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NITROGENASE OF *KLEBSIELLA PNEUMONIAE*

INHIBITION OF ACETYLENE REDUCTION BY ATP

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SUMMARY

1. The equilibria between Mg^{2+} and ATP^{4-} have been considered in the interpretation of acetylene reduction data for the purified nitrogenase from *Klebsiella pneumoniae*.

2. The data are consistent with $MgATP^{2-}$ being the substrate and with parabolic competitive inhibition by ATP^{4-}. Two cooperative binding sites are suggested for ATP^{4-}.

3. The regulation of in vivo nitrogenase activity by Mg^{2+} and ATP^{4-} levels are discussed.

An Fe-containing protein and a Fe–Mo-containing protein comprise nitrogenase [1]. A divalent metal ion and ATP are required for nitrogenase activity [1]. The most effective metal ion is Mg^{2+} [2–4]. Data from a number of techniques indicate that a Mg^{2+}–ATP^{4-} complex is the active substrate for nitrogenase [5–13]. It is therefore appropriate to consider the ATP and Mg^{2+} dependence of acetylene reduction in terms of the equilibria between Mg^{2+}, ATP^{4-}, $MgATP^{2-}$ and Mg_2ATP. This paper is concerned with the competitive inhibition by ATP^{4-} of acetylene reduction by the purified nitrogenase components from *Klebsiella pneumoniae*.

Growth, harvesting, purification and assay procedures used were as described by Eady et al. [4]. Kp1 and Kp2 proteins (nomenclature Eady et al. [4]) had specific activities of 1350 and 1000 nmoles acetylene reduced/mg/min at 30 °C, respectively. These specific activities refer to the concentration of whichever protein limited the rate of acetylene reduction. Creatine kinase and creatine phosphate were omitted from the assays in order to avoid complications arising from competing equilibria involving Mg^{2+} and ATP. Assays of 5, 10 and 15 min duration indicated that a linear rate of acetylene reduction occurred in all cases. The kinetics were studied at 15 °C because equilibrium data concerning Mg^{2+}–ATP^{4-} complexes were available at this temperature [14]. Acetylene assays were done using serum bottles (7.5 ml) capped with Subaseal (Griffin and George, Wembley, Middlesex, U.K.) rubber closures containing in 1.5 ml, 40 μmoles of 2-(*N*-2-hydroxyethylpiperazon-*N'*-yl)ethanesulphonic acid (HEPES) buffer (pH 7.8), 20 μmoles of $Na_2S_2O_4$, 0.09 nmole of Kp1 and 0.9 nmole of Kp2 proteins and various concentrations of ATP and $MgCl_2$ under 10^4 nmoles/m^2

(0.1 atm) of acetylene in argon (0.9 atm). ATP and $MgCl_2$ stock solutions were adjusted with NaOH to pH 7.8 before use. ATP (Sigma Chemical Co., Kingston-upon-Thames, Surrey, United Kingdom) was used without further purification. ADP was not detectable (less than 1%) in the ATP by thin-layer chromatography in isobutyric acid–aq. NH_3 (spec. gravity 0.88)–water (66:1:33, by vol.) on cellulose F. Low protein concentrations were used in order to minimise the accumulation of ADP during the assay and also to minimise the effects of equilibria involving non-specific binding of Mg^{2+} to the proteins. Doubling the concentrations of both proteins in an assay, which would have been sensitive to a change in Mg^{2+} concentration, doubled the rate of acetylene reduction.

The equilibria which need to be considered in order to calculate the concentrations of Mg^{2+}, ATP^{4-}, $MgATP^{2-}$ and Mg_2ATP are given in Eqns 1 and 2. Since these experiments were done at pH 7.8, equilibria involving $HATP^{3-}$ can be neglected. $K_1 = 112 \cdot 10^4\ M^{-1}$, $K_2 = 59\ M^{-1}$, at 15 °C in 0,1 M $NaNo_3$ [14].

$$Mg^{2+} + ATP^{4-} \underset{\longleftarrow}{\xrightarrow{K_1}} MgATP^{2-} \tag{1}$$

$$Mg^{2+} + MgATP^{2-} \underset{\longleftarrow}{\xrightarrow{K_2}} Mg_2ATP \tag{2}$$

Acetylene reduction as a function of $MgATP^{2-}$ concentration was determined at 15 °C, pH 7.8. An apparent K_m (MgATP) = 0.3 mM was obtained from a Lineweaver-Burk plot (Fig. 1) by keeping the total Mg^{2+} constant at 2.5 mM and varying the total ATP over the range 2.0–0.2 mM. Under these conditions the free ATP^{4-} concentration

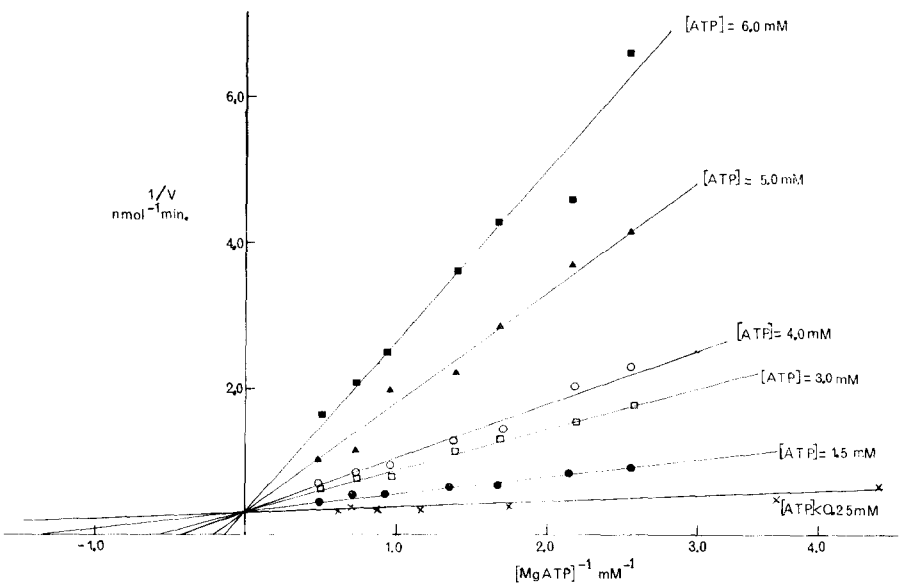

Fig. 1. Lineweaver–Burk plots for the rate of acetylene reduction as a function of [MgATP] at various concentrations of the competitive inhibitor, ATP^{4-}, pH 7.8, 15 °C. Assay conditions as described in the text.

was always less than 0.25 mM and no correction was made for the small amount of inhibition due to these low concentrations of ATP^{4-}. The Mg_2ATP complex is inactive as a substrate [13]. Inhibition by ATP^{4-} was demonstrated by varying the total Mg^{2+} from 2.2 to 0.4 mM with such an excess of ATP^{4-} as to give a constant level of free ATP^{4-} for any given series of Mg^{2+} concentrations. Lineweaver–Burk plots at various free ATP^{4-} concentrations (Fig. 1) indicated competitive inhibition by ATP^{4-} of $MgATP^{2-}$ during acetylene reduction. A linear secondary plot was obtained by plotting the slopes against $[ATP^{4-}]^2$, (Fig. 2).

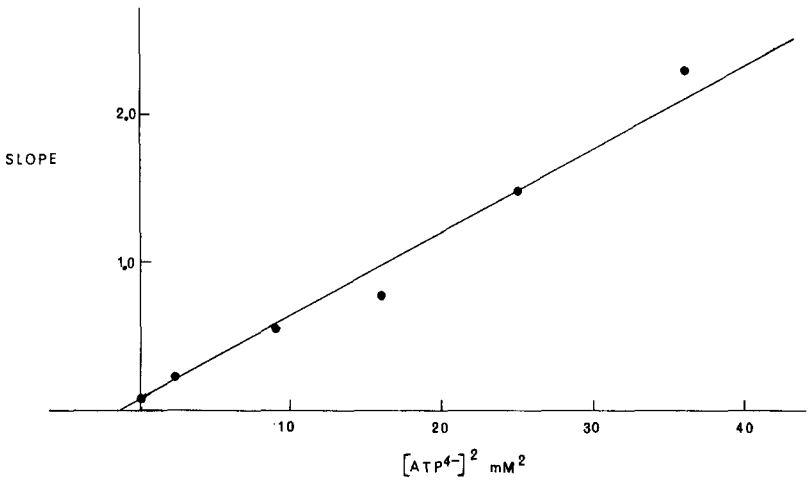

Fig. 2. Secondary plot of the slopes of Fig. 1 against $[ATP^{4-}]^2$.

These data are consistent with $MgATP^{2-}$ being the active substrate for nitrogenase and with ATP^{4-} being a competitive inhibitor. There is no evidence for the existence of $Mg(ATP)_2$ from kinetic [14, 15] or stability constant determinations [16]. The calculated levels of free Mg^{2+} at the various ATP^{4-} concentrations showed that the inhibition of acetylene reduction was not due to a decrease in Mg^{2+} concentration.

The interpretation of parabolic inhibition patterns is difficult [17]. However, the dependence in $[ATP^{4-}]^2$ is consistent with either two molecules of ATP^{4-} binding to a single site, with the second molecule binding much tighter than the first, or with two cooperative sites each binding one ATP^{4-}. Since ATP^{4-} is most likely to bind to the $MgATP^{2-}$ sites, the latter of these possibilities seems to be the more likely. Tso and Burris [10] have shown that Cp2 protein has two sites which bind $MgATP^{2-}$ but obtained no evidence for cooperativity.

The competitive nature of the inhibition between ATP^{4-} and $MgATP^{2-}$ explains why Eady et al. [4] observed no inhibition of acetylene reduction at 30 °C by up to 25 mM total ATP; the excess ATP^{4-} probably did not compete with the high levels of $MgATP^{2-}$. Thorneley and Willison [13] discussed the dangers of using total concentrations of ATP and Mg^{2+} in the interpretation of nitrogenase activity data.

Inhibition by ADP [18] and ATP^{4-} may be significant in the regulation of *K. pneumoniae* nitrogenase activity in vivo. Inhibition by ADP has previously been considered to be a potential control mechanism for other nitrogenases [19–21]. How-

ever, with both ADP and ATP^{4-} as inhibitors and with $MgATP^{2-}$ as a substrate, this could provide a range in the 'energy charge' [22] of the bacterium, over which nitrogenase would be functional. This range could be very sensitive to the Mg^{2+} concentrations since for certain ATP^{4-} concentrations increasing the Mg^{2+} concentration would not only remove an inhibitor but would also generate substrate MgATP. Atkinson [22] has discussed the relative free concentrations of Mg^{2+} and ATP^{4-} in cells and has concluded that substantial amounts of free ATP^{4-} could be present.

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