The role of Mg^{2+} and Mn^{2+} in the enzyme-catalysed activation of nitrogenase Fe protein from *Rhodospirillum rubrum*

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The activation of the Fe protein of nitrogenase (Rr2) from glutamate-grown *Rhodospirillum rubrum* by activating enzyme (AE) was investigated. AE is confirmed to have M_r about 20000 and is shown to operate catalytically. There is a role in activation for metal-ion–ATP, which can be met by either MnATP or MgATP. There is also a site of action for free metal ions. This site prefers Mn^{2+} (apparent K_d approx. 20 μM) over Mg^{2+} (apparent K_d approx. 20 mM) by a factor of 1000-fold. Non-activated Rr2 does not contain this binding site. MnATP is an inhibitor of C_2H_2 reduction, and excess Mg^{2+} inhibits both AE activity and C_2H_2 reduction, when each is studied independently under otherwise optimal conditions. The activity of AE is increased in normal reaction mixtures (in which AE activity and nitrogenase activity occur simultaneously) by Mg^{2+} concentrations in excess of ATP concentrations; this occurs because the excess Mg^{2+} prevents ATP from chelating the free Mn^{2+} necessary for optimal AE activity.

In the photosynthetic bacterium *Rhodospirillum rubrum*, nitrogenase activity is regulated in response to the availability of fixed nitrogen (reviewed by Yoch, 1978). This ability to regulate nitrogenase activity is confined to relatively few species, although regulation of *nif*-gene expression is widespread among nitrogen-fixing bacteria (reviewed by Brill, 1980).

The type of nitrogenase obtained in extracts from *R. rubrum* can be manipulated by variations in the nutritional regime. Ludden & Burris (1976) and Nordlund *et al.* (1977) discovered that inactive nitrogenase is obtained from *R. rubrum* grown on glutamate, but it can be activated by a component now generally designated as activating enzyme (AE). Carithers *et al.* (1979) found that *R. rubrum* grown on limiting NH_4^+ , in contrast with cells grown on glutamate, yields extracts containing fully active nitrogenase. The activation of nitrogenase by AE *in vitro* may be linked to the regulation of nitrogenase activity by *R. rubrum* *in vivo* in response to the availability of fixed nitrogen.

AE activates nitrogenase from glutamate-grown

cells by removing at least part of a modifying group found on Rr2_i (Ludden & Burris, 1979; Gotto & Yoch, 1982a). The fully active Rr2 from NH_4^+ -limited cells contains no part of the modifying group (Ludden *et al.*, 1982; Gotto & Yoch, 1982a), and its activity is not affected by AE. Activation of Rr2_i from glutamate-grown cells by AE is enhanced by 0.5 mM- Mn^{2+} and 20 mM- Mg^{2+} in addition to the 5 mM-MgATP that typically supports maximum nitrogenase activity (Burns & Bulen, 1966; Ludden & Burris, 1976; Zumft & Nordlund, 1981; Gotto & Yoch, 1982a). The physiological relevance of the Mn^{2+} requirement for activation is supported by the demonstration that Mn^{2+} is required for growth of *R. rubrum* on certain nitrogen sources and the consequent implication of a role for Mn^{2+} in the regulation of nitrogenase activity (Yoch, 1979). The role of excess Mg^{2+} has not been understood.

As this manuscript was in preparation, Gotto & Yoch (1982b) reported their studies on the role of Mn^{2+} and Mg^{2+} in AE activity. They concluded that Mg^{2+} in excess of the ATP concentration enhances AE activity in the presence of Mn^{2+} by competing with Mn^{2+} for the metal-ion-binding site on AE, thereby increasing the affinity of AE for Mn^{2+} .

In the present paper we explore the activation of Rr2_i by AE and examine the nature of AE and the role of each metal ion required for optimal AE activity. The conclusions reached by Gotto & Yoch (1982b) are discussed in the light of our results.

Abbreviations used: AE, activating enzyme; Rr1, MoFe protein from *R. rubrum*; Rr1_{glu}, Rr1 from glutamate-grown *R. rubrum*; Rr2_i, the inactive Fe protein from *R. rubrum* grown on glutamate (Rr2_i activated *in vitro* is distinguished from fully active Rr2 from NH_4^+ -limited cells, which is designated fully active Rr2).

Materials and methods

Preparation of nitrogenase

Rhodospirillum rubrum (A.T.C.C. 11170) was grown in batch cultures on Ormerod's medium (Ormerod *et al.*, 1961) with either 20 mM-glutamate or 2 mM-NH₄Cl as nitrogen source. Cell paste was stored in liquid N₂ until used. Nitrogenase components were purified as described by Ludden & Burris (1978), except for the following changes. Cells were broken by sonic oscillation. Rr1 was resuspended in 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH 7.5, after the precipitation with 30% poly(ethylene glycol) 4000. Rr2 was desalted by chromatography on Sephadex G-25 and then was kept in 20 mM-Hepes buffer, pH 7.5, after a final concentration step. No significant differences in behaviour were noted during purification of nitrogenase components from glutamate-grown or NH₄⁺-limited cells.

Purification of AE

The sediments from the centrifugation of broken cells at 120 000 g were resuspended in about 3 vol. of 0.5 M-NaCl in Tris/HCl buffer, pH 7.3, and subjected to a second 120 min centrifugation at 120 000 g. The supernatant from this salt wash was stored in liquid N₂ until used. Typically 20 ml of 0.5 M-NaCl wash was thawed and desalted on Sephadex G-10 into Tris buffer. The desalted AE preparation was passed over a 2.5 cm × 5 cm column of DEAE-cellulose that was washed with buffer and eluted with buffered 100 mM-NaCl. AE then was chromatographed on Sephacryl S-200; the fractions containing AE activity were combined and concentrated under Ar in an Amicon ultrafiltration apparatus fitted with a PM10 membrane. This final AE preparation was stored in liquid N₂ and was used for all studies reported.

Standard C₂H₂-reduction assay

Nitrogenase activity was measured exclusively by the C₂H₂-reduction method (Burris, 1972). Mixtures for the C₂H₂-reduction assay contained, in 1.0 ml, 5 μmol of ATP (Sigma Chemical Co.), 10 μmol of MgCl₂, 30 μmol of creatine phosphate (United States Biochemical Co.), 0.1 mg of creatine phosphokinase (EC 2.7.3.2, from Sigma Chemical Co.), 20 μmol of Na₂S₂O₄ (Sigma Chemical Co.), 50 μmol of Hepes (Research Organics), adjusted to pH 7.5, and nitrogenase proteins as indicated. Assays, run at 30°C, were initiated by addition of an anaerobic solution of ATP and were terminated by addition of 0.3 ml of 25% (w/v) trichloroacetic acid. The gas phase was 10% C₂H₂ (generated by addition of CaC₂ to water) in Ar. C₂H₄ was determined by gas chromatography.

Activation of Rr₂ *in vitro*

Purified Rr₂ (20–30 mg) was activated by incubation for 60 min with AE prepared from 20–30 g of wet cell paste in the presence of 4 mM-ATP, 5 mM-MnCl₂, 20 mM-creatine phosphate, 500 μg of creatine phosphokinase and 15 mM-Na₂S₂O₄ in 30 mM-Hepes buffer, pH 7.5 (5 ml), at 25°C. Activated Rr₂ was recovered from AE by passage of the activating mixture over a 0.6 cm × 3 cm column of DEAE-cellulose, elution of AE with buffered 100 mM-NaCl and elution of activated Rr₂ with buffered 400 mM-NaCl. Activated Rr₂ was then desalted into 20 mM-Hepes buffer, pH 7.5, to remove the ATP that eluted with Rr₂ from the DEAE-cellulose column. Typical recovery of activated Rr₂ was 70–80%, with a specific activity of 500–800 nmol of C₂H₄ produced/min per mg of protein in a standard assay.

Mn²⁺ binding

The gel-equilibration method (Hirose & Kano, 1971) adapted for use with nitrogenase proteins (Tso & Burris, 1973) was followed. In our gel-equilibration assay, 50 mg of dry Sephadex G-50 (coarse grade) was swollen for 4 h in 400 μl of 20 mM-Hepes buffer, pH 7.5, in 5 ml serum bottles. The bottles were made anaerobic before 5 mM-Na₂S₂O₄ and Rr₂ were added. A 10 min equilibration period was initiated by addition of anaerobic MnCl₂ containing about 60 000 c.p.m. of carrier-free ⁵⁴MnCl₂ (New England Nuclear) per assay, so that the final MnCl₂ concentration was 23 μM in 600 μl total volume. The equilibration was terminated by removal of two 50 μl samples of solution from the exterior of the gel with a 100 μl Hamilton syringe. The radioactivity of each sample was measured separately in a Packard Auto-Gamma 5220 scintillation spectrometer with window settings of 50 and 1000. As outlined by Tso & Burris (1973), an increase in the concentration of ligand outside the gel, caused by the addition of protein, can be used to estimate a dissociation constant between the ligand and protein.

Other procedures

Total protein was determined, after precipitation with trichloroacetic acid, by the micro biuret method of Goa (1953), with bovine serum albumin as standard. Iron as Fe₄S₄ centres in Rr₂ was determined by measuring the extent of ATP-dependent iron chelation with bathophenanthroline disulphonate as described by Ljones & Burris (1978).

Results

Purification and properties of AE

Our procedure for the purification of AE was similar to that used by Zumft & Nordlund (1981);

we obtained similar results. With 50g of wet cell paste as starting material, our final AE preparations contained roughly 1 mg of partially purified protein. This preparation contained at least ten different proteins, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. No single protein consistently appeared as the most prominent band. The preparation carried no detectable activity of either nitrogenase component. AE activity was stabilized by performing chromatography at 7°C. It was necessary to have dithionite in all buffers, because we confirmed that AE is irreversibly inactivated by O_2 (Nordlund *et al.*, 1977). We observed no stabilization of AE activity by 1 mM-dithiothreitol, but unlike Zumft & Nordlund (1981) we did not find that the addition of 0.5 mM-MnCl₂ to the buffer solutions markedly enhanced AE stability. During chromatography on Sephacryl S-200, AE showed an M_r value of about 20000, as was reported by Zumft & Nordlund (1981).

Catalytic nature of AE

Though most investigators refer to the activating component as activating enzyme, no evidence that AE acts catalytically has been presented. The following experiment shows that each molecule of AE will activate more than one molecule of Rr2_i.

We purified fully active Rr2 from NH_4^+ -limited cells and confirmed that no increase in specific activity could be obtained by treatment with AE, as was shown previously by Carithers *et al.* (1979). Our Rr2 preparations generally had a specific activity of 800–1100 nmol of C_2H_4 formed/min per mg of protein in a standard assay, and an Fe_4S_4 content of 0.45–0.65 Fe_4S_4 equivalents per molecule (60 500 Da).

In a series of otherwise standard reaction mixtures containing 25 mM-MgCl₂ and 0.5 mM-MnCl₂, 225 µg of Rr1glu was incubated with various amounts of fully active Rr2 for 10 min. Knowing the Fe_4S_4 content of this Rr2 preparation allowed us to construct a 'standard curve' of C_2H_2 -reduction activity as a function of active Rr2 (Fe_4S_4 equivalents). This activity was not linear with respect to Rr2, since Rr1 can be saturated with active Rr2.

A second set of otherwise standard reaction mixtures contained 25 mM-MgCl₂ and 0.5 mM-MnCl₂, but C_2H_2 was omitted. In one group of this second set of assay mixtures 225 µg of Rr1glu was incubated with 970 µg of Rr2_i, and in the second group 225 µg of Rr1glu was incubated with 970 µg of Rr2_i and 1.6 µg of AE preparation. After 30 min, C_2H_2 to 10% was added to both groups of this second set of assays; C_2H_2 reduction was terminated after an additional 10 min. We attributed the increased C_2H_4 produced in the second group of these reactions to the conversion of Rr2_i into active Rr2 by AE.

With the 'standard curve' obtained with Rr2 from NH_4^+ -limited cells, we estimated that 0.57 nmol of active Rr2 had been produced. (This increased the Rr2 activity about 2-fold over the background activity always found in Rr2_i preparations.) Since 0.57 nmol of pure 100%-active Rr2 would weigh 34 µg, only a small portion of this additional activity could have been due to contaminating Rr2 in 1.6 µg of AE preparation. Assuming the M_r of AE to be 20000, the maximum number of active AE molecules present in the activation mixture (1.6 µg) was 0.080 nmol. The minimum number of AE turnovers was therefore 7.1/30 min (0.57/0.080). Because this AE preparation was impure, this result should be taken primarily as evidence that AE acts catalytically and should not be used to estimate a turnover time for AE. This approach assumes that activated Rr2_i has the same intrinsic reactivity as Rr2 from NH_4^+ -limited cells. Comparisons of the different types of Rr2 by Gotto & Yoch (1982a) and by Ludden *et al.* (1982) support the validity of this assumption.

Stability of AE in the reaction mixture

The results illustrated in Fig. 1 show that activation of 100 µg of Rr2_i by 11 µg of AE preparation proceeds approximately linearly for at least 20 min. Point A (Fig. 1) establishes that AE is stable to incubation in the reaction mixture for at least 20 min. Fig. 1 also shows that the presence of Rr1glu has no effect on the activation reaction between AE and Rr2_i. Thus, if Rr2_i and Rr1glu interact at all, their interaction does not modify the rate of reaction between AE and Rr2_i.

Dependence on Mn^{2+} and Mg^{2+} when their effects on the activation reaction and C_2H_2 -reduction reaction are not distinguished

When C_2H_2 -reduction assays are initiated by adding ATP to mixtures containing Rr1, Rr2_i and AE, the reactions follow a non-linear time course of increasing slope, reflecting the time-dependent activation of Rr2_i (Munson & Burris, 1969). Ludden & Burris (1976) showed that 0.5 mM- Mn^{2+} and 25 mM- Mg^{2+} increased the rate of the activation reaction synergistically. Zumft & Nordlund (1981) found that the optimal concentration for each metal ion varied with the concentration of the other. These observations were confirmed by an experiment in which concentrations of Mg^{2+} and Mn^{2+} were varied, whereas the ATP concentration was constant, in a series of fixed-time assays (Fig. 2). We observed maximal C_2H_2 -reduction activity with 25 mM- Mg^{2+} and 0.5 mM- Mn^{2+} . In such an experiment, Mg^{2+} and Mn^{2+} exert their effects simultaneously on both the activation reaction between Rr2_i and AE, and the C_2H_2 -reduction reaction catalysed by the action of both active Rr2 and Rr1.

Fig. 1. Time course of $Rr2_i$ activation by AE

C_2H_2 -reduction assay bottles contained reagents as described for standard assays in the Materials and methods section except that they contained 25 mM- $MgCl_2$ and 0.5 mM- $MnCl_2$. Proteins were as follows: $Rr2_p$, 100 μg ; $Rr1glu$, 47.5 μg ; AE preparation, 11 μg . For one set of measurements (●) $Rr2_p$, $Rr1$ and AE were incubated in the reaction mixture for the time indicated. C_2H_2 was then added for a 5 min C_2H_2 -reduction period that was terminated with trichloroacetic acid. For a second set of measurements (○) $Rr2_i$ and AE were incubated in the reaction mixture for the time indicated. C_2H_2 and $Rr1$ then were added for a 5 min C_2H_2 -reduction period that was terminated with trichloroacetic acid. For point A (■), AE was incubated in the reaction mixture for 20 min. $Rr2_i$ then was added. After an additional 20 min, $Rr1$ and C_2H_2 were added for a 5 min C_2H_2 -reduction period.

Effects of Mg^{2+} and Mn^{2+} on the C_2H_2 -reduction reaction

Fig. 3 illustrates the effects of Mg^{2+} and Mn^{2+} on C_2H_2 reduction by nitrogenase derived from NH_4^+ -limited cells. Mg^{2+} at concentrations over 15 mM and all concentrations of Mn^{2+} tested inhibited C_2H_2 reduction by active nitrogenase. Inhibition of nitrogenase by Mg^{2+} is recognized and has been attributed to both ionic-strength effects and the formation of the inactive compound Mg_2ATP (Thorneley & Willison, 1974). We have explored the inhibition by Mn^{2+} further.

Fig. 4 shows how reduction of C_2H_2 by $Rr1glu$ and activated $Rr2_i$ (prepared from purified $Rr2_i$ as described in the Materials and methods section)

Fig. 2. Dependence of C_2H_2 reduction on Mg^{2+} and Mn^{2+} while $Rr2_i$ is being activated

Bottles for C_2H_2 -reduction assays contained reagents as described for standard assays in the Materials and methods section except that they contained $MnCl_2$ as shown and $MgCl_2$ at 5 mM (Δ), 15 mM (□), 25 mM (○) and 40 mM (●). Reaction vessels contained 83 μg of $Rr2_p$, 95 μg of $Rr1glu$ and 8 μg of AE preparation. The 15 min assays were initiated with ATP and terminated with trichloroacetic acid.

depends on $MgATP$ or $MnATP$. Identical results were obtained with crude nitrogenase from NH_4^+ -limited cells (results not shown). The apparent $K_m(MgATP)$ is about 1 mM, and inhibition is consistently observed with greater than 6 mM- $MgATP$. $MnATP$ is a much poorer substrate than is $MgATP$, and inhibition is dominant above 2 mM. That $MnATP$ is a poor substrate suggests that $MnATP$ is an inhibitor of $MgATP$ -supported C_2H_2 reduction. We found, in fact, that in the presence of 5 mM- $MgATP$ $MnATP$ inhibited fully active nitrogenase with an apparent K_i of 2 mM (not shown). Thus the inhibition of C_2H_2 reduction by Mn^{2+} (Fig. 3) is due to formation of inhibitory $MnATP$ and to the decrease in the concentration of $MgATP$ below the optimal value of 5 mM. Because the stability constant of $MnATP$ is about $10^{4.8}$ compared with $10^{4.2}$ for $MgATP$ (Sillén & Martell, 1946), the presence of $MnATP$ is significant even when Mg^{2+} is in moderate excess of ATP. Increasing the concentration of creatine phosphokinase does not relieve the inhibition of C_2H_2 -reduction activity caused by Mn^{2+} and Mg^{2+} (results not shown). This indicates

Fig. 3. Effects of Mg^{2+} and Mn^{2+} on C_2H_2 reduction by fully active nitrogenase

A 94 μg portion of Rr2 from NH_4^+ -limited cells and 47.5 μg of Rr1glu were assayed for 10 min in otherwise standard assay mixtures containing $MnCl_2$ as shown and $MgCl_2$ at 5 mM (Δ), 15 mM ($\square$), 25 mM ($\circ$) or 40 mM ($\bullet$).

that the inhibition of C_2H_2 -reduction activity shown in Fig. 3 is not caused by effects of metal ions on the ATP-regenerating system.

Effects of Mg^{2+} on the activation reaction

To observe the effects of metal ions specifically on the activating reaction, the following procedure was followed. Rr₂ first was activated during 15 min of preincubation. The preincubation mixtures contained Rr₂, AE and various concentrations of metal ions and ATP. They did not contain Rr₁ or C_2H_2 . A sample of each preincubation mixture then was diluted 4-fold into assay bottles containing Rr₁ and C_2H_2 . The contents of the assay bottles were prepared so that, after addition of the preincubation mixtures, the contents of all assay bottles were identical (except for the amount of active Rr₂). C_2H_2 -reduction reactions lasted for 5 min. We assume that the equilibria between all species were reached quickly compared with the 5 min reaction time. Thus any differences in the amounts of C_2H_2 reduced during the reaction period reflected differences in the rates of activation during the preincubation period. Note that the 5 min reaction seldom was under optimal conditions for C_2H_2 reduction, because the 4-fold dilution of the preincubation mixture usually did not decrease the concentrations of Mg^{2+} and Mn^{2+} to

Fig. 4. Dependence of activated nitrogenase on MnATP and MgATP for reduction of C_2H_2

Standard assay mixtures contained MnATP ($\circ$) or MgATP ($\bullet$) as shown with metal ions always in 1 mM excess over ATP. The 10 min reactions with 44 μg of activated Rr₂, prepared as described in the Materials and methods section and 71 μg of Rr1glu were initiated with ATP.

non-inhibitory values. Some activation often occurred during the 5 min C_2H_2 -reduction assay. This method might be improved by including EDTA in the 5 min assay to chelate Mn^{2+} and thereby inhibit AE activity.

The dependence of the activation reaction on MnATP or MgATP is illustrated in Fig. 5. MnATP alone supports AE activity more effectively than does MgATP alone. The apparent K_m (MnATP) of 0.2 mM for the activation reaction is about 5-fold lower than the apparent K_m (MnATP) of 1 mM for C_2H_2 reduction, indicating that MnATP has a greater affinity for Rr₂ than does MgATP. Note that the apparent saturation of AE activity by 1 mM-MnATP (Fig. 5) does not result from completion of activation or from the saturation of Rr₁ by active Rr₂, because a higher concentration of AE in the preincubation mixture produces a higher rate of C_2H_2 reduction by the same amount of nitrogenase (Fig. 5).

When 3 mM-MnATP and 1 mM excess Mn^{2+} are present in the preincubation mixture, all concen-

Fig. 5. Dependence of activation of $Rr2_i$ on $MnATP$ and $MgATP$

AE activity as determined by preincubation was measured as described in the Materials and methods section and elsewhere in the text. A $100\mu\text{g}$ portion of $Rr2_i$, $11\mu\text{g}$ of AE preparation and $MnATP$ (○) or $MgATP$ (●) as shown (metal ions were in 1 mM excess of ATP) were preincubated for 15 min. Then 0.3 ml of each 0.5 ml mixture was diluted 4-fold into a reaction vial containing $47\mu\text{g}$ of $Rr1glu$, 10% C_2H_2 , metal ions and ATP such that all reaction mixtures contained 1 mM- $MnCl_2$, 10 mM- $MgCl_2$ and 5 mM-ATP. Reactions were terminated after 5 min. In one assay (□), $22\mu\text{g}$ of AE preparation was included in the preincubation mixture.

trations of Mg^{2+} inhibit AE activity (Fig. 6, upper curve). In the absence of Mn^{2+} , however, high concentrations of Mg^{2+} will enhance the rate of activation (Fig. 6, lower curve), although the rate with Mg^{2+} alone never approaches the rate of activation with Mn^{2+} alone. Above 50–60 mM- Mg^{2+} , the presence of Mn^{2+} during preincubation has no effect on the rate of activation. Thus Mg^{2+} will fully substitute for Mn^{2+} in the activation reaction, but only at much greater concentrations, which are otherwise inhibitory.

$NaCl$ inhibits activation supported by Mn^{2+} and $MnATP$ (results not shown), presumably through ionic-strength effects. $NaCl$ (50 mM or 150 mM) does not increase the rate of activation when 5 mM- $MgATP$ is included in the preincubation mixture (results not shown), implying that the Mg^{2+} -enhanced activation illustrated in Fig. 6 is not attributable to an effect of ionic strength.

Thus optimal activation can be obtained with

Fig. 6. Effects of Mg^{2+} on the activation of $Rr2_i$ by AE. AE activity was measured by the preincubation method described in the Materials and methods section and in the legend to Fig. 5. Preincubation mixtures contained $150\mu\text{g}$ of $Rr2_i$, $17\mu\text{g}$ of AE preparation, 3 mM-ATP either 4 mM- $MnCl_2$ (●) or no $MnCl_2$ (○); $MgCl_2$ concentration was varied as shown. Reaction mixtures for 5 min C_2H_2 -reduction reactions contained 1 mM- $MnCl_2$, 27.5 mM- $MgCl_2$, 5 mM-ATP, $47\mu\text{g}$ of $Rr1glu$ and 10% C_2H_2 .

Mn^{2+} and ATP, and Mg^{2+} (or $NaCl$) inhibits this optimal rate. Yet, with 0.5 mM- Mn^{2+} and 5 mM-ATP, Mg^{2+} up to 25 mM significantly enhances the rate of C_2H_2 reduction (Fig. 2). Because excess Mg^{2+} inhibits the C_2H_2 -reduction reaction by active nitrogenase (Fig. 3), the enhancement of C_2H_2 reduction by excess Mg^{2+} (Fig. 2) must arise from an increase in the rate of activation. We propose that this enhancement occurs because Mg^{2+} prevents ATP from chelating Mn^{2+} , so that Mn^{2+} will then be free to interact with AE and $Rr2_i$. The excess Mg^{2+} is not necessary unless ATP is present in excess of Mn^{2+} . If Mn^{2+} is present in excess of ATP, Mg^{2+} exerts only an inhibitory effect on $Rr2_i$ activation by AE (Fig. 6).

Fig. 7 shows that, when ATP is in substantial excess over Mn^{2+} , activation is dramatically inhibited, presumably because ATP chelates the free Mn^{2+} . Mg^{2+} at 15 mM weakens, and 50 mM- Mg^{2+} abolishes, the inhibition by ATP. Thus relatively high concentrations of Mg^{2+} are needed to overcome the inhibition of AE activity by ATP. This suggests the inhibition is caused, not by free ATP itself, but by the high affinity of ATP for Mn^{2+} . It seems likely that excess Mg^{2+} both enhances the activation reaction (Fig. 2) and reverses ATP inhibition of the activation reaction (Fig. 7) by preventing the chelation of Mn^{2+} by ATP.

Fig. 7. Inhibition of AE activity by ATP and its reversal by MgCl_2

AE activity was measured by the preincubation method described in the Materials and methods section. Preincubation mixtures contained $34 \mu\text{g}$ of AE preparation, $150 \mu\text{g}$ of Rr2_1 , 2.5 mM-MnCl_2 , ATP as shown and no MgCl_2 (●), 15 mM-MgCl_2 (□) or 50 mM-MgCl_2 (○). Reaction mixtures for 5 min C_2H_2 -reduction assays contained 0.62 mM-MnCl_2 , 12.5 mM-MgCl_2 , 5 mM-ATP , 47 mg of Rr1glu and 10% C_2H_2 .

Estimation of the K_d for Mn-E and for Mg-E

We have shown that there is a role for free metal ions in the activation reaction. Their most obvious role is to facilitate the reaction between AE and Rr2_1 by binding to either or to a complex between them; metal-ion-ATP also may be part of such a complex. The dissociation constant (K_d) between Mn^{2+} and the complex, 'E', to which it binds can be estimated as follows.

Assuming 1 Mn^{2+} binds per 'E' to form $\text{E}\cdot\text{Mn}$, the species competent to carry out the activation reaction:

where

$$K_d = \frac{[\text{E}][\text{Mn}^{2+}]_{\text{free}}}{[\text{E}\cdot\text{Mn}]} \quad \text{at equilibrium.}$$

When $[\text{E}] = [\text{E}\cdot\text{Mn}]$, $[\text{Mn}^{2+}]_{\text{free}} = K_d$, assuming that $[\text{Mn}^{2+}]_{\text{free}} \gg [\text{E}]$.

Table 1. Binding of $^{54}\text{Mn}^{2+}$ by dinitrogenase reductase of *Rhodospirillum rubrum* and by other proteins

The procedure described in the Materials and methods section was followed. The 5 ml serum bottles were made anaerobic before addition of protein, except for the experiment with O_2 -inactivated protein, in which Rr2_1 was added 10 min before the vials were evacuated and made anaerobic. MnCl_2 was $23 \mu\text{M}$. Background radioactivity was 625 c.p.m. Experiments 1 and 2 were performed simultaneously, and the radioactivities of two samples from each vial were counted.

Protein added	Radioactivity counts in 2 min	
	Expt. 1	Expt. 2
None	10883	10265
	10484	10740
Rr2_1 (0.59 mg)	11232	10998
	10973	11347
O_2 -inactivated Rr2_1 (0.59 mg)	10743	11034
	10655	10888
Bovine serum albumin (0.5 mg)	11763	11379
	11348	11285
Enolase (1 mg)	10461	10372
	10469	10442
Enolase (1 mg) and 0.5 mM-2-phosphoglycerate	16715	15883
	16944	16216

Fig. 7 shows that, when $[\text{ATP}]_{\text{total}} = 4.5 \text{ mM}$ and $[\text{Mn}^{2+}]_{\text{total}} = 2.5 \text{ mM}$, activation is 50% inhibited. Under these conditions, therefore, $[\text{E}] = [\text{E}\cdot\text{Mn}]$, and $K_d = [\text{Mn}^{2+}]_{\text{free}}$. From the known binding constant of MnATP of $10^{4.8}$, $[\text{Mn}^{2+}]_{\text{free}}$, and therefore K_d , can be estimated to be $20 \mu\text{M}$.

The K_d for $\text{E}\cdot\text{Mg}$ can be estimated from Fig. 6. The upper curve of Fig. 6 can be taken to be the maximal rate of activation in the presence of various inhibitory concentrations of Mg^{2+} . The difference between the lower curve and the upper curve represents the degree of saturation of 'E' by Mg^{2+} . This difference is half-maximal at about $20\text{--}25 \text{ mM-Mg}^{2+}$. Thus the K_d of $\text{E}\cdot\text{Mg}$ can be estimated to be $20\text{--}25 \text{ mM}$, which is about 1000-fold greater than the K_d of $\text{E}\cdot\text{Mn}$.

Mn^{2+} does not bind to Rr2_1 alone

A gel-equilibration method for measuring ligand binding by proteins was used to measure the binding of Mn^{2+} to Rr2_1 (Table 1). The presence of phosphate, ribose and 'Ad' in a modifying group attached to Rr2_1 (Ludden & Burris, 1978) suggested the possibility that Rr2_1 might contain the Mn^{2+} -binding site. The data in Table 1 demonstrate that the Mn^{2+} -binding site is not on Rr2_1 . If there were a single binding site for Mn^{2+} on Rr2_1 with a K_d of $20 \mu\text{M}$, the presence of 0.59 mg of Rr2_1 in the gel-

equilibration assay would increase the ^{54}Mn concentration outside the gel by 28.7%. The observed 4% increase in ^{54}Mn concentration is consistent with the existence of a binding site for ^{54}Mn with a K_d of $340\mu\text{M}$. This binding is weaker than the 'non-specific' binding of $^{54}\text{Mn}^{2+}$ to bovine serum albumin (Table 1). One could argue that, since ATP is required for AE activity, ATP may create a Mn^{2+} -binding site on native Rr2_p . Because O_2 -inactivated Rr2_i is a substrate for AE in the presence of Mn^{2+} but in the absence of ATP (Ludden & Burris, 1979), ATP cannot be required for the formation of an Mn^{2+} -binding site on O_2 -denatured Rr2_p , if such a site exists. We therefore measured the strength of Mn^{2+} binding to O_2 -inactivated Rr2_i and found it unchanged from that measured for native Rr2_i (Table 1).

The formation of the ternary complex of Mn^{2+} , 2-phosphoglycerate and yeast enolase (Cohn, 1963; Nowak & Mildvan, 1970) was exploited as a positive control for this experiment (Table 1). The presence of $500\mu\text{M}$ -2-phosphoglycerate and 12.5nmol of enolase in the gel-equilibration assay caused the ^{54}Mn concentration outside the gel to increase by 57.4% from $23\mu\text{M}$ to $36.2\mu\text{M}$. One can calculate that, under these conditions, there were 0.68Mn^{2+} bound per enolase molecule with a K_d of $5\mu\text{M}$, which is in the expected range (Nowak *et al.*, 1973).

Discussion

We have demonstrated that AE is indeed an enzyme. Since there is a bond-breaking step during activation of Rr2_i (Ludden & Burris, 1979), there must be a net hydrolysis during the activation of Rr2_p . AE may catalyse this hydrolysis itself, or AE may produce a re-arranged species that subsequently reacts with water.

We have investigated the roles of Mg^{2+} and Mn^{2+} in the AE-catalysed activation of Rr2_i and have discerned a requirement for free metal ions that can be met by either Mn^{2+} (K_d approx. $20\mu\text{M}$) or Mg^{2+} (K_d approx. 20mM). The site of action of free metal ions is not on Rr2_i (Table 1); it is probably on AE or an AE- Rr2_i complex. Since MnATP is an effective inhibitor of nitrogenase activity, the concentration of Mn^{2+} must be kept low in reactions measuring nitrogenase activity and AE activity simultaneously. However, when the Mn^{2+} concentration is low compared with the ATP concentration, excess Mg^{2+} is necessary to prevent chelation of Mn^{2+} by ATP (Fig. 7). Using dissociation constants of $10^{-4.8}$ for MnATP and $10^{-4.2}$ for MgATP [and ignoring the ion-binding effects of creatine phosphate and buffer, and the formation of (metal ion) $_2$ -ATP complexes], one can calculate that raising the Mg^{2+} concentration from 5mM to 25mM in the presence of 0.5mM - Mn^{2+} and 5mM -ATP increases the free Mn^{2+}

concentration from $14\mu\text{M}$ to $240\mu\text{M}$. This change in the free Mn^{2+} concentration is in the general range expected to influence a reaction with a K_d for Mn^{2+} near $20\mu\text{M}$. A more reliable estimate of the K_d for Mn^{2+} -E will require either a thorough consideration of the metal-ion-binding properties of all components of nitrogenase reaction mixtures, or the isolation of enough active AE to perform binding studies.

Gotto & Yoch (1982b) have reported studies on the roles of Mg^{2+} and Mn^{2+} in the AE-catalysed activation of Rr2_p . They confirmed the work of Ludden & Burris (1976) and of Zumft & Nordlund (1981); all three groups found (a) that Mg^{2+} enhances AE activity independently of its role in forming an MgATP complex, (b) that Mn^{2+} is used preferentially over free Mg^{2+} as an effector for AE activity, and (c) that Mg^{2+} enhanced activation even in the presence of Mn^{2+} . These conclusions led Zumft & Nordlund (1981) and Gotto & Yoch (1982b) to agree with the suggestion by Ludden & Burris (1979) that metal ions complex with ATP and also bind at some other site on AE or Rr2_p .

Gotto & Yoch (1982b) measured the effects of free Mg^{2+} and total Mn^{2+} in reactions that measured AE and nitrogenase activities simultaneously. They found that a plot of $1/(\text{C}_2\text{H}_2 \text{ reduction})$ versus $1/[\text{Mn}^{2+}]$ at different Mg^{2+} concentrations yielded straight lines that intersected on the vertical axis, with increased Mg^{2+} concentrations causing greater activity. The effects of these ions on C_2H_2 -reduction activity were attributed to effects on AE activity; within the range of metal ion concentrations reported, this approximation is probably warranted. However, their conclusion that 'the observation that the K_a for Mn^{2+} decreased as the free Mg^{2+} concentration increased, and the lines intersecting a common point on the $1/v$ axis, is indicative of activation by a competitive interaction of Mn^{2+} and Mg^{2+} at a single site' needs to be assessed in light of our work. The interactions of the lines on the vertical axis means that, in the presence of saturating Mn^{2+} , up to 11.8mM -free Mg^{2+} (the maximum Mg^{2+} concentration used by them) has very little effect on AE activity. Above this concentration of Mg^{2+} , the maximum rate of activation should decrease (Fig. 6) and cause the vertical intercept of double-reciprocal plots to increase. In addition, Mn^{2+} above the 0.5mM used by Gotto & Yoch (1982b) would cause apparent substrate inhibition (Fig. 3). The different slopes Gotto & Yoch (1982b) obtained with different Mg^{2+} concentrations means that the apparent K_a for Mn^{2+} is dependent on Mg^{2+} concentration; we believe this is because Mg^{2+} increases the free Mn^{2+} concentration by competitively removing Mn^{2+} from ATP. We do not believe that Mg^{2+} increases the affinity of AE for Mn^{2+} by binding to the Mn^{2+} -binding site on AE, as suggested by Gotto & Yoch

(1982*b*). Thus Mg^{2+} and Mn^{2+} both can support AE activity, probably by reacting at the same site, but we see no evidence for the two ions interacting competitively at this site to enhance AE activity.

Gotto & Yoch (1982*b*) also concluded that Mn^{2+} and ATP in the absence of Mg^{2+} were only weak effectors of AE activity. They used 0.5 mM- Mn^{2+} and 5 mM-ATP in their experiments; under these conditions one can calculate from a K_d ($MnATP$) of $10^{-4.8}$ that only 1.7 μM free Mn^{2+} and about 0.5 mM- $MnATP$ are present. In our experiments (Figs. 5 and 6), we demonstrate that Mn^{2+} and ATP alone are capable of supporting maximal AE activity, if there is enough Mn^{2+} to allow for the presence of both substantial free Mn^{2+} and greater than 1 mM- $MnATP$.

This conclusion that Mg^{2+} and Mn^{2+} interact competitively leads Gotto & Yoch (1982*b*) to suggest that only the inhibitory effects of high concentrations of Mg^{2+} prevent maximal AE activity from being achievable with Mg^{2+} in the absence of Mn^{2+} . Our results confirm this conclusion and show that free Mg^{2+} concentrations must be roughly 1000-fold greater than those needed for AE activity with free Mn^{2+} (Fig. 6).

Fe^{2+} (Nordlund *et al.*, 1978; Yoch, 1979) and Co^{2+} (Yoch, 1979) have been reported to function in the activation reaction; they are most effective at concentrations higher than needed for Mn^{2+} (Yoch, 1979). Studies comparable with those reported in the present paper would help to distinguish the effects that these ions have on nitrogenase activity from their effects on AE activity. Such information should aid in the assessment of the physiological relevance of the observed metal-ion requirements for optimal AE activity *in vitro*.

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