NITROGENASE OF *KLEBSIELLA PNEUMONIAE*: A PRE-STEADY STATE BURST OF ATP HYDROLYSIS IS COUPLED TO ELECTRON TRANSFER BETWEEN THE COMPONENT PROTEINS

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1. Introduction

Nitrogenase, the enzyme responsible for biological nitrogen fixation is comprised of two redox proteins, both of which contain Fe–S clusters and one of which, the Mo–Fe protein, additionally contains Mo. Substrate reduction requires both proteins and is obligatorily coupled to the hydrolysis of MgATP to MgADP and P_i [1,2]. Pre-steady state EPR [3,4] and stopped flow spectrophotometric studies [5,6] together with Mössbauer studies [7] have shown that MgATP is required for the rapid transfer of electrons from the Fe protein to the Mo–Fe protein. Binding of MgATP to the Fe protein results in a lowering of its redox potential [8,9] from −290 to −400 mV [8]. The question arises whether MgATP acts merely as an activator of the Fe protein, allowing electron transfer to the Mo–Fe protein, or whether MgATP hydrolysis is coupled to this electron transfer. Kinetic data presented here demonstrate a pre-steady state burst of MgATP hydrolysis which occurs with the same time constant as does electron transfer between the nitrogenase proteins. These observations, and their implications for the role of MgATP 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

2. Materials and methods

The *Klebsiella pneumoniae* nitrogenase component proteins were purified as in [10] with final additional chromatography on DEAE cellulose [11]. Nitrogenase assays [10] at 30°C showed that the Kp1 and Kp2 proteins used had spec. act. 1345 and 1026 nmol C_2H_4 produced/min/mg protein, respectively. Stopped-flow spectrophotometry was performed as in [5]. Samples were prepared using the rapid reaction apparatus in [12] and quenched at 0°C by positioning the jet of the rapid reaction apparatus just below the surface of 0.6 ml 30% trichloroacetic acid in a 5 ml glass vessel; this gives a quenching time of about 2 ms [13]. Inorganic phosphate was determined as in [14] after initial centrifugation to remove precipitated protein. Biochemicals were obtained from Sigma (London) and salts from BDH, Poole.

The time constant, 'τ', for the rate of P_i formation was estimated from a least squares fit to a single exponential; errors were estimated from the variation in τ required to give a 25% increase in the sum of squares of the errors.

3. Results

Electron transfer from Kp2 to Kp1 at 23°C, when monitored by stopped-flow spectrophotometry, occurs with τ = 8.5 ms with 4 mM MgATP. At this temperature, the electron transfer reaction is too fast to enable a precise correlation with the early time

Fig.1a

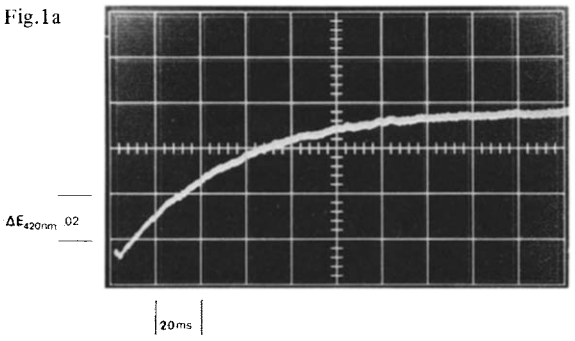

Fig.1b

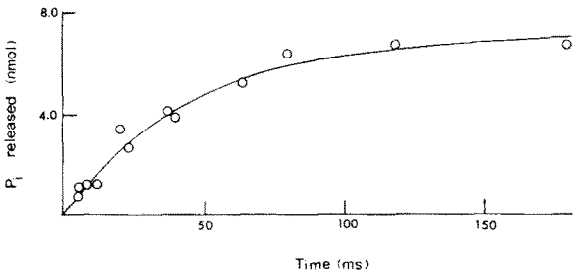

Fig.1. Correlation between electron transfer from Kp2 to Kp1 and release of phosphate from ATP. Kp1 (10 μM) was reduced by Kp2 (50 μM) at 10°C in 25 mM Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer (pH 7.4) in the presence of $Na_2S_2O_4$ (10 mM), ATP (5 mM) and $MgCl_2$ (10 mM). (a) Stopped-flow oscillograph of ΔA_{420}, $\tau = 42 \pm 3$ ms (b) time course of phosphate release. The curve is the best fit exponential, $\tau = 44 \pm 4$ ms.

course of P_i formation because of the restricted number of short times available with the particular rapid quench apparatus used. In order to overcome this difficulty the reaction temperature was lowered to 10°C to increase the value of τ.

The rate of the MgATP-dependent electron transfer from Kp2 to Kp1 was studied by stopped-flow spectrophotometry. A typical oscilloscope trace of ΔA_{420} due to the oxidation of Kp2 after initiation of the reaction by mixing with MgATP is shown in fig.1a. At ≤250 ms a single exponential process was observed with $\tau = 42 \pm 3$ ms, independent of Kp2 : Kp1 ratio and absolute Kp2 concentration (10–50 μM). The dependence of the amplitude of this process on [Kp2] at fixed [Kp1] is shown in fig.2. The amplitude increased with increasing [Kp2] to a limiting value with [Kp2] = 50 μM when [Kp1] = 10 μM.

These data are similar to those at 23°C [5] except that τ has increased from 8 ms to 42 ms. The amplitude of the optical change however, increased beyond a 3:1 ratio of [Kp2] : [Kp1] at 10°C in contrast to the data obtained at 23°C. A 5-fold excess of Kp2 was therefore used in experiments to measure P_i formation in order to obtain maximal oxidation of Kp2.

The pre-steady state rate of MgATP hydrolysis was measured in the rapid quench apparatus. At short times after initiation of the reaction by mixing with MgATP, 0.6 ml solution was quenched and P_i determined. The amount of P_i obtained at different times is shown in fig.1b. A pre-steady state burst in P_i formation was observed with $\tau = 44 \pm 4$ ms, indistinguishable from that obtained for the electron transfer process under comparable conditions (fig.1a). The amount of P_i released in this burst phase corresponded to 2.3 mol P_i/mol Kp1 in the assay. Samples prepared using Kp1 that had been inactivated by stirring in air for 25 min showed no release of P_i at ≤70 ms. Phosphate analysis showed that neither Kp1 nor Kp2 contained associated phosphate (S.M.R. Imam and R.R.E., unpublished).

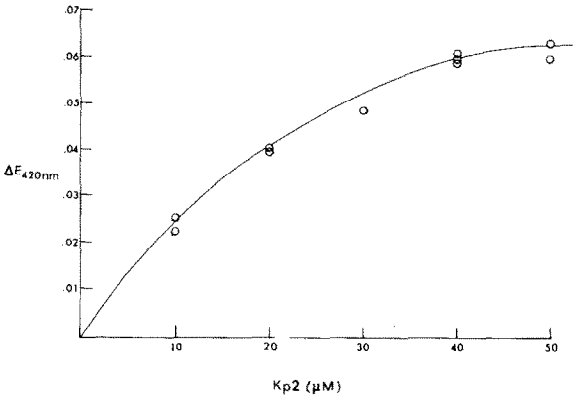

Fig.2. Variation of amplitude of stopped-flow oscillograph with Kp2 : Kp1 ratio. Conditions as for fig.1 except that the Kp1 concentration (10 μM) was held constant, and the Kp2 concentration varied.

4. Discussion

Our finding that the initial reduction of Kp1 by Kp2 follows the time course of P_i formation indicates that this electron transfer is coupled to the hydrolysis of MgATP. Although MgATP is hydrolysed at this point in the catalytic cycle, we cannot conclude from our data that the resulting MgADP and P_i then rapidly dissociate from the enzyme. The release of MgADP from reduced Kp2, or a coupled conformational change has been shown [16] to occur at a rate comparable with that of electron transfer. We cannot, exclude the possibility that dissociation of MgADP or P_i from oxidised Kp2 occurs at a slower rate, than electron transfer. Whether these dissociation reactions are rate limiting in the overall reaction (turnover time ~15 s for Kp1 at 10°C) is yet to be determined.

The coupling of MgATP hydrolysis to electron transfer from Kp2 to Kp1 may enable a centre in Kp1 with a redox potential considerably lower than that of the isolated Kp2–MgATP complex (~–400 mV) to become significantly reduced. Since the first order rate constant for electron transfer is independent of the concentration of Kp1 and Kp2 and since neither the oxidised nor the reduced Kp2–MgATP complexes alone hydrolyse MgATP, the ability to effect this hydrolysis is a property of the Kp1–Kp2–MgATP complex. Whether the MgATP that is hydrolysed is bound to the Kp2 or Kp1 or bridges both proteins is still not known.

It is interesting to note that the 2.3 mol MgATP hydrolysed/mol Kp1 in the burst phase is half that required to transfer 2 e^- from reductant (dithionite) to oxidizing substrate (H^+) under the optimum steady state conditions at 30°C [1], i.e., we have shown that at 10°C MgATP hydrolysis is not uncoupled from electron transfer from Kp2 to Kp1. However, it was shown [17] that MgATP hydrolysis is uncoupled from total electron flux from dithionite ion to reducible substrates at 10°C. These observations are consistent with a second role for MgATP which becomes uncoupled from electron transfer at 10°C [6,18].

The data reported here are of more general interest since this is the first example of ATP hydrolysis being coupled to an electron transfer reaction in a soluble system.

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