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THE NITROGENASE SYSTEM FROM AZOTOBACTER

ACTIVATION ENERGY AND DIVALENT CATION REQUIREMENT*

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SUMMARY

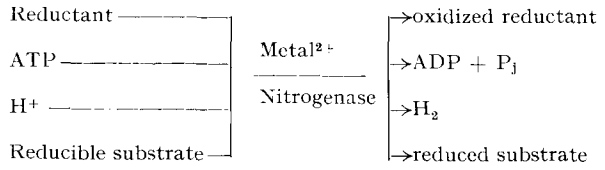
1. The Arrhenius plot for nitrogenase from *Azotobacter vinelandii* is biphasic with an inflection at about 21°; the activation energy was calculated to be 14 600 cal/mole above this temperature and 39 000 cal/mole below.

2. Values of $9 \cdot 10^{-3}$ M and $3 \cdot 10^{-4}$ M were determined for the Michaelis constants for hydrosulfite and for ATP, respectively, both above and below 21°.

3. Mn^{2+}, Co^{2+}, Fe^{2+}, and Ni^{2+} (in that order of effectiveness) replaced Mg^{2+} in supporting nitrogenase activity; Cu^{2+} and Zn^{2+} were inhibitory, while Ca^{2+} was without effect.

INTRODUCTION

Numerous reaction schemes have been presented recently to illustrate the catalytic activity of nitrogenase[1-4]. These differ in certain particulars, but all conform to the simplified formulation shown below.



Suitable reductants include hydrosulfite, reduced ferredoxin, and reduced flavodoxin; reducible substrates include N_2, N_2O, N_3^-, cyanide and other nitriles, acetylene and certain analogs, and methyl- and ethylisonitrile.

Enzyme activity appears to be absolutely dependent on ATP, reductant, and a divalent metal cation. None of the products indicated is observed in the absence

Abbreviation: TES, potassium *N*-tris (hydroxymethyl)methyl-2-aminoethane sulfonate.

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of any of these components. Because H^+ functions as an electron acceptor, forming H_2, added reducible substrates are not essential for enzyme activity. In the presence of added electron acceptors, H_2 evolution and substrate reduction occur concomitantly.

Available evidence indicates that the amount of ATP hydrolyzed per electron pair transferred is independent of the nature of the electron acceptor, but there is as yet no general agreement on the stoichiometry of ATP hydrolysis and electron transfer. Values of from two to five have been reported for the ratio of ATP hydrolyzed per electron pair transferred[4–7]. The high level of energy expenditure represented by this magnitude of ATP hydrolysis suggests that peculiarities may exist in the energetics of electron transfer by nitrogenase and has prompted an evaluation of the activation energy of the nitrogenase from *Azotobacter vinelandii*.

The requirement for a divalent metal cation by azotobacter nitrogenase was demonstrated by BURNS AND BULEN[8], who observed that nitrogenase preparations supplied hydrosulfite and substrate levels of ATP failed to evolve H_2 unless Mg^{2+} was added. Using similar reaction mixtures, HARDY AND KNIGHT[5] showed that no ATP was hydrolyzed, over and above control levels, in the absence of Mg^{2+}. They also reported that Mn^{2+} was 20% as effective as Mg^{2+} and that Ca^{2+} was inhibitory. DILWORTH *et al.*[9] examined the effect of divalent metal cations on the nitrogenase activity of preparations from *Clostridium pasteurianum*, using reaction mixtures in which ATP was supplied by an acetylphosphate acetokinase (ATP:acetate phosphotransferase, EC 2.7.2.1) generating system; in spite of the ambiguity introduced by the divalent metal cation requirement of acetokinase, they were able to conclude that Mg^{2+} was required for the ATP hydrolysis associated with nitrogenase activity. Their data also indicated that Mn^{2+}, Co^{2+}, and Fe^{2+} could replace Mg^{2+}, but were less effective.

METHODS

The culture of *A. vinelandii* O in nitrogen-free medium and the preparation of cell-free extracts using a French pressure cell were described previously[10]. Procedures for preparation of reagents, manometric analysis of H_2 evolution, and separation and colorimetric determination of ammonia were the same as used earlier[8,11]. Nitrogenase preparations were purified by a modification of the method of BULEN AND LECOMTE[6] through the polyacrylamide gel filtration step. Purified preparations were stable for 3–5 days when kept at room temperature under H_2 or argon, but lost activity upon exposure to air or overnight storage at 0°.

Hydrosulfite concentration was estimated by the method of CHARLOT AND BEZIER[12]. Inorganic phosphate released by the action of nitrogenase was determined by a modification of the method of FISKE AND SUBBAROW[13] in reaction mixtures quenched with cold trichloroacetic acid (final concentration, 10%) after first converting remaining creatine phosphate and ATP to creatine and ADP by the addition of excess glucose, creatine kinase and hexokinase. Protein was determined with the biuret reagent of GORNALL, BARDAWILL AND DAVID[14] with deoxycholate added to solubilize particulate material.

RESULTS

Activation energy of nitrogenase

The Arrhenius plot for nitrogenase over the temperature range 15–35° is shown in Fig. 1. Initial velocity values were determined as percentages of the initial velocity at 30°. At each temperature several concentrations of enzyme were assayed to verify a linear relationship between activity and enzyme concentration. The reactions were linear with time during the periods used to measure initial velocities. Activities were

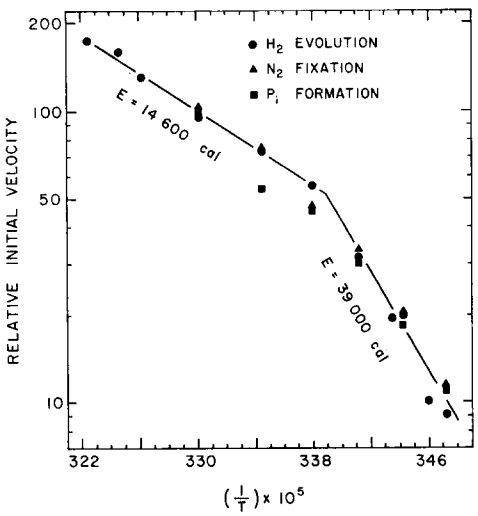


Fig. 1. Arrhenius plot of nitrogenase activities. Reaction mixtures of 2–5 ml contained (in μmoles per ml) 5 ATP, 5 $MgCl_2$, 45 creatine phosphate, 25 potassium cacodylate (pH 7.0), 20 $Na_2S_2O_4$, 0.1 mg creatine kinase, and 0.8–8.3 mg nitrogenase protein. Atmosphere: argon for measurement of H_2 evolution (●) and P_i (■); N_2 for measurement of NH_3 (▲). Enzyme preparations were stored at room temperature, but were preincubated at assay temperature for 5 min prior to assay. Activities were determined as nmoles H_2 evolved, N_2 reduced or P_i released per min per mg protein, and are expressed as percentage of activity observed at 30°.

assayed in terms of H_2 evolution, N_2 reduction, and P_i release, the former assay being used most frequently because of the ease in measuring reaction rates manometrically. Doubling the amount of creatine kinase in assays made at 16° did not affect activity, as measured by H_2 evolution.

A break in the Arrhenius plot occurs at about 21°, indicating a change in the activation energy of the enzyme at about this temperature. Activation energies, E, were calculated from the slopes of the plot of $\log_{10} v_i$ *vs.* $1/T$ according to the relationship,

$$\mathrm{d} \log_{10} v_i = \mathrm{d}\left(\frac{1}{T}\right) \frac{E}{2.303\,R}$$

From the slopes of Fig. 1, activation energy values of 14 600 cal/mole above 21° and 39 000 cal/mole below 21° were calculated.

Michaelis constants for ATP and for hydrosulfite were determined at temperatures above and below the point of inflection in the Arrhenius curve from plots of

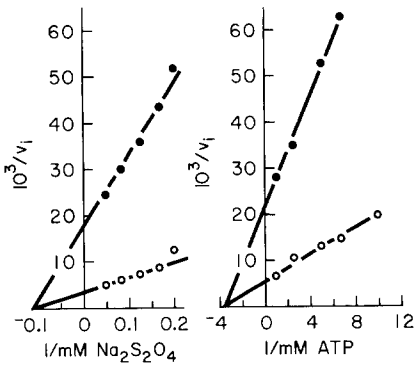


Fig. 2. Reciprocal plots for determination of Michaelis constants for $Na_2S_2O_4$ and ATP. Reaction mixtures were the same as described in Fig. 1, except that $Na_2S_2O_4$ and ATP were varied as indicated. v_i = initial velocity of H_2 evolution as nmoles H_2 per min per mg protein. Temp., 26° (○) or 16° (●).

reciprocal rate of H_2 evolution *vs.* reciprocal substrate concentration (Fig. 2). A value of about $9 \cdot 10^{-3}$ M was obtained for the K_m of hydrosulfite at both 16° and 26°. Similarly, the K_m for ATP appeared to be unaffected by whatever phenomenon causes the change in activation energy, and a value of about $3 \cdot 10^{-4}$ M was obtained for this constant at both temperatures. In determining the K_m for ATP, 0.005 M $MgCl_2$ was used over the entire ATP concentration range employed. Varying the $MgCl_2$ concentration from 0.002 to 0.010 M had no effect on activity at ATP concentrations in this range, nor did doubling the amount of creatine kinase.

A comparison of the inhibitory effects of high buffer and salt concentrations on H_2 evolution at 16° and 26° showed greater inhibition by potassium *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonate (TES) (ref. 15), cacodylate and phosphate at the lower temperature, but no apparent temperature effect on inhibition by NaCl or K_2SO_4 (Table I).

Divalent cation specificity

Of eight divalent cations tested, Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, and Ni^{2+} (in that

TABLE I

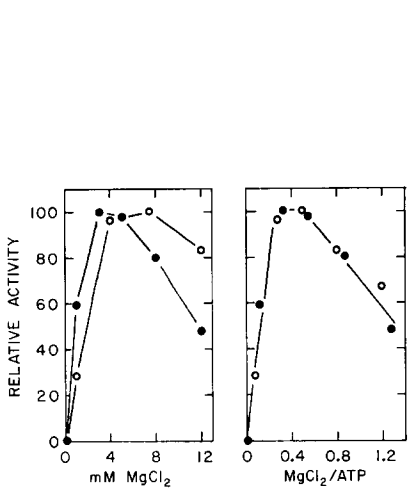
EFFECT OF TEMPERATURE ON INHIBITION OF H_2 EVOLUTION BY BUFFERS AND SALTS

Reaction mixtures were the same as described for Fig. 1 for H_2 evolution, except that NaCl and K_2SO_4 were added as indicated; TES, potassium cacodylate and potassium phosphate were added as indicated in place of 0.025 M potassium cacodylate, and buffers were at pH 7.25; incubation time was 30 min.

Compound	*Inhibition (%)*		
	16°	*26°*	*30°*
None	0	0	0
0.20 M TES	39	17	—
0.20 M cacodylate	55	17	—
0.05 M phosphate	57	44	46
0.10 M NaCl	35	31	—
0.50 M K_2SO_4	30	31	—

order of effectiveness) supported nitrogenase activity (Figs. 3, 4). No activity was observed with Ca^{2+}, Cu^{2+}, or Zn^{2+}. The results presented here are based on assays of H_2 evolution; qualitatively similar results were obtained in assays of N_2 reduction, but the small amounts of NH_3 formed under the conditions used and the high ammonia background from an apparent ADP deaminase in the enzyme preparations precluded quantitation of these data.

In this study nitrogenase was supported by substrate levels of ATP. The more conventional method of supplying ATP by means of an added phosphatide-kinase ATP generating system was not employed because of the difficulty in assessing the divalent cation effect on nitrogenase *per se* in the presence of a kinase which requires a divalent cation. As a result, the linear reaction rates and relatively high levels of product formation obtainable with an ATP generating system were not observed. As



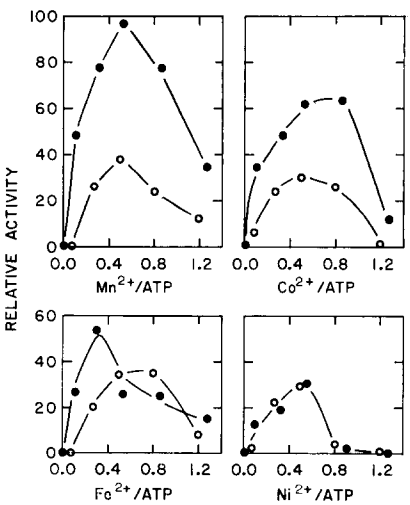


Fig. 3. Effect of $MgCl_2$ on H_2 evolution. Reaction mixtures of 2.0 ml contained (in μmoles/ml) 50 potassium cacodylate (pH 7.0), 20 $Na_2S_2O_4$, 9.3 (●) or 15 (○) ATP, Mg^{2+} added as $MgCl_2$ as indicated, and 3.6 mg nitrogenase protein. Atmosphere, argon; temp., 30°. Activities expressed as percent of maximum activity observed at each concentration of ATP.

Fig. 4. Effect of Mn^{2+}, Co^{2+}, Fe^{2+}, and Ni^{2+} on H_2 evolution. Conditions were the same as described for Fig. 3. Mn^{2+}, Co^{2+}, and Ni^{2+} were added as the chloride salts. Fe^{2+} was generated from the reduction of Fe^{3+}, as $FeCl_3$, by $Na_2S_2O_4$ prior to adding enzyme. Activities are expressed as percentages of optimal activity observed with Mg^{2+} in the presence of 9.3 mM (●) and 15 mM (○) ATP.

shown previously[8], the use of substrate levels of ATP results in nonlinear reaction rates of apparently mixed order kinetics; in addition, the amount of product obtained is reduced because of the low tolerance of nitrogenase for high levels of ATP or for the ADP which accumulates[11].

A range of concentrations of each divalent cation was tested at two levels of ATP. The results indicate that optimal enzyme activity is a function of the divalent cation:ATP ratio (M^{2+}:ATP) rather than of the absolute concentration of divalent cation. This is illustrated for Mg^{2+} in Fig. 3, which shows a different optimal Mg^{2+} concentration for each level of ATP, but an optimal Mg^{2+}:ATP ratio of about 0.5 at

both ATP concentrations. A similar relationship between M^{2+}:ATP and activity is observed with Mn^{2+}, Co^{2+}, Fe^{2+}, and Ni^{2+} (Fig. 4).

Various combinations of two active cations supported activity at levels between those observed for either cation alone, and no synergistic effects were observed. H_2 evolution supported by 1–14 mM $MgCl_2$ was unaffected by the addition of sufficient $CaCl_2$ to bring the total divalent cation concentration to 15 mM. In contrast, almost complete inhibition of H_2 evolution was observed by 1 mM $CuCl_2$ or $ZnCl_2$, even in the presence of an optimal level of $MgCl_2$.

DISCUSSION

The activation energy data obtained in this study show identical values for nitrogenase activity as measured by N_2 reduction, P_i release, and H_2 evolution. Since the latter two activities were measured in the absence of N_2, it may be concluded that the activation energy of nitrogenase does not relate directly to activation of the inert N_2 molecule, but rather is a characteristic of the catalysis of the overall concerted reaction described earlier. Because of the complexity of the nitrogenase reaction system, the activation energy values of 14 600 cal/mole and 39 000 cal/mole should be considered as maximum values for the upper and lower temperature ranges. The break in the Arrhenius plot may simply reflect this complexity, but since the factors which give rise to biphasic Arrhenius plots are difficult to assess in complex systems, the explanation for this phenomenon with nitrogenase may have to await the development of a more simplified reaction system.

The present survey of divalent cation effects on azotobacter nitrogenase activity demonstrates the non-specific nature of this requirement. In this respect nitrogenase is similar to numerous other ATP-utilizing enzymes (see ref. 16, p. 422). Nitrogenase differs from most enzymes which require a divalent metal cation with respect to its lack of response to Ca^{2+}, since this cation is generally either active or inhibitory with other enzymes, but seldom plays a neutral role. The lack of a Ca^{2+} effect reported here is not in agreement with the inhibitory role assigned to Ca^{2+} by HARDY AND KNIGHT[5] based on their observation that, in azotobacter reaction mixtures containing Ca^{2+}, ATP, and hydrosulfite, less ATP was hydrolyzed than in control reaction mixtures which lacked hydrosulfite. The ability of Mn^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+}, in addition to Mg^{2+}, to function in the azotobacter nitrogenase system indirectly supports a conclusion that the effects of Mn^{2+}, Fe^{2+}, and Co^{2+} observed by DILWORTH *et al.*[9] with clostridial nitrogenase are direct effects on nitrogenase *per se* and not on the acetokinase present in their reaction mixtures.

The optimal M^{2+}:ATP ratio of about 0.5 observed with nitrogenase supported by substrate levels of ATP is not observed with nitrogenase supported by an ATP generating system. This difference may be due to the effect of ADP, which accumulates only in the former case. Since ADP or the ADP–M^{2+} complex has been shown to be inhibitory[11], it is suggested that the optimal ratio reflects those concentrations of the various free and bound species of M^{2+}, ATP and ADP which provide the maximum favorable balance between support of the reaction by an ATP species and inhibition by an ADP species.

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