Citrate Substitutes for Homocitrate in Nitrogenase of a *nifV* Mutant of *Klebsiella pneumoniae*†

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ABSTRACT: An organic acid extracted from purified dinitrogenase isolated from a *nifV* mutant of *Klebsiella pneumoniae* has been identified as citric acid. H_2 evolution by the citrate-containing dinitrogenase is partially inhibited by CO, and by some substrates for nitrogenase. The response of maximum velocities to changes in pH for both the wild-type and the $NifV^-$ dinitrogenase was compared. No substantial differences between the enzymes were observed, but there are minor differences. Both enzymes are stable in the pH range 4.8–10, but the enzyme activities dropped dramatically below pH 6.2.

Biological nitrogen fixation is conducted by a number of free-living bacteria or bacteria in symbiotic association with plants. Nitrogenase, isolated from any N_2-fixing bacteria, is the enzyme catalyzing the reduction of N_2 to ammonium. Nitrogenase is composed of two proteins, dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein) (Hageman & Burris, 1978). Dinitrogenase reductase is a dimer of two identical subunits that bridge an Fe_4S_4 cluster (Hausinger & Howard, 1984). Dinitrogenase reductase transfers one electron at a time to dinitrogenase in a MgATP-dependent process. Reduced dinitrogenase catalyzes the reduction of substrates. Dinitrogenase is an $\alpha_2\beta_2$ tetramer, and it carries a unique prosthetic group [iron–molybdenum cofactor (FeMoco)] that contains Fe, Mo, and S (Shah & Brill, 1977). Recently, homocitrate has been demonstrated to be a component of FeMoco (Hoover et al., 1989).

In addition to N_2, nitrogenase also reduces other low molecular weight compounds, e.g., C_2H_2, N_2O, NaN_3, NaCN, CH_3NC, cyclopropene, and H^+ (Burgess, 1985). All the substrates compete for the same electron pool; therefore, they are mutually inhibitory, and all the reactions catalyzed by nitrogenase, with the exception of H_2 evolution, are inhibited by CO (Rivera-Ortiz & Burris, 1975).

At least 17 genes are required for biological nitrogen fixation in *Klebsiella pneumoniae* (Postgate, 1982); 6 of them (*nifQ*, *nifB*, *nifV*, *nifN*, *nifE*, and *nifH*) are involved in the biosynthesis of FeMoco (Hoover et al., 1988c). The *nifQ* gene product is apparently required for early steps in the processing of Mo for FeMoco synthesis (Imperial et al., 1984). Strains of *K. pneumoniae* with mutations in *nifB*, *nifN*, and *nifE* accumulate a dinitrogenase lacking FeMoco that can be activated in vitro with purified FeMoco (Hoover et al., 1988c; Shah & Brill, 1977).

Dinitrogenase from *nifV* mutants of *K. pneumoniae* differs from the wild-type enzyme in its substrate specificity and inhibitor susceptibility. It reduces N_2 poorly (Liang & Burris, 1989; McLean & Dixon, 1981; McLean et al., 1983). This is evidenced by the low V_{max} and high K_m for N_2 (Liang & Burris, 1989). Extrapolation of the experimental data indicated that at infinitely high pN_2, only about 50% of the total electron flux through the enzyme would be allocated to reduction of N_2 and that the rest of the electrons would be dissipated in H_2 evolution. The K_m for N_2, which was estimated at 24 kPa, is twice the K_m of the wild-type enzyme (12 kPa N_2). Therefore, under atmospheric N_2, *nifV* mutants fix less N_2 than the wild-type cells. The mutant enzyme also exhibits lower activity for N_2O reduction (Liang & Burris, 1989). Much lower rates were observed for HD formation than by the wild-type enzyme either with partially purified or homogeneous dinitrogenase from the *nifV* mutant of *K. pneumoniae* (Hoover et al., 1988a,b; Liang & Burris, 1989). The $NifV^-$ dinitrogenase is more effective at reducing HCN than is wild-type dinitrogenase (Liang & Smith, 1984). Reduction of cyclopropene by $NifV^-$ dinitrogenase gave a product ratio of 1/1.4 (cyclopropane/propene) which differs from the ratio of 1/2 of the wild-type enzyme (Gemoets et al., 1989).

Efforts have been made to determine the differences between the $NifV^-$ and wild-type dinitrogenases. Electron paramagnetic resonance (EPR) (Eidsness et al., 1986; McLean et al., 1987), Mossbauer (McLean et al., 1985), low-temperature magnetic circular dichroism (Smith et al., 1985), and Mo-extended X-ray absorption fine structure (EXAFS) (Eidsness et al., 1986; McLean et al., 1985) techniques did not reveal any difference. ^{57}Fe, ^{1}H, and ^{95}Mo electron nuclear double resonance (ENDOR) (McLean et al., 1987) studies showed that the molybdenum site is perturbed in the $NifV^-$ dinitrogenase, possibly because of an alteration of the non-sulfur ligands at molybdenum. When the FeMoco extracted from dinitrogenase of a *nifV* mutant combined with apodinitrogenase (the enzyme without FeMoco), the resultant holodinitrogenase had the substrate-reducing properties of the enzyme from the *nifV* mutant, indicating that the defect in *nifV* mutants is in the FeMoco (Hawkes et al., 1984).

Homocitrate has been identified as a component of FeMoco in dinitrogenase (Hoover et al., 1989). N_2-fixing cells of wild-type *K. pneumoniae* produce and accumulate homocitrate, but *nifV* mutant cells lack this organic acid (Hoover et al., 1986). Addition of homocitrate to the medium of a *nifV* mutant reversed the phenotype; i.e., dinitrogenase from such cells exhibited the substrate-reducing properties of the wild-type enzyme (Hoover et al., 1988a). When citrate was substituted for homocritrate in the in vitro FeMoco synthesis system, the resulting dinitrogenase had the substrate reduction properties of $NifV^-$ dinitrogenase (Hoover et al., 1988a), suggesting that in the absence of homocitrate, *nifV* mutants

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incorporate citrate into FeMoco. Here we report the extraction of the organic acid from NifV$^-$ dinitrogenase and its identification as citric acid.

Materials and Methods

Growth of Bacteria and Purification of Nitrogenase. *K. pneumoniae* mutant strain UN1991 (*nifV4945*) (MacNeil et al., 1978) was grown at 30 °C in a 300-L fermenter as described (Imperial et al., 1984; MacNeil et al., 1978). Cells were harvested by centrifugation under N_2 and stored in liquid N_2. The cells were broken in a French pressure cell, and the nitrogenase proteins were purified by a published method except that both component proteins were finally purified by preparative polyacrylamide gel electrophoresis (Hageman & Burris, 1980). The purified proteins had specific activities of 1037 and 639 nmol of ethylene produced/(min·mg) for dinitrogenase and dinitrogenase reductase, respectively. The dinitrogenase from wild-type *K. pneumoniae* was a generous gift from Dr. Robert Lowery.

Nitrogenase Assays. The nitrogenase reactions were performed in 5-mL bottles fitted with vaccine stoppers and filled with experimental gaseous substrates or inhibitors to the desired pressures. Each reaction bottle held 0.5 mL of enzyme reaction mixture which contained the following in 1 mL: 5.9 μmol of ATP (Sigma Chemical Co.), 15.4 μmol of magnesium acetate (Matheson Coleman and Bell), 27.8 μmol of creatine phosphate (United States Biochemical Co.), 0.1 mg of creatine phosphokinase (EC 2.7.3.2; Sigma Chemical Co.), 20 μmol of $Na_2S_2O_4$ (Sigma Chemical Co.), 50 μmol of Pipes (Sigma Chemical Co.) adjusted to pH 6.8, 53 μg of dinitrogenase, and 229 μg of dinitrogenase reductase. Reactions were initiated by addition of MgATP and were run at 30 °C with shaking for the time indicated in figure legends. Addition of 80 μL of 25% trichloroacetic acid terminated the reactions.

H_2 was determined in a 0.5-mL gas sample by gas chromatography with a thermal conductivity detector and a column of molecular sieve 5 Å. Argon was used as carrier gas. The column temperature was 50 °C. The instrument was calibrated with known amounts of H_2 in argon.

Variation in pH from 4.0 to 10.0 was achieved with a mixed buffer system that contained 25 mM each of the following: succinic acid, MES [2-(*N*-morpholino)ethanesulfonic acid], MOPS (morpholinopropanesulfonic acid), glycylglycine, and boric acid. In pH stability experiments, dinitrogenase and dinitrogenase reductase (in a ratio of 1/13) were first incubated in 0.1 mL of buffer at various pH values for 10 min; then 0.5 mL of the same buffer at pH 6.9 (containing 12 mg of ATP and 3.2 mg of dithionite) was added to the incubation mixture to start the enzymatic reaction. The pH of the reaction solution was brought to the range of 6.5–7.2 by the addition of pH 6.9 buffer. In experiments to examine variation of enzyme activity with pH, the assays simply were performed in buffers of various pH.

Isolation and Purification of Citrate from Dinitrogenase. Organic acid was extracted from NifV$^-$ dinitrogenase as described previously (Hoover et al., 1989). In brief, about 11 mL of dinitrogenase (10 mg/mL) which was in 20 mM Tris buffer (pH 7.4 and containing 300 mM NaCl) was diluted to 50 mL with 10 mM phosphate buffer (pH 7.4; contained 1 mM dithionite). The diluted enzyme then was loaded onto a 2 × 10 cm DE52 column that had been washed and equilibrated with phosphate buffer. The column was washed with 200 mL of the same phosphate buffer. The enzyme was eluted with the same buffer containing 300 mM NaCl. All operations were conducted anaerobically. The enzyme solution was added dropwise with stirring to 200 mL of acetone containing 1.5 mL of 4 N HCl at room temperature. The mixture was stirred for an additional 30 min. Precipitated protein was removed by filtration, and the filtrate was evaporated to dryness with a rotary evaporator under vacuum at 50 °C. The residue was dissolved in 2 mL of distilled water, the pH was adjusted to 8.0 with NaOH, and the resultant mixture was purified on an AG1-X8 formate ion-exchange column (0.75 × 8 cm). The column was eluted in three successive 6-mL portions of 0.25, 2, and 6 N formic acid. The eluted fractions were evaporated to dryness, dissolved in 1 mL of distilled water, and used in citrate analysis.

Table I: H_2 Evolution by NifV$^-$ Nitrogenase and Its Inhibition[a]

gas phase	nmol of H_2/(mg·min)	% activity
Ar	1369	100
Ar + CO	718	52
C_2H_2	202	15
C_2H_2 + CO	585	43
N_2	812	59
N_2 + CO	574	42
N_2O	372	27
N_2O + CO	779	57

[a] Experiments were performed as described under Materials and Methods, under 150 kPa substrate gas and 5 kPa CO.

^1H NMR Study. The fractions (including the first flow-through) eluted from the AG1-X8 column were evaporated to dryness, redissolved in D_2O, and analyzed with a Bruker AM500 spectrometer operating at 500.13-MHz ^1H frequency.

Enzymatic Assay for Citrate. The citrate assay kit used (Boehringer-Mannheim Biochemicals, Indianapolis, IN) consists of two solutions. Solution 1 contains, in 1 mL, 11.3 units of malate dehydrogenase, 23.3 units of L-lactate dehydrogenase, and 0.5 mg of NADH; solution 2 contains 1 unit of citrate lyase/mL. The pH of the samples was adjusted to 7.8 with NaOH. The assay was conducted in 1-mL cuvettes with a light path of 1 cm. OD_{340} was measured on a Beckman 40536 quartz spectrophotometer. After addition of 0.3 mL of solution 1 and 0–0.4 mL of sample solution, glass-distilled water was added to make up to 0.99 mL. The cuvette was covered with a piece of parafilm, and the contents were mixed by gentle inversion. OD_{340} was recorded when a steady reading was established. Then 0.01 mL of solution 2 was added to the cuvette followed by gentle mixing; a second OD_{340} reading was recorded after 3–5 min. The difference between the two readings was used to calculate the concentration of citrate in solution. A standard curve was prepared with authentic citric acid provided with the assay kit.

Results and Discussion

Dinitrogenase purified from a *nifV* mutant of *K. pneumoniae* (UN1991) was tested for H_2 evolution and inhibition of H_2 evolution by other substrates of nitrogenase and by CO. Table I summarizes the results. The activity of H_2 evolution is comparable to that of the wild-type enzyme. It is obvious that H_2 evolution is inhibited by CO. All substrates tested in this experiment inhibited H_2 evolution. Note that 150 kPa N_2O reduced H_2 evolution to 27% of that in the absence of N_2O. This value appears different from that we reported previously (Liang & Burris, 1989). This discrepancy is attributed to the difference in component protein ratios. In this study, a ratio of 13/1 of dinitrogenase reductase to dinitrogenase was used instead of 6/1 as in the previous work. Since a high component ratio favors N_2O reduction, fewer electrons were allocated to H_2 in this than in the previous study.

In contrast to the wild-type enzyme, in which CO completely reverses substrate inhibition of H_2 evolution, H_2 evolution by

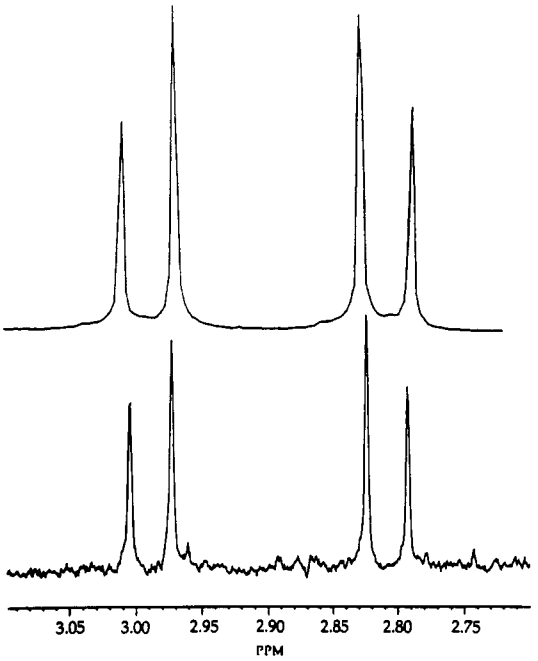

FIGURE 1: Proton NMR spectra of commercially obtained citric acid (top) and citric acid isolated from dinitrogenase of a *nifV* mutant of *K. pneumoniae* (bottom). Spectra were recorded as described under Materials and Methods on a Bruker AM500 spectrometer operating at 500.13-MHz ^1H frequency.

NifV⁻ nitrogenase was only partially restored by CO from substrate inhibitions by C_2H_2 and N_2O. With N_2 as substrate, addition of 5% CO increased the inhibition of H_2 evolution.

Homocitrate has been purified from dinitrogenase of *K. pneumoniae* (Hoover et al., 1989). It seemed very likely that there is an organic acid associated with NifV⁻ dinitrogenase, because the purified enzyme had a specific activity of 1369 nmol of H_2 produced (mg of dinitrogenase)$^{-1}$ min^{-1}, whereas no activity was observed with the in vitro synthesized FeMoco in the absence of organic acid (Hoover et al., 1989). Furthermore, this organic acid had been suggested to be citric acid (Hoover et al., 1988a). We employed a method similar to that of Hoover et al. for organic acid extraction from dinitrogenase to ascertain whether citric acid was a constituent of NifV⁻ dinitrogenase. The enzyme was extracted with acidified acetone, and after removal of precipitated proteins by filtration, the extract was further purified on an AG1-X8 column. Fractions were collected including the first flow-through, and eluates with 0.25, 2.0, and 6.0 N formic acid. All these fractions were evaporated to dryness, and the residues were examined for the presence of an organic acid by proton NMR. The spectra were compared with those of homocitrate, citrate, (*R*)-citramalate, and D-malate that had been shown to support significant acetylene reduction activity following in vitro FeMoco synthesis. Nothing similar to these standard spectra was detected in the earlier fractions from the column, but the fraction eluted by 6 N formic acid exhibited two doublets that appeared with 2.8 and 3.0 ppm chemical shifts. As shown in Figure 1, the spectra obtained with the 6 N formic acid fraction and with standard citric acid appear identical. On the basis of the signal to noise ratio, about 25 μg of citrate was estimated to be present in the 6 N formic acid fraction. The samples also were analyzed for citrate with an enzyme-coupled citrate lyase assay system. Citrate lyase is very specific for citrate, and no activity was detected with 15 analogues of citrate (Moellering & Gruber, 1966). The enzyme analysis revealed that 21 μg of citric acid was contained in the 6 N formic acid fraction. The amount of citrate in the NifV⁻ dinitrogenase determined by the enzyme assay was about 12% of what was expected. The level of citrate may have been low because (a) only a fraction of the whole population of the enzyme molecules contained citrate; this is unlikely because the specific activity of the enzyme for H^+ and C_2H_2 reduction is very high; (b) conditions for citric acid extraction were not optimal, and a portion of the citric acid remained associated with the protein; (c) a large amount of iron and molybdenum was coextracted with citric acid from the protein; the citric acid then may have chelated with these ions so that NMR spectroscopy and the citrate lyase assay did not detect citrate remaining in the complexed form. It seems most probable that (c) explains the lower than expected recovery of citric acid. NMR analysis of ferric citrate did not show characteristic citric acid spectra. Citrate in the form of ferric citrate cannot be estimated by the citrate lyase assay (data not shown). The citrate lyase assay failed after addition of $FeCl_3$ to a standard citric acid solution. In a separate experiment, 50 pCi of ^{14}C-labeled citric acid was added as a tracer to partially purified NifV⁻ dinitrogenase and subjected to acidified acetone extraction, and the supernatant was evaporated to dryness. The residue was dissolved in a minimal quantity of distilled water, and the pH was adjusted to <1.0. This solution was passed through a 0.75 × 8 cm column of strong cation-exchange resin (Dowex 50X8-400). The column was eluted with 1 N HCl, and a considerable amount of color remained bound to the column. Fractions containing ^{14}C label were pooled, evaporated to dryness, and dissolved in D_2O for ^1H NMR analysis. NMR spectroscopy did not show any peaks diagnostic of citric acid; therefore, paramagnetic iron atoms still could be chelated by citrate. On this assumption, the NMR sample was acidified to pH <1.0 and passed through another Dowex 50X8-400 column (0.75 × 8 cm), this time preequilibrated in 1 N HCl. Fractions containing ^{14}C label were pooled, evaporated to dryness, and redissolved in D_2O for NMR analysis. The NMR sample was also colorless and showed the two sets of doublets characteristic of citric acid.

FeMoco that includes homocitrate as a component has been demonstrated as the site of substrate reduction (Hawkes et al., 1984; Hoover et al., 1989). Replacement of homocitrate by citrate in the NifV⁻ nitrogenase caused substantial changes in substrate specificity and inhibitor susceptibility (Hoover et al., 1988a). To elucidate the role of homocitrate in enzyme function, we have performed comparative studies on pH stabilities and pH variation of the maximum velocity of H_2 evolution by both NifV⁻ and wild-type dinitrogenases. The pH profile of the maximum velocity for an enzymatic reaction often is useful in determining the mechanism of an enzyme that carries pH-sensitive functional group(s) active in catalysis or in substrate binding (Viola & Cleland, 1978). However, a study of pH responses for nitrogenase can be particularly complicated, because the reaction system includes steps that may be pH sensitive other than the actual substrate binding and reduction at the FeMoco center. One complicating step involves the ATP regenerating system (creatine phosphate/creatine phosphokinase). To minimize variables, we have excluded the ATP regenerating mixture from the reaction system, and instead have increased the MgATP concentration to substrate levels and have shortened the reaction time to avoid the accumulation of inhibitory levels of ADP. The experiments shown in Figures 2 and 3 were conducted with dinitrogenases from the wild-type and from the *nifV* mutant of *K. pneumoniae*. A high component ratio (dinitrogenase reductase/dinitrogenase = 12/1) and high MgATP (34 mM) and high dithionite concentrations (26 mM) have been used

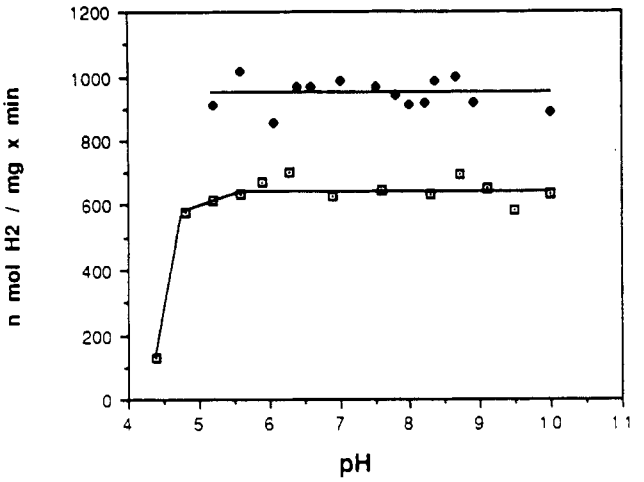

FIGURE 2: pH stabilities of wild-type (♦) and NifV⁻ (⊡) nitrogenases. Experiments were performed as described under Materials and Methods.

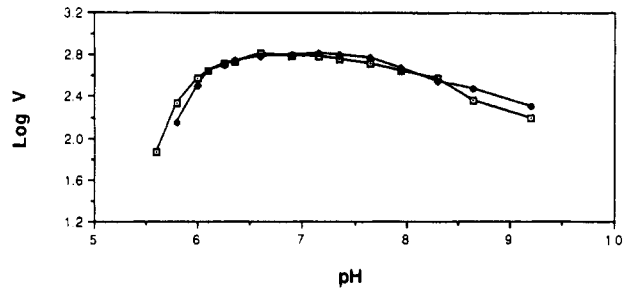

FIGURE 3: Variation with pH of maximum velocities of H_2 evolution by wild-type (♦) and NifV⁻ (⊡) nitrogenases. The reaction mixture contained a 25 mM aliquot of each of the five buffer components adjusted to different pH values, 34.3 mM ATP and equimolar magnesium acetate, and 26.3 mM dithionite. Reactions were initiated by addition of 47 μg of dinitrogenase and 138 μg of dinitrogenase reductase. Reactions ran 10 min before termination with trichloroacetic acid. The reaction pH was measured on mixtures of the same components used in the actual reaction but without the nitrogenase proteins.

to assure that the reaction proceeded at its maximum rate under the experimental conditions. Figure 2 clearly indicates that both wild-type and NifV⁻ nitrogenases are stable in the pH range 4.8–10. The enzyme activity decreased by 80% at pH 4.4, and was completely inactivated at pH 4.0. Figure 3 indicates how the maximum velocity of H_2 evolution varies with pH for both the wild-type and the mutant enzymes. For ease of comparison, the curve for the wild-type enzyme is superimposed on that of the NifV⁻ enzyme. The overall shapes of the curves are similar in spite of minor differences. For both enzymes, the effect of pH on the maximum velocity is rather unusual in the high-pH range. The slope of less than unity for decrease in V_m is difficult to explain with our present knowledge of nitrogenase. More than one factor may govern the response. In the low-pH range, the maximum velocity decreased sharply with a slope of 2 below pH 6.16 and 6.24 for the NifV⁻ and wild-type enzymes, respectively, indicating that the protonation of two groups leads to the decrease in activity. These groups could be involved in protein–protein interaction between dinitrogenase and dinitrogenase reductase, or in electron transfer between the two components with concomitant ATP hydrolysis. The groups may function in substrate binding and/or catalysis of substrate reduction. The possibility exists that one of these groups that causes a dramatic decrease of enzyme activity when protonated is derived from homocitrate in the wild-type enzyme or from citrate in

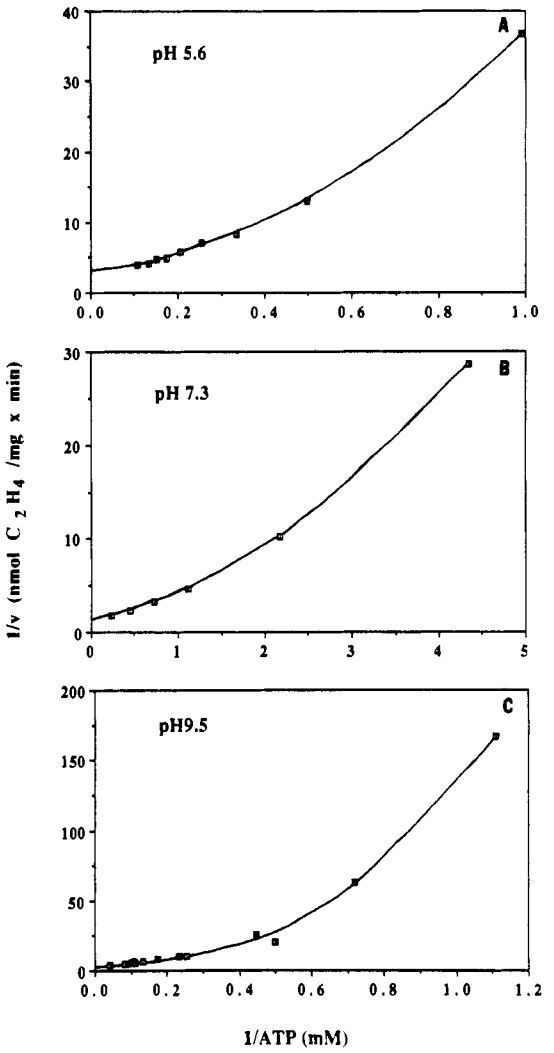

FIGURE 4: Dependence of the rate of C_2H_2 reduction by NifV⁻ nitrogenase on the concentration of ATP at different pHs. Reaction mixture contained a 25 mM sample of each of the five buffer components adjusted to pHs of 5.6, 7.3, and 9.5, 30 mM dithionite, 47 μg of NifV⁻ dinitrogenase, and 137 μg of dinitrogenase reductase. The reaction was initiated by addition of different volumes of ATP containing an equimolar amount of magnesium acetate. Reactions were stopped by addition of 25% trichloroacetic acid after 5 min.

the NifV⁻ nitrogenase. The third pK_a of citrate is 6.4, and this closely resembles the pK_a estimated for the functional group(s) on the NifV⁻ dinitrogenase.

To establish that the observed pH variation of V_m was not caused by a limitation of ATP, we determined the K_m values of NifV⁻ nitrogenase for ATP at different pH values. Figure 4A–C shows the plots of $1/v$ vs $1/[ATP]$ at three pH values. The reactions had sigmoidal dependence on MgATP concentration and supported parabolic double-reciprocal plots. Because two MgATP molecules are bound to dinitrogenase reductase (Hageman et al., 1980), the data were fitted to the equation

$$V = \frac{V_m[MgATP]^2}{K_A K_B + K_B[MgATP] + [MgATP]^2}$$

to evaluate the two binding constants (K_A and K_B) for ATP. The fitted equation gave K_A and K_B of 1.79 and 4.42 mM at pH 5.6, 0.50 and 1.75 mM at pH 7.3, and 2.90 and 6.1 mM at pH 9.5. The ATP concentration (34.4 mM) used in our experiments on the variation of V_m with pH was as high as 7.8-fold the higher K_m at pH 5.6, and 5.6-fold over the higher K_m at pH 9.5; these concentrations should be high enough to

support a valid apparent V_m. Thus, the ATP concentration apparently was not responsible for the decrease in activity at low and high pHs, and observed decreased activity must have been caused by protonation of a functional group(s) on the enzyme.

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