to yield H_2 gas. In addition to these substrates, however, nitrogenase catalyzes the reduction of a large number of alternate substrates (e.g. C_2H_2, CH_3NC, N_3^-, N_2O, HCN, and N_2H_4) involving 2–14 electrons (Yates, 1992; Burgess, 1993, 1985). Most of these reducible substrates contain NN, NO, NC, or CC triple or potential triple bonds. In addition to these alternate substrates, the similar molecules CH_3NC and CO serve as specific inhibitors of nitrogenase, the former causing inhibition of electron flow while the latter inhibits the reduction of all substrates except protons. Despite this great diversity in nitrogenase substrate reactivity, the reductions of all nitrogenase substrates have at least two features in common. All are reduced by multiples of two electrons, and with rare exceptions, all reductions require equivalent numbers of electrons and protons. Although the immediate electron donor to substrate is believed to be the FeMo cofactor (Smith & Eady, 1992; Burgess, 1990; Newton, 1992), the nature of the proton donor is unknown and might be different for different substrates.

Very recently a major breakthrough in nitrogenase research occurred with the determination of the X-ray structures of the *Azotobacter vinelandii* Fe protein (Georgiadis et al., 1992), the MoFe protein (Kim & Rees, 1992a), and its P-cluster and FeMo cofactor centers (Kim & Rees, 1992b; Chan et al., 1993). These data now shift the focus of inquiry away from structure and toward mechanistic studies. Here we report the development of a simple buffer and assay system that

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* To whom correspondence should be addressed. Phone (714) 856-4297, FAX (714) 856-8551.

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allows pH titrations to be performed on this complex system in the range pH 5.0–9.8 and the use of that system to study the effects of pH on the substrate reactions of native *A. vinelandii* molybdenum nitrogenase.

MATERIALS AND METHODS

Reagents and Chemicals. ATP,[1] creatine phosphate, creatine phosphokinase, $MgCl_2$, Tes,[1] Bis-Tris,[1] HEPPS,[1] CHES,[1] *o*-phthalaldehyde, NaCl, NaOH, HCl, and EDTA[1] were from the Sigma Chemical Co. The specialty gases 99.997% Ar, 99.997% N_2, 99.997% CO, 10% C_2H_2 in Ar, and 1.05% H_2 in Ar (gravimetric standard) were provided by Liquid Carbonic. $Na_2S_2O_4$ was from E-M Chemicals (a division of E. Merck). The extra long glass body combination electrode–pH probe, Accu-pHast, was from Fisher Scientific.

Nitrogenase Assays. A. vinelandii MoFe and Fe proteins were purified and analyzed as described elsewhere (Burgess et al., 1980). Specific activities of the proteins were ca. 1800 nmol of H_2 min^{-1} (mg of Fe protein)$^{-1}$ and 2800 nmol of H_2 min^{-1} (mg of MoFe protein)$^{-1}$. Two separate batches of each protein were used. All assays were performed at 30 °C in 9.5-mL calibrated vials, fitted with butyl rubber serum caps and aluminum serum bottle seals, containing the appropriate gas mixture. The 1.0-mL reaction mixture contained 2.5 mM ATP, 5.0 mM $MgCl_2$, 30 mM creatine phosphate, 75 mM Bis-Tris, 38 mM HEPPS, 38 mM CHES (B75H38C38), 20 mM pH-adjusted $Na_2S_2O_4$ in a B75H38C38 buffer at 30 °C, and 10 units of creatine phosphokinase. It should be noted that much higher concentrations of HEPPS and CHES are inhibitory.

The pH values for each assay vial were adjusted individually at 30 °C by adding either 1 N HCl or 2 N NaOH. Each assay vial was also adjusted to a constant ionic strength value, equivalent to 75 mM NaCl, by adding aliquots of a 1 M NaCl stock solution. The amounts of HCl and NaOH needed for each pH and the amount of NaCl needed to adjust the ionic strength were initially calculated using the program for calculating the ionic strength of a three-buffer system mixture (Ellis & Morrison, 1982). A 75 mM concentration of NaCl was the minimum that could be added to keep the ionic strength equal at all pH values. The actual titration curve is shifted about 0.5 pH unit higher relative to the theoretical curve (data not shown). This is probably because of the large creatine phosphate concentration in the reaction mixture which has a pK of ~4.6 (Fiske & Subbarow, 1929). The experimental values were used for subsequent experiments.

The quantity of creatine phosphokinase used in these assays was a 4-fold increase over that of our usual assays (Burgess et al., 1980). This amount was based on the pH profile of creatine phosphokinase and was chosen to ensure an excess amount in each vial at different pH values. A 50% increase in this amount did not result in any increase in activity. The amount of $Na_2S_2O_4$ was similarly shown to be in excess at each pH value. The vials containing the reaction mixture were degassed and filled with the appropriate gas mixture, $Na_2S_2O_4$ was added, the mixture was incubated at 30 °C for 2 min, and the MoFe protein was added. Then, the reaction was initiated 10 s later by adding Fe protein to give 1 mg/mL total protein at the Fe protein/MoFe protein molar ratio of 5.0. The assays were run for 2 min with shaking at 30 °C, and the reactions were quenched with 0.3 mL of 0.25 M EDTA, pH 7.9. All reactions were linear with time for at least 4 min in the pH range 6.2–8.75. Above and below these pH values, there was deviation from linearity due to irreversible inhibition of the MoFe protein (see Results).

Preincubation Assays. A protocol similar to the one described above was utilized for preincubation assays with the exception that all vials were assayed at the optimal pH for nitrogenase of 7.5. For preincubation assays containing either the MoFe protein or the Fe protein, the preincubation buffer contained 92 mM Bis-Tris, 46 mM HEPPS, and 46 mM CHES (B92H46C46) (0.9 mL) at pH 7.5. For preincubation assays containing the creatine phosphate/creatine phosphokinase–ATP generating system (composed of ATP, $MgCl_2$, creatine phosphate, and creatine phosphokinase) the preincubation buffer contained 50 mM Bis-Tris, 25 mM HEPPS, and 25 mM CHES (B50H25C25) (2.0 mL) at pH 7.5. The concentrations of B92H46C46 and B50H25C25 were chosen to maintain a constant internal concentration of B75H38C38 buffer in the final reaction mixture. From pH 7.5, the B92H46C46 and B50H25C25 buffer systems were adjusted to various pH values with the addition of 1 N HCl or 2 N NaOH. Then the vials containing the buffer system were degassed and filled with the appropriate gas mixtures, and the correct amounts of MoFe protein, Fe protein, or degassed ATP generating system were added. These mixtures were then preincubated at 30 °C for 3 min while being shaken. After preincubation, each vial was adjusted back to the optimal pH 7.5. At this point, the proper amount of preincubated MoFe protein, Fe protein, or ATP generating system was added to the rest of the nitrogenase assay vials to give either 1 mg of total protein or the same concentration of the ATP generating system as stated above. These vials were assayed for 2 min at 30 °C, and the reactions were quenched with 0.25 M EDTA, pH 7.9, as stated in the protocol for nitrogenase assays.

Product Analysis. All products were measured in the same reaction vial. All data represent means of, typically, triplicate determinations with standard deviations less than 10% (e.g. shown by the error bars in the figures). Gas samples, 0.2 mL at bottle pressure, were taken with a pressure-lock syringe (Precision Sampling, Baton Rough, LA) and were analyzed with a Varian 3700 gas chromatograph with a thermal conductivity detector with a molecular sieve 13X column (Ar) for H_2 analysis. Ammonia was determined by an HPLC[1] fluorescence method described elsewhere (Corbin, 1984) and analyzed by a Waters 510 HPLC. C_2H_2 reduction assays and data calculations for H_2 and NH_3 were performed as described previously (Robinson et al., 1986).

RESULTS

H_2 Evolution under Ar. Figure 1 shows the pH profile for H_2 evolution by *A. vinelandii* nitrogenase, under an Ar atmosphere, in the absence of added reducible substrate. The bell-shaped curve gives an optimal pH for H_2 evolution of ca. 7.5 with a 50% loss of activity observed by ca. pH 6.3 and pH 9.0. To determine if the loss of activity above and below pH 7.5 was caused by irreversible inactivation of the enzyme or by active-site groups being in the wrong state of protonation/deprotonation, we performed the preincubation experiments shown in Figure 2. In these experiments either the Fe protein, the MoFe protein, or the creatine phosphokinase based ATP generating system were first preincubated at the indicated pH values for 1 min longer than the assays shown in Figure

[1] Abbreviations: ATP, adenosine 5′-triphosphate; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-propanesulfonic acid acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; EDTA, ethylenediaminetricarboxylic acid; HPLC, high-performance liquid chromatograph.

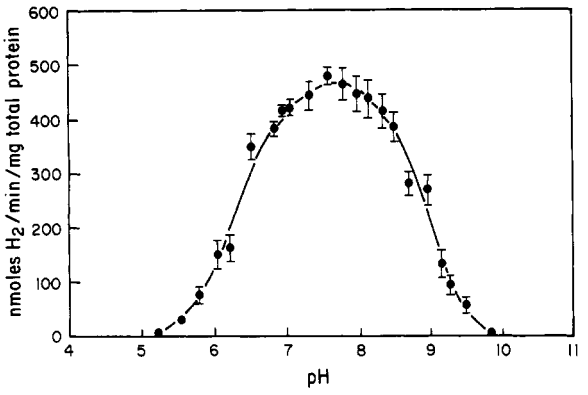

FIGURE 1: pH profile of H_2 evolution under Ar. Assays were performed as described under Experimental Procedures.

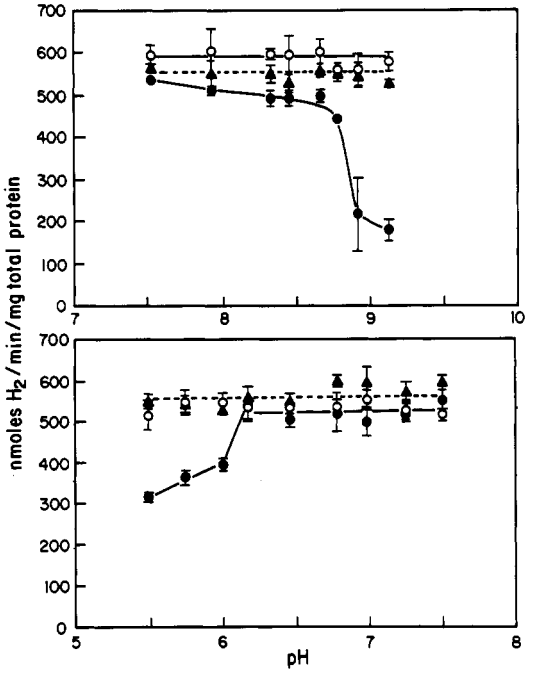

FIGURE 2: The reaction mixture and Fe protein are unaffected by preincubation in the pH range 5.5–9.2 while the MoFe protein is irreversibly damaged by preincubation above pH = 8.65 and below pH = 6.3. The top panel shows the high-pH region, while the botton panel shows the low-pH region. The symbols are for the components present during preincubation: O, the creatine phosphokinase system referred to as Rxn Mix for reaction mixture; ▲, the Fe protein, ●, the MoFe protein. Preincubation assays were performed as described under Experimental procedures. All activity measurements were performed at pH = 7.5.

1. Then, the H_2 evolution under Ar assays were carried out at the optimal pH of 7.5. The data in Figure 2 clearly show that the Fe protein and creatine phosphokinase system were not affected by the preincubation in this pH range. As shown in Figure 2, the MoFe protein is similarly unaffected by the preincubation in the pH range 6.2–8.65. However, above and below those pH values, the *A. vinelandii* MoFe protein undergoes irreversible inactivation.

Nitrogen Reduction and H_2 Evolution under N_2. Figure 3 shows the pH dependence of N_2 reduction to ammonia and of H_2 evolution under N_2. The bell-shaped profile for N_2 reduction is similar to that observed for H_2 evolution under Ar, with a broad optimal pH of ca. 7.5; however, it appears to fall off somewhat more steeply at both high and especially low pH. These trends are more easily visualized in Figure 4, which plots the percentage of electrons being using to reduce N_2 and evolve H_2, as a function of pH under 1 atm of N_2. The enzyme is maximally efficient for N_2 reduction in the broad

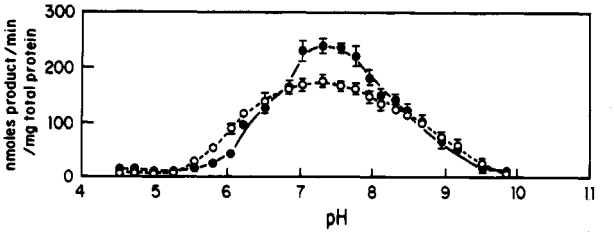

FIGURE 3: pH profiles of (●) N_2 reduction to ammonia and (O) H_2 evolution under N_2.

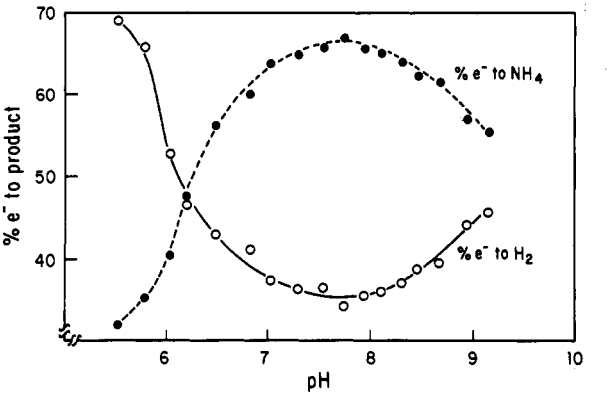

FIGURE 4: Nitrogenase is optimally efficient for N_2 reduction in the pH range 7.0–8.2 and becomes a better hydrogenase above and below these values. The percentages of electrons forming products were calculated using total electron flow = 2 × nmol of H_2 + 3 × nmol of NH_3. Symbols: ●, electrons to NH_3; O, electrons to H_2.

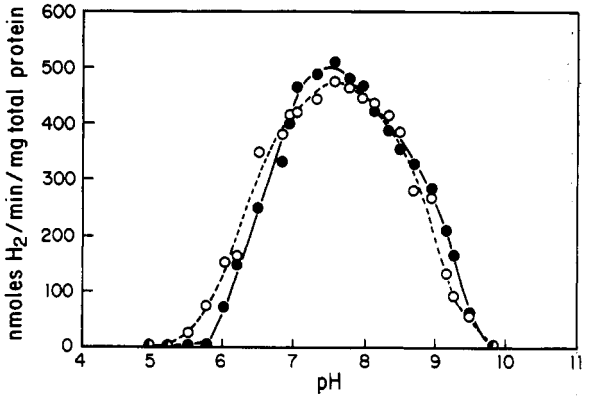

FIGURE 5: H_2 evolution under Ar and H_2 evolution under N_2 have very similar pH profiles: O, H_2 evolution under Ar from Figure 2; ●, H_2 evolution under N_2. For comparative purposes, the H_2 evolution under N_2 numbers from Figure 4 have been multiplied by 3.

pH range 7.0–8.2; above and below these pH values, the enzyme increasingly favors the H_2 evolution reaction. Figure 5 compares the pH profiles of H_2 evolution under Ar and H_2 evolution under N_2. Because so much less H_2 is evolved under N_2, these data have been multiplied by a factor of 3 for comparative purposes. Within the errors of these measurements, there are no obvious differences in the pH profiles of the two reactions.

H_2 Evolution under CO. CO, which is a potent noncompetitive inhibitor of N_2 reduction and all alternative substrate reductions, does not inhibit H_2 evolution by native nitrogenase (Hardy, 1979; Burris, 1979; Huang et al., 1973). Figure 6 compares the pH profiles of H_2 evolution under Ar and H_2 evolution under CO. The two profiles below the optimal pH of 7.5 are indistinguishable from each other. In contrast, the profile above pH 7.5 obtained under CO is greatly shifted in the acid direction relative to the profile under Ar.

C_2H_2 Reduction. In 1966, using crude extracts of *Clostridium pasteurianum*, Dilworth (1966) first demonstrated the

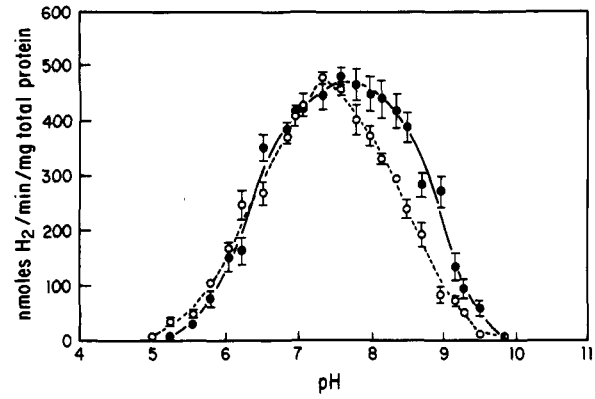

FIGURE 6: CO shifts the pH profile of the H_2 evolution reaction in the high-pH region but not in the low-pH region: ●, H_2 evolution under Ar taken from Figure 2; ○, H_2 evolution under CO.

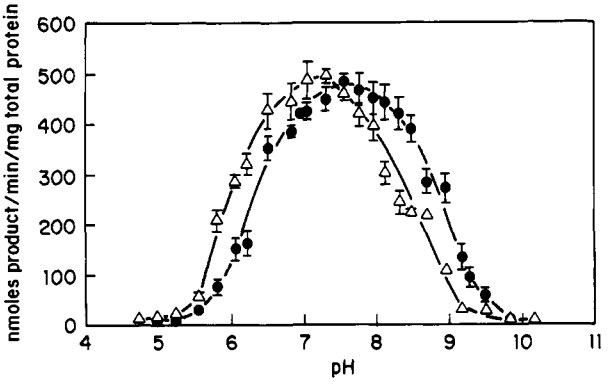

FIGURE 7: C_2H_2 shifts the pH profile in all regions in the acid direction: ●, H_2 evolution under Ar from Figure 2; △, C_2H_2 reduction.

reduction of C_2H_2 to C_2H_4 by nitrogenase as shown in eq 1.

$$C_2H_2 + 2e^- + 2H^+ \rightarrow C_2H_4 \quad (1)$$

This reaction subsequently became the most commonly used assay for nitrogenase both *in vivo* and *in vitro*. Figure 7 shows the pH profile of C_2H_2 reduction compared to that of H_2 evolution under Ar. The entire profile is now shifted in the acid direction. In the high pH region, the data are indistinguishable from the data shown in Figure 6 for H_2 evolution under CO. Figure 7 further shows however that, unlike N_2 or CO, C_2H_2 also appears to cause a shift of ca. 0.4 pH unit in the acid direction at pH values below the optimum.

DISCUSSION

With the exceptions of the NO_2^- and N_3^- cases, all nitrogenase substrate reductions incorporate an equal number of protons and electrons (Burgess, 1985). Currently no information is available concerning how protons are transferred from the solvent through the protein matrix to the FeMo cofactor site, which is now known to be buried ca. 10 Å from the surface of the protein (Kim & Rees, 1992a,b). In addition, there is no information available concerning where those protons residue at the FeMo cofactor site prior to their transfer to substrate. FeMo cofactor is a metal cluster of stoichiometry $MoFe_7S_9^{2-}$ that has an endogenous organic component, homocitrate, and that is attached to the protein by one cysteine and one histidine ligand (Kim & Rees, 1992a,b). Thus, in addition to the possibility of metal hydrides (Thorneley & Lowe, 1985), the bridging sulfide atoms on FeMo cofactor could be protonated directly, or the protonation could involve homocitrate, the protein ligands on FeMo cofactor, or nearby amino acid residues.

The pH variation of kinetic parameters is often used to gain information about the involvement of acid–base groups in enzymatic reactions. Unfortunately such studies of nitrogenase are greatly complicated by the fact that, in addition to active-site components, several other components of the assay system are likely to be pH dependent. These include the interaction between the Fe protein and the MoFe protein, MgATP hydrolysis by nitrogenase (Imam & Eady, 1980), and the creatine phosphate–creatine phosphokinase ATP generating system as well as the reductant, dithionite. Consequently, the main use of pH variability in nitrogenase mechanistic studies has been to examine substrates (e.g. cyanide (Li et al., 1982) and azide (Dilworth & Thorneley, 1981; Rubinson et al., 1985) that exist in two forms depending upon the pH. These studies by necessity have focused on a relatively narrow pH range where overall nitrogenase activity shows little change with pH.

As described under Experimental Procedures, we have now developed and optimized a mixed buffer system, composed of 75 mM Bis-Tris, 38 mM HEPPS, and 38 mM CHES, that is compatible with nitrogenase and the creatine phosphate–creatine phosphokinase ATP generating system and with the methods available for product analysis. This system eliminates variables of buffer type, ionic strength, ATP availability, and dithionite availability while allowing pH-dependent substrate reduction studies to be carried out in the range pH 5.0–9.8. Here we report pH studies of the two physiological reactions, H_2 evolution and N_2 reduction, as well as studies of C_2H_2 reduction and CO inhibition.

Hydrogen Evolution under Ar. In the absence of added reducible substrate (e.g. N_2), nitrogenase catalyzes an ATP-dependent H_2-evolution reaction (Bulen et al., 1965). Studies of the ratios of H_2:HD:D_2 evolved from H_2O–D_2O mixtures (Jackson et al., 1968) demonstrate that hydronium ions are the ultimate source of this hydrogen, as shown in eq 2.

$$2H_3O^+ + 2e^- \rightarrow H_2 + 2H_2O \quad (2)$$

At present, the chemical mechanism of H_2 evolution by nitrogenase in unknown, but one possibility is that a metal hydride or dihydride is involved which can produce H_2 either by reaction with protons (e.g., eq 3) or by reductive elimination (e.g. eq 4). It has also been suggested that E–H could represent

$$E + H^+ + 2e^- \longrightarrow E\text{–}H \xrightarrow{+H^+} E + H_2 \quad (3)$$

$$E^{2-} + 2H^+ + 2e^- \longrightarrow E\begin{matrix}H\\H\end{matrix} \longrightarrow E^{2-} + H_2 \quad (4)$$

a protonated ligand of a reduced metal site on the enzyme which could, by a coupled proton–electron transfer process, react in a manner stoichiometrically equivalent to that of a hydride (Stiefel, 1973).

Figure 1 shows the pH profile for H_2 evolution by *A. vinelandii* nitrogenase in the absence of added reducible substrate, and Figure 2 shows the results from preincubation studies performed under the same conditions. The latter were performed to determine if the loss of activity observed in Figure 1 above and below pH 7.5 was due to irreversible inactivation of some component of the assay system. The data in Figure 2 clearly show that activity losses in the pH range 6.2–8.65 are not due to irreversible inactivation of any component of the system. Some of the decrease in activity above and below this pH range can be attributed to inactivation of the MoFe protein.

The steepness of inactivation in the region pH 8.75–8.9 (Figure 2) suggests that there is a critical group on the MoFe protein with a pK in that range whose deprotonation leads either to cluster destruction or to an irreversible change in the structure of some critical part of the protein. The inactivation observed below pH 6.2 is less steep and could represent another protonation-induced irreversible change in structure or the loss of acid-labile sulfide from the P-clusters. It is unlikely, however, to arise from destruction of the FeMo cofactor, because that cluster is known to be stable for 3 min at much lower pH values (Smith & Eady, 1992; Burgess, 1990; Newton, 1992).

The preincubation experiments shown in Figure 2 establish that the 70% loss of activity between pH 7.5 and 6.2, and the 30% loss of activity between pH 7.5 and 8.65, observed in Figure 1, cannot be attributed to irreversible inactivation of the enzyme. The simplest explanation for these losses of activity is that there is a group on the MoFe protein with a pK of ca. 6.3 that must be deprotonated for the H_2 evolution reaction to occur and that there is a group on the MoFe protein with a pK of ca. 9.0 that must be protonated for H_2 evolution to occur. That there are two groups and that they are at or near the active, FeMo cofactor, site of the MoFe protein are evidenced by the fact that both pK's can be individually perturbed by addition of nitrogenase substrates and inhibitors (see below).

Nitrogen Reduction and H_2 Evolution under N_2. Because the rate-limiting step in nitrogenase turnover occurs prior to substrate reduction (Thorneley & Lowe, 1985), the addition of a reducible substrate to the complete nitrogenase system does not generally alter the rate of electron flow through the enzyme. Rather, the same number of electrons become distributed between the H_2 evolution reaction and the substrate reduction reaction. In the case of the physiological substrate N_2, under optimal conditions, the H_2 evolution reaction continues to consume ca. 25% of the electrons in the presence of saturating N_2, with the remainder being used for the $6e^-$ reduction of N_2 to $2NH_4^+$ (Yates, 1992; Thorneley & Lowe, 1985; Burgess, 1985; Simpson & Burris, 1984). As for the H_2 evolution reaction, the mechanism of protonation of N_2 reduction intermediates has not been established. It has been suggested that a metal atom(s) in FeMo cofactor could be protonated to form a metal hydride species which is then transferred to substrate (Thorneley & Lowe, 1985) or that a protonated ligand of the reduced metal site could serve as the proton donor during N_2 reduction (Stiefel, 1973). A bridging sulfide atom(s), an amino acid R group at the active site not directly ligated to the metal cluster, or homocitrate could presumably also serve as a proton donor.

Figure 3 shows the pH dependence of N_2 reduction to ammonia and of H_2 evolution under N_2 while Figure 4 shows the distribution of electrons to the two products as a function of pH. These data show that N_2 reduction falls off more steeply at both high and especially low pH, and thus N_2 reduction appears to be more sensitive to the required protonation/deprotonation of the critical groups than does H_2 evolution.

In previous work, a number of hypotheses have been put forward to explain the observation that, even under optimal conditions, H_2 evolution cannot be eliminated during N_2 reduction (Burgess, 1985, and references therein). Some of these require a minimum stoichiometry of one H_2 evolved per N_2 fixed, and some do not. For example, it is possible that H_2 evolution under N_2 and H_2 evolution under Ar occur by the same mechanism and that the residual H_2 evolution observed under N_2 represents either a leakage of electrons at a more oxidized state of the enzyme or a simple competition for electrons and protons at a highly reduced state of the enzyme (Thorneley & Lowe, 1985; Wherland et al., 1981; Guth & Burris, 1983). Another suggestion is that the two H_2-evolution reactions occur via different mechanisms, with H_2 evolution under N_2 occurring by an obligatory displacement of a metal dihydride by N_2 (Thorneley & Lowe, 1985; Newton et al., 1976). Figure 5 compares the pH profiles of H_2 evolution under Ar and H_2 evolution under N_2. Within the errors of these measurements there are no obvious differences in the pH profiles of the two reactions. Thus, it appears likely that both depend upon the protonation of a group with p$K \approx 9.0$ and the deprotonation of a group with p$K \approx 6.3$ and that N_2 does not significantly alter these pK values. It is therefore not necessary to evoke two distinct mechanisms for H_2 evolution in order to explain these results.

H_2 Evolution under CO. Although CO is a potent noncompetitive inhibitor of N_2 reduction, it has not been reported to inhibit H_2 evolution by native nitrogenase (Hardy, 1979; Burris, 1979; Huang et al., 1973). Figure 6 shows that the pH profiles of H_2 evolution under Ar and H_2 evolution under CO are indistinguishable from each other below the optimal pH of 7.5, indicating that both depend on the deprotonation of a group with p$K \approx 6.3$. In contrast, the profile above pH 7.5 obtained under CO is greatly shifted in the acid direction relative to the profile under Ar. One possible explanation for this result would be if the H_2 evolution under CO occurs via a mechanism different from that of H_2 evolution under Ar, relying on the protonation of a different group with a p$K \approx 8.5$. Another simpler possibility, however, is that CO binding to FeMo cofactor causes a 0.5 pH unit shift of the pK of this critical group from ca. pH 9 to pH 8.5.

Another way to describe the data in Figure 6 would be to say that CO inhibits H_2 evolution by native nitrogenase at high pH. CO inhibition of H_2 evolution has not previously been reported for native nitrogenase; however, it was recently observed in some cases where the FeMo cofactor site of the enzyme was perturbed either by alteration of the homocitrate ligand (McLean & Dixon, 1981) or by site-directed mutagenesis of nearby residues (Scott et al., 1992). For example, *Nif*V$^-$ mutants, which have citrate substituted for homocitrate (Liang et al., 1990), exhibit substantial CO inhibition of H_2 evolution (McLean & Dixon, 1981). The pH dependence of that reaction has been reported (McLean et al., 1983). In that case, although CO inhibits H_2 evolution throughout the pH range, as shown for the native protein in Figure 6, CO is a much better inhibitor of H_2 evolution by the citrate-substituted enzyme at high pH, again causing a shift of the pH profile toward acid pH (McLean et al., 1983). It would be interesting to known if the substitution of citrate for homocitrate shifted the pH profile of the H_2 evolution under Ar reaction. Unfortunately, there are conflicting data in the literature on this point. For example, one group that observed no difference in the pH profiles of the native versus the *nif*V$^-$ proteins from *Klebsiella pneumonia* also reported only a very small decrease in activity for either enzyme in the pH range 7–8.5 (Liang et al., 1990). In contrast, a second group that also studied the *nif*V$^-$ protein from *K. pneumonia*, but not the native protein, reported an 80% decrease in activity between pH 7 and 8.5 (McLean et al., 1983). Thus, examining the possibility that the pK of an essential group is shifted in the acid direction in an additive fashion by both CO and specific substitutions at homocitrate must await further investigation.

CO inhibition of H_2 evolution has also been reported to occur for a site-directed mutant variant of *A. vinelandii* nitrogenase that has a homocitrate-containing FeMo cofactor (Scott et al., 1992). The substitution of α subunit glutamine 191, which interacts with homocitrate in the native protein (Kim & Rees, 1992a), by a lysine results in a protein that exhibits CO inhibition of H_2 evolution at neutral pH. The pH dependence of this reaction has unfortunately not yet been reported.

C_2H_2 Reduction. C_2H_2 reduction is the most commonly used assay for nitrogenase both *in vivo* and *in vitro*. Figure 7 shows that the entire profile of C_2H_2 reduction is shifted in the acid direction compares to that of H_2 evolution under Ar. In the high-pH region these data are indistinguishable from the data shown in Figure 6 for H_2 evolution under CO. Again, one possible explanation for this result would be that C_2H_2 reduction occurs via a protonation mechanism different from that of H_2 evolution under Ar, relying on the protonation of a different group with p$K \approx 8.5$. However, a much simpler possibility is that C_2H_2 binding to the FeMo cofactor causes a 0.5 pH unit shift of the pK of this critical group from ca. pH 9 to pH 8.5. In this case, the same shift occurs for CO and C_2H_2 binding but not for N_2 binding.

These data are further consistent with conclusions drawn from an early study of nitrogenase which monitored the $S = {}^3/_2$ electron paramagnetic resonance (EPR) signal exhibited by the FeMo cofactor site of the MoFe protein as a function of pH and substrate (Smith et al., 1973). That study found that there was a slight change in the shape of the EPR signal as a function of pH, with a pK for the *K. pneumonia* protein of 8.8. That pK was shifted 0.5 pH unit in the acid direction by addition of C_2H_2, and this is likely to be correlation with the activity data presented here.

Possible Groups Being Titrated. The data described above establish that the 30% loss of activity between pH 7.5 and 8.65 and the 70% loss of activity between pH 7.5 and 6.2 are not due to irreversible inactivation of the enzyme. These data are consistent with the view that there is a group on the MoFe protein with a pK of ca. 6.3 that must be deprotonated for substrate reduction to occur and a group with a pK of ca. 9.0 that must protonated for substrate reduction to occur. The p$K \approx 6.3$ is not perturbated by addition of N_2 or CO but is shifted to ca. pK 5.9 in the presence of C_2H_2. The p$k \approx 9.0$ is not affected by addition of N_2 but appears to be shifted to ca. pK 8.5 by both CO and C_2H_2. The substrate dependence of these pK's strongly suggests that the groups being titrated are located at or near the FeMo cofactor site of the MoFe protein.

Recent studies of much simpler [Fe-S] proteins called ferredoxins, have shown that $[3Fe\text{-}4S]^+$ cluster reduction, which increases the negative charge on the cluster, may be facilitated by protonation of the cluster itself with pK's in the physiological range (George et al., 1984; Stephens et al., 1991; our unpublished results). In this case, since the protons may reside on sulfide atoms in the cluster, the pK of this process is likely to vary with cluster type and oxidation state. Certainly direct protonation of the cluster is one possibility for the p$K \sim 9.0$ group identified here. If the p$K \sim 9.0$ group, however, is an amino acid residue, possibilities include strictly conserved α subunit Cys275 and α subunit Ser278, which is hydrogen-bonded to the Sγ of Cys α275 (Kim & Rees, 1992a). Cys275 is a ligand on one of the Fe atoms in FeMo cofactor (Kim & Rees, 1992a,b). There are no other cysteine residues in the vicinity of the FeMo cofactor.

Two general types of mechanisms have been proposed for proton transfers from solvent to the buried active sites of metalloproteins that involved protonatable groups (Okamura & Feher, 1992; Nagle & Morowitz, 1978; Deamer & Nicholas, 1989; Warshel et al., 1989). In one cases, the protons are proposed to hop from one base to another in a sequential manner whereas, in the other case, they are proposed to be conducted in a concerted fashion through a chain of hydrogen-bonded proton donor groups. In this case, the p$K \sim 6.3$ group could be one of the carboxylates of homocitrate, or strictly conserved α subunit residue His195, which has already been shown to be required for N_2 reduction (Scott et al., 1992). In either case, the deprotonated group could form part of a hydrogen-bonding network required for the efficient translation of protons to the active site. Future studies of the pH dependence of substrate reduction by site-directed mutant variants of the MoFe protein using the system reported here, combined with further refinement of the X-ray structure, should establish the nature of the groups involved in proton transfer to the FeMo cofactor site and in protonation of the substrate reduction intermediates.

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