Nitrogenase of *Klebsiella pneumoniae*

INHIBITION OF ACETYLENE REDUCTION BY MAGNESIUM ION EXPLAINED BY THE FORMATION OF AN INACTIVE DIMAGNESIUM-ADENOSINE TRIPHOSPHATE COMPLEX

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Acetylene-reducing activity of purified nitrogenase from *Klebsiella pneumoniae* was studied over a range of ATP and Mg^{2+} concentrations at 15°C, pH 7.8. Inhibition at Mg^{2+} concentrations of 2.5–30 mM was due to the formation of the inactive complex, Mg_2ATP . At higher Mg^{2+} concentrations an additional inhibitory effect was observed. The results were consistent with a $MgATP$ complex being the active substrate with an apparent $K_m(MgATP) = 0.4$ mM.

A bivalent metal ion and ATP are required for nitrogenase activity (Burris, 1971). The most effective metal ion is Mg^{2+} (Dilworth *et al.*, 1965; Burns, 1969; Eady *et al.*, 1972). A Fe-containing protein and a Fe Mo-containing protein comprise the nitrogenase enzyme (Burris, 1971). ATP in the presence of Mg^{2+} has been shown by e.p.r. (electron-paramagnetic-resonance) spectroscopy to interact with Kp2 protein* (Smith *et al.*, 1973), Cp2 protein (Zumft *et al.*, 1973), Cp2 and Av2 proteins (Orme-Johnson *et al.*, 1972) and Cv2 protein (Evans *et al.*, 1973). In the absence of Mg^{2+} no evidence was obtained for the binding of ATP to Cp2 protein (Zumft *et al.*, 1973). Bui & Mortenson (1968) showed that $[8-^{14}C]ATP$ and $[8-^{14}C]ADP$ bound to Cp2 protein, and Tso & Burris (1973) have determined these binding constants by a rapid gel-equilibration method. The binding of ATP changed the thiol-group reactivity of Kp2 protein (Thorneley & Eady, 1973) and the reactivity of the Fe of Cp2 protein with $\alpha\alpha$ -bipyridyl (Walker & Mortenson, 1973). These interactions have been interpreted as an $MgATP$ -induced conformation change of the protein, which changes the redox potential, in the case of Cp2 protein, from -270 to -400 mV (L. E. Mortenson, personal communication).

Thus data from a number of techniques indicate that a $MgATP$ complex is the active substrate for nitrogenase. It is therefore appropriate to consider the ATP- and Mg^{2+} -dependence of acetylene

reduction in terms of the equilibria between Mg^{2+} , ATP, $MgATP$ and Mg_2ATP . This approach may help to explain a number of observations in the literature where only total added concentrations of either Mg^{2+} or ATP or the optimum ratio of Mg^{2+}/ATP have been used as a function of acetylene-reduction activity. The present paper is concerned with the effect of high Mg^{2+} concentrations on acetylene reduction by purified nitrogenase components from *Klebsiella pneumoniae*. The formation of an inactive Mg_2ATP complex at the expense of the active $MgATP$ complex was shown to account for the inhibition of acetylene reduction at Mg^{2+} concentrations in the range 2.5–30 mM.

Materials and Methods

K. pneumoniae strain M5a1 was a gift from Dr. P. W. Wilson, Department of Bacteriology, University of Wisconsin, Madison, U.S.A. Growth, harvesting, purification and assay procedures used during the preparation of the component nitrogenase proteins from *K. pneumoniae* were as described by Eady *et al.* (1972). Kp1 and Kp2 proteins had specific activities of 1350 and 1000 nmol of acetylene reduced per min/mg of protein at 30°C respectively. These specific activities refer to the concentration of whichever protein limited the rate of acetylene reduction.

The acetylene assay procedure used for the steady-state kinetics was as described by Eady *et al.* (1972) except the creatine kinase and creatine phosphate were omitted in order to avoid complications arising from competing equilibria involving Mg^{2+} and ATP. The absence of an ATP-generating system necessitated a shorter assay time (5 min) in order to avoid the accumulation of inhibitory concentrations of ADP. Assays over the range of ATP and Mg^{2+}

* The nitrogenase components of various organisms are denoted by a capital letter indicating the genus and the number 1 or 2 indicating which of the protein components is referred to. The number 1 indicates the Fe Mo-containing protein and number 2 the Fe-containing protein. Kp is *Klebsiella pneumoniae*, Cp is *Clostridium pasteurianum*, Av is *Azotobacter vinelandii*, Cv is *Chromatium vinosum*.

concentrations used were linear for at least 5 min (checked by assays of 1, 2, 3 and 4 min duration). The kinetics were studied at 15°C because equilibrium data about the Mg_2ATP complex were available at this temperature (Frey *et al.*, 1972). This lower temperature also helped in the prevention of the accumulation of inhibitory concentrations of ADP. Acetylene assays were done with serum bottles (7.5 ml) capped with Suba-seal (Griffin and George, Wembley, Middx., U.K.) rubber closures containing, in 1.5 ml, 40 μmol of Hepes [2-(*N*-2-hydroxyethyl-piperazin-*N'*-yl)ethanesulphonic acid] buffer, pH 7.8, 20 μmol of $\text{Na}_2\text{S}_2\text{O}_4$, 0.09 nmol of Kpl and 0.9 nmol of Kp2 proteins and various concentrations of ATP and MgCl_2 under 10^4 Pa (0.1 atm) of acetylene in argon [0.9×10^5 Pa (0.9 atm)]. ATP and MgCl_2 stock solutions were adjusted with NaOH to pH 7.8 before use. ATP [Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.] was used without further purification.

Results

The equilibria that need to be considered for calculation of the concentrations of Mg^{2+} , ATP^{4-} , MgATP^{2-} and Mg_2ATP are given in eqns. (1)–(4).

$K_1 = 1.12 \times 10^4 \text{ M}^{-1}$, $K_2 = 59 \text{ M}^{-1}$, $K_3 = 3.7 \times 10^6 \text{ M}^{-1}$, $K_4 = 1.51 \times 10^2 \text{ M}^{-1}$ at 15° in 0.1 M- NaNO_3 (Frey *et al.*, 1972).

Since these experiments were done at pH 7.8, the contributions from eqns. (3) and (4) can be neglected. However, earlier studies concerned with effects of Mg^{2+} and ATP on nitrogenase have been at lower pH values, e.g. pH 6.5 (Tso & Burris, 1973), pH 6.7 (Dilworth *et al.*, 1965), pH 7.2 (Burns, 1969), and eqns. (3) and (4) may be relevant under these conditions.

Acetylene reduction as a function of MgATP concentration was determined at 15°C, pH 7.8 (Fig. 1). The total $[\text{Mg}^{2+}]$ was kept constant at 2.5 mM and the total $[\text{ATP}]$ varied from 0.05–1.5 mM (total concentrations refer to the sum of the concentrations of free and complexed Mg^{2+} or ATP). The concentration of MgATP was calculated by using the equilibrium constants defined in eqns. (1) and (2); 90–95% of the total ATP was present at the MgATP complex and 5–10% was present as free ATP and Mg_2ATP . Since low protein concentrations

Fig. 1. Dependence of acetylene-reduction activity on the concentration of the MgATP complex

□, Total $[\text{Mg}^{2+}]$ was kept constant at 2.5 mM and total $[\text{ATP}]$ varied from 0.05–1.5 mM. ●, Data taken from Table 1 with constant total $[\text{ATP}] = 0.6 \text{ M}$ and $[\text{Mg}^{2+}]$ varied from 2.5–50 mM. Assay conditions were as described for Table 1 and in the text.

were used, the amount of Mg^{2+} and ATP bound to the proteins was considered to be negligible compared with the total Mg^{2+} and ATP. A double-reciprocal plot of the data of Fig. 1 gave an apparent $K_m(\text{Mg-ATP})$ of 0.4 mM. Some deviation from linearity was observed at the lowest concentrations of MgATP ; however, under these conditions the contribution to the total ethylene measured from ethylene contaminating the reagents (determined from assay blanks from which enzyme was omitted) was considerable (approx. 50%) and no significance can be given to this deviation.

The effect on acetylene reduction of increasing the total $[\text{Mg}^{2+}]$ from 2.5 to 50 mM at constant total $[\text{ATP}]$ (0.6 mM) was determined (Table 1 and Fig. 1). Increasing the total $[\text{Mg}^{2+}]$ increases the proportion of the total ATP which is in the form of the Mg_2ATP complex at the expense of the MgATP concentration (Table 1). The inhibition caused by increasing the total $[\text{Mg}^{2+}]$ (Table 1) was seen to reflect the decrease in the concentration of MgATP . Acetylene reduction as a function of MgATP concentration for assays with up to 30 mM total Mg^{2+} followed the activity curve obtained by varying the total ATP at constant, but low total $[\text{Mg}^{2+}]$ (2.5 mM) (Fig. 1). At the highest total concentrations of Mg^{2+} (40 and 50 mM), acetylene reduction was only 50% and 30% respectively of that predicted from the concentration of MgATP . This indicated that at total $[\text{Mg}^{2+}]$ above 30 mM, there was an additional inhibitory effect associated with Mg^{2+} . Assays with total ATP (1.0 mM) gave results consistent with those at 0.6 mM total ATP. When these data were plotted as in Fig. 1 the points at total $[\text{Mg}^{2+}]$ in the range 2.5–30 mM, where the MgATP concentration changes from 0.9 to 0.4 mM, also followed the dependence of acetylene reduction

Table 1. *Dependence of acetylene-reduction activity on Mg^{2+} at a constant ATP concentration*

Assays contained 0.06 μ M-Kp1 protein, 0.6 μ M-Kp2 protein, 13 mM- $Na_2S_2O_4$, 25 mM-Hepes, pH 7.8, and the indicated concentrations of ATP and $MgCl_2$. Assays at 15°C for 5 min under 10^4 Pa (0.1 atm) of acetylene were used. The equilibrium concentrations of Mg^{2+} , MgATP and Mg_2ATP were calculated by using the equilibrium constants defined in eqns. (1) and (2).

Total [ATP] (mM)	Total [Mg^{2+}] (mM)	[Mg^{2+}] (mM)	[MgATP] (mM)	[Mg_2ATP] (mM)	Ethylene produced (nmol/min)
0.6	2.5	1.84	0.54	0.06	0.39
0.6	5.0	4.28	0.48	0.12	0.40
0.6	10.0	9.19	0.39	0.21	0.35
0.6	15.0	14.13	0.33	0.27	0.32
0.6	20.0	19.08	0.28	0.32	0.30
0.6	25.0	24.05	0.25	0.35	0.27
0.6	30.0	29.02	0.22	0.38	0.23
0.6	40.0	38.98	0.18	0.42	0.11
0.6	50.0	48.95	0.15	0.45	0.05

on MgATP concentration. Deviation from this curve again occurred at total [Mg^{2+}] above 30 mM.

Discussion

These data were consistent with MgATP being the active substrate for nitrogenase and with Mg_2ATP being inactive and responsible for inhibition by Mg^{2+} up to total concentrations of 30 mM. However, to prove this it would be necessary to show that all alternative explanations are inconsistent with these data. This has been done for the binding of MgATP to yeast hexokinase (Noat *et al.*, 1970). An extremely detailed steady-state kinetic analysis showed that Mg^{2+} inhibition could only be accounted for by the formation of an Mg_2ATP complex ($K_2 = 25 M^{-1}$ at 30°C) and that Mg^{2+} did not bind to the enzyme. It was also shown that yeast hexokinase did not bind Mg_2ATP . A similar detailed investigation of the nitrogenase enzyme system would be extremely difficult and probably not conclusive because of the multi-substrate site, multi-binding site and two-protein nature of the enzyme. However, the excellent agreement obtained for the data at high Mg^{2+} concentrations, together with those obtained at low concentrations of Mg^{2+} , when plotted in terms of the concentration of MgATP is good evidence for Mg_2ATP being an inactive substrate and for MgATP being the active substrate for nitrogenase.

The deviation from the MgATP-acetylene-reduction dependence at total Mg^{2+} above 30 mM for total concentrations of ATP of 0.6 mM and 1.0 mM indicated that this additional inhibition is not caused by the binding of Mg_2ATP to nitrogenase. The separation of Kp1 and Kp2 proteins on G-200 Sephadex gel requires 50 mM- $MgCl_2$ although their

molecular weights are 220 000 and 67 000 respectively (Eady *et al.*, 1972). In addition, the 1:1 complex of Kp1 and Kp2 proteins observed in the ultra-centrifuge (Eady, 1973) was dissociated by 50 mM- $MgCl_2$ (R. R. Eady & R. N. F. Thorneley, unpublished work). Hence inhibition of acetylene reduction by total Mg^{2+} above 30 mM is probably an effect of Mg^{2+} on complex formation between Kp1 and Kp2 proteins. An interaction of Mg^{2+} with Kp2 protein has been concluded from small changes seen in the e.p.r. spectrum at $g = 1.94$ at total [Mg^{2+}] above 20 mM (B. E. Smith, R. C. Bray & D. J. Lowe, unpublished work).

If MgATP is accepted as the reactive substrate, certain published data can be reinterpreted. Smith *et al.* (1973) used e.p.r. spectroscopy to determine a dissociation constant of 0.4 mM for the ATP complex with Kp2 protein, based on the total concentration of ATP. However, their experiments were done with 50 mM total Mg^{2+} present, which resulted in only 25% of the ATP being in the form MgATP, the other 75% being present as Mg_2ATP . The dissociation constant was recalculated in terms of MgATP concentration and a value of 0.1 mM obtained, which is similar to that for Cp2 protein (Bui & Mortenson, 1968).

Bergersen & Turner (1973) obtained curved double-reciprocal plots for acetylene reduction as a function of total ATP concentration for partially purified nitrogenase from soya-bean bacteroids. These experiments were done in the presence of 40 mM-creatine phosphate, which complexes Mg^{2+} with $K = 40 M^{-1}$ (O'Sullivan & Perrin, 1964) and this equilibrium has to be considered along with those defined by eqns. (1)–(4) in the calculation of MgATP concentrations; for example at 10 mM total Mg^{2+} and ATP, 85% of the total ATP is present as MgATP, but at 0.6 mM total Mg^{2+} and ATP only

50% is in the form MgATP. When the data of Bergersen & Turner (1973) were replotted, a linear double-reciprocal plot was obtained for acetylene reduction as a function of MgATP concentration with an apparent $K_m(\text{MgATP}) = 4.0\text{mM}$. Their data thus provide no evidence for multiple binding sites for MgATP.

The conclusions of Silverstein & Bulen (1970) for H_2 and NH_3 evolution as a function of ATP concentration are not affected by these considerations. The presence of 50mM-creatine phosphate in their case fortuitously prevented the formation of Mg_2ATP by successfully competing for the excess Mg^{2+} and caused the MgATP concentration essentially to equal the total ATP concentration.

Clearly it is important to define the equilibria, and then calculate the concentrations of the substrates present in an assay system. Experiments with high concentrations of enzyme or crude cell extracts, where it is often impossible to calculate substrate concentrations, in particular Mg^{2+} , which is likely to be bound to proteins, and ATP, which can be deaminated (Yates, 1969), should be interpreted with caution. The situation for Kp nitrogenase is further complicated by ATP^{4-} being an inhibitor of acetylene reduction (R. N. F. Thorneley, unpublished work). This inhibition and the equilibria between Mg^{2+} and ATP may be sufficient to explain some of the unusual dependences of acetylene reduction on total ATP concentration in the literature (e.g. Kennedy, 1970).

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