NITROGENASE OF *KLEBSIELLA PNEUMONIAE*: REDUCTANT-INDEPENDENT ATP HYDROLYSIS AND THE EFFECT OF pH ON THE EFFICIENCY OF COUPLING OF ATP HYDROLYSIS TO SUBSTRATE REDUCTION

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1. Introduction

A pre-steady state study of nitrogenase of *Klebsiella pneumoniae* has shown that electron transfer from the Fe protein (Kp2) to the Mo–Fe protein (Kp1) is coupled to the hydrolysis of MgATP [1]. Steady state studies on nitrogenase from a number of bacteria have shown that the efficiency of coupling of MgATP hydrolysis to substrate reduction (the ATP/2 e^- ratio) depends on several factors, including temperature pH and the ratio of the Mo–Fe and Fe proteins [2–4].

In addition to the ATPase activity accompanying substrate reduction, nitrogenases of *Clostridium pasteurianum* and *Azotobacter vinelandii* have been shown to catalyse the hydrolysis of ATP in the absence of reductant. This so-called reductant-independent ATPase activity has been reported variously as 6% [5] or 30–50% [6,7] the rate in the presence of reductant ($Na_2S_2O_4$), when hydrolysis is coupled to substrate reduction.

Data are presented here on the properties of the reductant-independent ATPase activity of highly-purified nitrogenase components of *K. pneumoniae* and on the effect of pH on the efficiency of coupling of ATP hydrolysis to substrate reduction. These data and their implications for the mechanism of nitrogenase are discussed.

Abbreviations and nomenclature: The component proteins of nitrogenase from various organisms are designated by a capital letter for the genus and a lower case letter for the species, the numeral 1 for the Mo–Fe protein and 2 for the Fe protein. Thus, in this paper, the Mo–Fe and Fe proteins from *K. pneumoniae* are Kp1 and Kp2 respectively, Av is *Azotobacter vinelandii*, Ac is *Azotobacter chroococcum* and Cp is *Clostridium pasteurianum*

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2. Methods and materials

2.1. *Nitrogenase*

The *K. pneumoniae* nitrogenase component proteins were purified as in [8] with a final additional purification of Kp1 on DEAE-cellulose [9]. Hydrogen evolution and acetylene reduction were measured as in [8], and the specific activities when measured under these conditions were 1500 and 1038 nmol C_2H_4 produced/min/mg protein for Kp1 and Kp2, respectively.

2.2. *Reductant-independent ATPase assays*

Reductant-independent ATPase activity was assayed in a round-bottom flask (50 ml) capped with a rubber closure and fitted with a side arm and tap to allow the reaction mixture to be degassed on an all-glass vacuum line, and purified Ar to be admitted before the flask was sealed by closing the tap. Trace amounts of O_2 were removed from the Ar by bubbling the gas through a photoreduced methyl viologen solution [10]. The reaction mixture (6 ml) contained ATP (0.8 mM), $MgCl_2$ (1.8 mM), the required buffer (30 mM) and methyl viologen (50 μM). The reaction was carried out at 30°C in a shaking water bath after the syringe addition of nitrogenase components to start the reaction. The residual dithionite in the nitrogenase solutions (added to protect them from oxygen inactivation) resulted in a transient blue colour of reduced methyl viologen. Soon after the blue colour was discharged on exhaustion of the dithionite,

samples (0.5 ml) were removed at intervals by syringe transfer to ice-cold tubes containing 0.1 ml 10% (w/v) trichloroacetic acid to stop the reaction. Phosphate in the samples was measured colourimetrically by the methods in [11]. Linear rates of P_i release were obtained until 200 nmol ATP had been hydrolysed.

2.3. *Reductant-dependent ATPase assays*

Assays were carried out at 30°C using an ATP-regenerating system as in [8] except that ATP was 1 mM and Mg^{2+} was 2 mM. At the end of the assay period 0.2 ml reaction mixture was mixed with 3 ml butanol to stop the reaction before estimation of phosphate [12].

2.4. *Buffers*

The buffers used in this work were succinate (pH 5.3–6); maleate (pH 6.25–6.75); BES–OH (pH 6.75–7.25); HEPES–OH (pH 7.25–7.75) and Tris–HCl (pH 8–8.7). PIPES was avoided since at 30 mM and pH 6.75 it produced a 40% inhibition of nitrogenase activity.

3. Results

3.1. *Reductant-independent ATPase activity*

Under our assay conditions reductant-independent hydrolysis of ATP required the presence of both Kp1 and Kp2. No ATP hydrolysis was observed when Kp1 (0.5 mg/ml), or Kp2 (0.2 mg/ml) were assayed alone, or when a mixture of O_2-inactivated proteins were assayed. When Kp1 and Kp2 were assayed alone, after a suitable incubation period $S_2O_4^{2-}$ and the complementary protein were added to check that the lack of activity was not due to inactivation of the proteins when incubated in the absence of $S_2O_4^{2-}$. The fact that both proteins are required for ATPase activity is the same as for substrate reduction and makes it very unlikely that the ATPase activity is due to contamination of the Kp1 or Kp2 with a non-specific ATPase.

The activity showed a marked pH dependence, increasing rapidly as the pH was decreased below 7.0. Maximum activity was observed at pH 5.4 (fig.1), at lower pH values the activity decreased due to enzyme inactivation. When the activity over pH 5.4–8.7 was plotted logarithmically as a function of pH, a straight line was obtained indicating that the increase in activity was exponential, and corresponded closely to the increase in concentration of MgHATP [13].

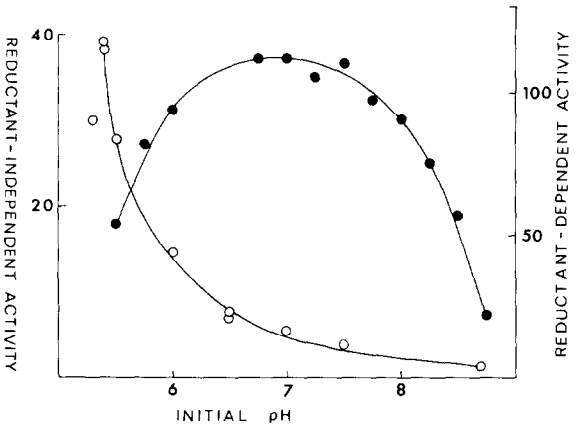

Fig.1. pH dependence of ATPase activity of nitrogenase components of *K. pneumoniae* nitrogenase. Assay conditions are in section 2. (•) Reductant-dependent activity; (○) reductant-independent activity, units of activity are nmol P_i formed/min.

The effect of ATP concentration on activity was investigated at pH 6.5. Under conditions of a 1 mM excess of Mg^{2+} over ATP an 0.8–1 mM ATP optimum was found. At higher ATP concentrations inhibition was observed, at 5 and 10 mM ATP activity was inhibited by 32% and 61%, respectively. At 1 mM ATP increasing Mg^{2+} to 6 mM or 10 mM resulted in 20% or 30% inhibition, respectively.

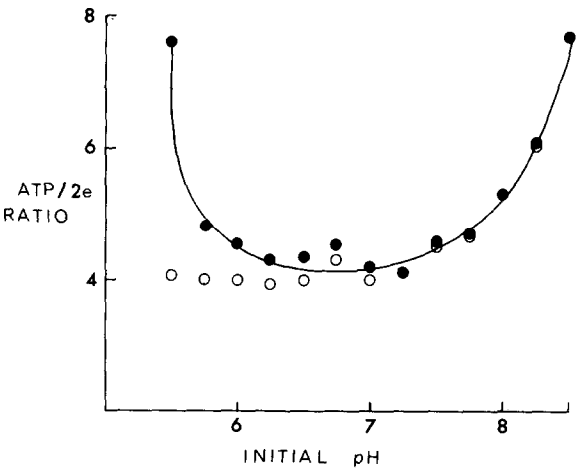

Fig.2. The pH dependence of ATP/2 e^- ratio for *K. pneumoniae* nitrogenase. Hydrogen evolution and ATP hydrolysis were measured as in section 2. (•) ATP/2 e^- ratio; (○) ATP/2 e^- ratio after subtraction of the contribution of reductant-independent ATPase activity to the total ATPase activity in the presence of $S_2O_4^{2-}$.

3.2. *Reductant-dependent ATPase activity and ATP/2 e⁻ ratio*

Reductant-dependent ATPase activity showed a broad pH optimum over 6.5–7.5 (fig.1). Similar data for phosphate formation were obtained when C_2H_2 rather than H^+ was the reducible substrate. The pH profile for hydrogen evolution and acetylene reduction were both similar to the curve for phosphate formation except that at the extremes of pH, substrate reduction decreased more rapidly than ATP hydrolysis. The data obtained with protons as the reducible substrate were used to calculate the ATP/2 e^- over the pH range investigated (fig.2). In the pH region of optimum activity the ATP/2 e^- value was ~4.4, both above and below this region the ratio increased to ~7.5. Figure 2 also shows the effect on the ATP/2 e^- ratio of subtracting the contribution of reductant-independent ATPase activity to phosphate formation measured under the same conditions. The effect is most marked on the acid side of the pH optimum where the ratio decreased from 7.6 to ~4. The ATP/2 e^- ratio remained at this value over the pH range of optimum activity up to pH 7.25. In contrast, subtracting the reductant independent activity had little effect on the ratio at higher pH values.

4. Discussion

We have shown that, as with the highly-purified nitrogenases of *C. pasteurianum* and *A. vinelandii*, nitrogenase of *K. pneumoniae* catalyses reductant-independent ATP hydrolysis. Over pH 7–8.7 the activity is ~5% that of the reductant-dependent activity. This value is similar to those reported for Cp nitrogenase [5] and Av nitrogenase [14] but rather lower than the 20–54% which has also been reported for Cp [6,7]. However the reductant-independent activity increases rapidly with decreasing pH and at pH 5.4 the rate of ATP hydrolysis is 90% that of the reductant-dependent activity. A similar pH dependence has been reported for Cp nitrogenase [6].

Under conditions where reductant-independent activity is high, the question arises to what extent it contributes to the increase in ATP/2 e^- ratio observed at low pH values (fig.2). Recent estimates of the ATP/2 e^- ratios for purified Cp and Av nitrogenases over pH 7–8 have been reported as 4–8 uncorrected for reductant-independent activity [5,14,15]. We find an uncorrected value of ~4.4 for Kp nitrogenase over pH 6.25–7.25.

Figure 2 shows the effect on the ATP/2 e^- ratio of subtracting the reductant-independent ATP hydrolysis from the rate observed in the presence of dithionite. Over pH 5.5–7.2 a constant value of 4 was obtained for the ATP/2 e^- ratio. In our experiments using a dithionite exhausted system, the oxidation states of the components catalysing reductant-independent ATP hydrolysis are $Kp2_{ox}$ and $Kp1_{semireduced}$ [16]. In the presence of ATP and dithionite steady-state EPR experiments have show the predominant oxidation states to be $Kp2_{ox}$ and $Kp1_{superreduced}$, although some 25% of the Kp1 remains in the semireduced state [16]. The constant value of the ATP/2 e^- ratio obtained when the reductant-independent ATPase activity is subtracted from the total ATPase activity suggests that the oxidation state of the Kp1 does not effect the ability of the nitrogenase complex to catalyse reductant-independent ATP hydrolysis. These data are consistent with the uncoupling observed at low pH being due to the increasing contribution of reductant-independent ATPase activity to the total ATPase activity. This is not so for the data obtained at the high pH range, where the perturbation which results in the increase in the ATP/2 e^- ratio may arise from a change in the rate-limiting step leading to substrate reduction.

The requirement for both proteins for reductant-dependent and reductant-independent ATPase activity can be rationalised in terms of a bridging site between the two proteins formed on complex formation. Interaction of ATP with the Fe protein of nitrogenase is well documented and is often assumed to be the only site of interaction with the enzyme system [17]. However it has recently been shown that highly-purified preparations of Ac1 and Kp1 catalyse the exchange of $^{32}P_i$ into both ATP and ADP and that Kp1 binds 4 ATP molecules/tetramer [18].

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