Kinetics of Nitrogenase of *Klebsiella pneumoniae*

HETEROTROPIC INTERACTIONS BETWEEN MAGNESIUM-ADENOSINE 5'-DIPHOSPHATE AND MAGNESIUM-ADENOSINE 5'-TRIPHOSPHATE

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The effects of MgADP and MgATP on the kinetics of a pre-steady-state electron-transfer reaction and on the steady-state kinetics of H₂ evolution for nitrogenase proteins of *K. pneumoniae* were studied. MgADP was a competitive inhibitor of MgATP in the MgATP-induced electron transfer from the Fe-protein to the Mo-Fe-protein. A dissociation constant $K'_1 = 20 \mu\text{M}$ was determined for MgADP. The release of MgADP or a coupled conformation change in the Fe-protein of *K. pneumoniae* occurred with a rate comparable with that of electron transfer, $k \sim 2 \times 10^2 \text{ s}^{-1}$. Neither homotropic nor heterotropic interactions involving MgATP and MgADP were observed for this reaction. Steady-state kinetic data for H₂ evolution exhibited heterotropic effects between MgADP and MgATP. The data have been fitted to symmetry and sequential-type models involving conformation changes in two identical subunits. The data suggest that the enzyme can bind up to two molecules of either MgATP or MgADP, but is unable to bind both nucleotides simultaneously. The control of H₂ evolution by the MgATP/MgADP ratio is not at the level of electron transfer between the Fe- and Mo-Fe-proteins.

The nitrogenase from the facultative anaerobe *Klebsiella pneumoniae* consists of two proteins: a dimeric protein (Kp2; * mol.wt. 67000) containing four non-haem iron atoms, and a tetrameric protein (Kp1, mol.wt. 218000) containing 22 ± 2 non-haem iron atoms and 2 molybdenum atoms (Smith *et al.*, 1976a). Both proteins, an anaerobic environment, MgATP and a source of electrons, probably flavodoxin *in vivo* (Yates, 1972; Yoch, 1974) or sodium dithionite *in vitro* (Bulen *et al.*, 1965), are required for activity. E.p.r. spectroscopy (Smith *et al.*, 1972, 1973; Orme-Johnson *et al.*, 1972; M. N. Walker & Mortenson, 1974), Mössbauer spectroscopy (Smith & Lang, 1974) and stopped-flow spectrophotometry (Thorneley, 1975; Thorneley *et al.*, 1976) have all shown that one role for the Fe-protein is to transfer electrons in an MgATP-dependent reaction to the Mo-Fe protein. Stopped-flow studies (Thorneley, 1975; Thorneley *et al.*, 1976) showed that this electron

transfer occurred within a tight complex of the two proteins with rate constants of $k = 2.0 \times 10^2 \text{ s}^{-1}$ (*K. pneumoniae*) and $2.5 \times 10^2 \text{ s}^{-1}$ (*A. chroococcum*). The dependence of the observed first-order rate constant for this reaction on MgATP concentration gave a dissociation constant for MgATP, $K'_{\text{DMgATP}} = 0.4 \text{ mM}$ (*K. pneumoniae*) and 1.0 mM (*A. chroococcum*). These values are very similar to the apparent K_m values for MgATP determined previously for the rate of acetylene reduction under steady-state conditions (Thorneley & Willison, 1974; Thorneley, 1974b). None of these studies indicated co-operative interactions involving multiple binding sites for MgATP, although the inhibition by ATP⁴⁻ did exhibit kinetics consistent with positive homotropic effects (Thorneley, 1974b). Previously reported sigmoid dependences of the rates of acetylene reduction and H₂ evolution on MgATP concentration (Bergersen & Turner, 1973; Moustafa & Mortenson, 1967) can probably be explained by inhibitory effects of MgADP (see below) or insufficient attention to the equilibria between Mg²⁺ and ATP⁴⁻ (Thorneley & Willison, 1974; Storer & Cornish-Bowden, 1976). Ljones (1973) measured the rate of dithionite oxidation as a function of ATP concentration at various fixed concentrations of ADP with *C. pasteurianum* nitrogenase. Linear Lineweaver-Burk (1934) plots were obtained in the absence of ADP, but, as the ADP concentration was increased, the plots became

* Abbreviations: The nitrogenase components of the various organisms are denoted by a capital letter indicating the genus, a lower-case letter indicating the species and the number 1 or 2 denotes which of the protein components is referred to; the number 1 indicates the Mo-Fe-containing protein and the number 2 the Fe-containing protein; Ac, *Azotobacter chroococcum*; Kp, *Klebsiella pneumoniae*; Cp, *Clostridium pasteurianum*; Av, *Azotobacter vinelandii*; e.p.r., electron paramagnetic resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

increasingly curved. No analysis of these data was attempted by Ljones (1973). These data were qualitatively similar to those reported below for H₂ evolution by *K. pneumoniae* nitrogenase. Heterotropic interactions between MgADP and MgATP have been reported by Tso & Burris (1973) using a gel-equilibration technique to measure the binding of these nucleotides to Cp2 protein. They found that MgATP bound to two sites on Cp2 protein, with a dissociation constant of 17 μ M, and that MgADP could only bind to one of these sites, with a dissociation constant of 5 μ M. The binding of MgADP at one site caused MgATP to be bound tighter at the other. Heterotropic effects involving MgADP, an inhibitor of all nitrogenase functions investigated to date, could be involved in the control of activity *in vivo*, as discussed by Atkinson (1970) for other ATP-dependent enzymes. In the present paper the kinetics of the inhibition by MgADP of the MgATP-induced electron transfer between the component proteins are now reported and compared with steady-state data for the MgATP-dependent H₂-evolution reaction.

Materials and Methods

The component proteins of *Klebsiella pneumoniae* nitrogenase were purified as described by Eady *et al.* (1972), with an additional final DEAE-cellulose chromatography (Smith *et al.*, 1976a). The complex Kp1Kp2 component proteins at a 1:1 molar ratio had a specific activity, when assayed under the conditions described by Eady *et al.* (1972), of 790 nmol of ethylene produced/min per mg of Kp2 protein. Each protein solution contained 25 mM-Hepes/NaOH, pH 7.4, and 10 mM-MgCl₂.

Protein concentrations were measured by the Folin-Ciocalteu method (Lowry *et al.*, 1951), with bovine serum albumin, previously dried for 24 h over P₂O₅, as a standard; dry-weight measurements have shown that no correction factor is necessary for *K. pneumoniae* proteins (Eady *et al.*, 1972).

All biochemicals were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K., and salts from BDH Chemicals, Poole, Dorset, U.K.

Stopped-flow experiments were done at 23°C, and monitored at 425 nm in a 1 cm-light-path cell. Anaerobic dilutions of stock solutions were done with gas-tight syringes into N₂- or Ar-flushed vessels fitted with rubber serum caps. Modifications to a commercial stopped-flow apparatus (American Instrument Co., Silver Spring, MD, U.S.A.), necessary to improve the temperature control and the anaerobicity, have been described (Thorneley, 1974a). Absorbance changes were recorded on a Tektronix 549 storage oscilloscope and time-constants of exponential traces were evaluated as previously described (Thorneley, 1975).

H₂ evolution was determined by the established technique of Eady *et al.* (1972) at 23°C. No ATP-generating system (phosphocreatine/creatine kinase, EC 2.7.3.2), was used in these assays, since the inhibitory effects of MgADP were under investigation. Assays of 2–40 min duration were used, depending on conditions; linear rates of H₂ evolution were obtained in all cases, indicating that additional inhibition by MgADP produced in the course of the assay was negligible.

Non-linear regression was carried out by the method of Cornish-Bowden & Koshland (1970), modified as described by Wharton *et al.* (1974). Each initial velocity *v* was assigned a weight 1/*v*², because the reproducibility of replicate observations indicated that the coefficient of variation of *v* was independent of *v*, i.e. that the standard deviation of *v* was proportional to *v*.

Results and Discussion

MgATP-induced electron transfer from Fe-protein to Mo-Fe-protein: inhibition by MgADP

MgATP has previously been shown to promote a unimolecular electron transfer within a tight complex of the component proteins of Kp nitrogenase (Thorneley, 1975) and Ac nitrogenase (Thorneley *et al.*, 1976). The reaction was monitored in the stopped-flow apparatus at 425 nm, where the oxidation of the Fe-protein resulted in an increase in absorbance in a single exponential process. Scheme 1 is consistent with the results obtained when mixtures of MgATP and MgADP were mixed in the stopped-flow apparatus with a 1:1 molar mixture of Fe- and Mo-Fe-proteins of Kp nitrogenase in the presence of sodium dithionite.

The observed first-order rate constant for the oxidation of Fe-protein (*k*_{obs.} = τ^{-1}) in Scheme 1 as a function of MgATP and MgADP concentrations is given by eqn. (1):

$$\frac{1}{k_{\text{obs.}}} = \tau = \frac{1}{k_{+2}} \frac{K_{\text{DMgATP}}}{[\text{MgATP}]} \left(1 + \frac{[\text{MgADP}]}{K'_1} \right) + \frac{1}{k_{+2}} \quad (1)$$

where *K*_{DMgATP} and *K*'₁ are dissociation constants for MgATP and MgADP respectively and *k*₊₂ is the first-order rate constant for electron transfer. Plots of τ against [MgADP] at various fixed MgATP concentrations (analogous to Dixon plots for steady-state kinetics) are presented in Fig. 1. The plots were linear in all cases; a value of *K*'₁ = 20 μ M was determined. The values of *K*_{DMgATP} (0.4 mM) and *k*₊₂ (2.0 × 10² s⁻¹) were in agreement with those previously reported (Thorneley, 1975). No evidence was obtained for either homotropic or heterotropic interactions involving multiple binding sites for MgATP or MgADP in these reactions. This is in contrast with

Scheme 1. Inhibition by MgADP of the MgATP-induced electron transfer between the component proteins. The term MgATP or MgADP+P_i has been included, since it has not been established whether electron transfer from protein Kp2 to protein Kp1 (k_{+2}) is coupled to MgATP hydrolysis.

Fig. 1. Dependence of τ for electron transfer from the Fe-protein to the Mo-Fe-protein of *K. pneumoniae* on MgADP concentration at various MgATP concentrations

Concentrations after mixing: [Kp1] = [Kp2] = 10 μM ; Na₂S₂O₄, 10mM; MgCl₂, 10mM; Hepes/NaOH, 25mM; pH 7.4; 23°C; monitored at 420nm. The Kp1 and Kp2 proteins were pre-mixed in the presence of MgCl₂ and Na₂S₂O₄, the other syringe containing ATP, ADP, MgCl₂ and Na₂S₂O₄. From the abscissa co-ordinate of the intercept, $K_i = 20 \mu\text{M}$ was calculated. MgATP concentrations were: $\square$, 0.1mM; $\circ$, 0.7mM; $\triangle$, 1.1mM; $\bullet$, 2.0mM; $\blacksquare$, 6.0mM.

the heterotropic interactions between MgADP and MgATP for steady-state H₂ evolution discussed below.

The equilibria involving the binding of MgADP and MgATP in Scheme 1 were assumed to be rapid relative to the rate of electron transfer in the derivation of eqn. (1). Fig. 2 shows that this assumption is not strictly valid. Since the final concentrations of all reactants are the same in Figs. 2(a) and 2(b), the time-constants for the oxidation of Kp2 protein by Kp1 protein should have been the same in both cases if the rapid-equilibrium assumption in Scheme 1 is valid under all conditions. However, in Fig. 2(b), in which protein Kp2 is saturated with MgADP before mixing, the time constant is clearly longer ($\tau = 15\text{ms}$) than in Fig. 2(a) ($\tau = 7.7\text{ms}$), in which MgATP competes effectively with the MgADP in the pre-

Fig. 2. Effect of preincubation of the Fe-protein of *K. pneumoniae* with MgADP on the rate of electron transfer to the Mo-Fe-protein

Trace (a)

Syringe A	Syringe B
[Kp1] = 20 μM	[Kp2] = 50 μM
[ADP] = 200 μM	[ADP] = 200 μM
	[ATP] = 10mM

Trace (b)

Syringe A	Syringe B
[Kp1] = 20 μM	[Kp2] = 50 μM
[ADP] = 200 μM	[ADP] = 200 μM
[ATP] = 10mM	

Both syringes contained 10mM-MgCl₂, 25mM-Hepes/NaOH, 10mM-Na₂S₂O₄, pH 7.4; 23°C; monitored at 420nm. Preincubation of protein Kp1 with ADP alone (syringe A) and of protein Kp2 with ADP+ATP (syringe B) results in rapid electron transfer ($\tau = 7.7\text{ms}$) in trace (a). However, preincubation of protein Kp2 with ADP alone causes slower electron transfer ($\tau = 15\text{ms}$, trace b).

incubation of protein Kp2. Therefore either the release of MgADP or a coupled conformation change occurs at a rate comparable with that of electron transfer, i.e. with $k \sim 2 \times 10^2 \text{s}^{-1}$. We also conclude that the binding sites for MgATP and MgADP that control electron transfer between the component proteins are on Kp2 protein. This conclusion cannot be drawn from binding studies involving isolated Fe-protein with MgATP and MgADP (Tso & Burris, 1973). Neither have previous e.p.r. studies

Fig. 3. Dependence of the steady-state rate of H_2 evolution on MgATP concentration at various MgADP concentrations. The curves drawn through the data points are a computer fit according to eqn. (2) for Scheme 2. The insert shows the same data presented in the form of Lineweaver-Burk plots for comparison with the published data for the rate of dithionite oxidation (Ljones, 1973). Concentrations in the assays were: protein $Kp1 = Kp2 = 0.3 \mu M$; $MgCl_2$, 10 mM; Hepes/NaOH, 25 mM; pH 7.4; 23°C. MgADP concentrations were: ●, 0.0; ▲, 0.2 mM; ■, 0.4 mM; ○, 0.6 mM; △, 0.8 mM.

shown that the site for MgADP inhibition of inter-protein electron transfer is on the Fe-protein. This latter point is important, since a second role for MgATP concerned with substrate reduction has been proposed (Smith *et al.*, 1976b), possibly involving additional MgATP- and MgADP-binding sites on the Mo-Fe-protein.

Steady-state kinetics of H_2 evolution: inhibition by MgADP

Lineweaver-Burk (1934) plots for the rate of H_2 evolution as a function of MgATP concentration at a series of fixed MgADP concentrations are shown in the insert to Fig. 3. No curvature is detectable in the absence of MgADP, but as the MgADP concentration increases the plots become increasingly curved.

Data for the rate of H_2 evolution at a fixed high MgATP concentration (4 mM) at different MgADP concentrations are presented in Fig. 4 in the form of a Hill (1910) plot. At low MgADP concentrations the

slope approaches -1 and at high MgADP concentrations approaches -2. The plot exhibits smooth curvature, with no evidence for sharp breaks as the slopes change from -1 to -2.

These results were obtained with low concentrations of protein ($Kp1 = Kp2 = 0.3 \mu M$) to minimize the amount of MgADP produced in the course of the assay. This low concentration of component proteins is close to the region where the 'dilution effect' (i.e. dissociation of the $Kp1Kp2$ complex) occurs (Thorneley *et al.*, 1976). Burns & Hardy (1975), working with crude nitrogenase preparations from *A. vinelandii*, reported that the dilution effect is enhanced by MgADP. The data of Fig. 5 show that the dilution effect with purified proteins was enhanced by MgADP, but, more importantly, that the protein concentration used for H_2 -evolution data even at the highest MgADP concentration used was outside the dilution-effect range. It was important to establish this fact for relevant comparison of the data for the MgATP- and MgADP-dependence of the rate of electron transfer between the component proteins,

Fig. 4. Hill plot for steady-state rate of H_2 evolution as a function of MgADP concentration for *K. pneumoniae* nitrogenase

v is rate of H_2 evolution in the absence of MgADP; v_i is rate of H_2 evolution at various MgADP concentrations. Concentrations in the assays were: protein $K_{p1} = K_{p2} = 0.3 \mu M$; $MgCl_2$, 10 mM; Hepes/NaOH, 25 mM; ATP, 5 mM; pH 7.4; 23°C. Broken lines are theoretical slopes of 1 and 2.

Fig. 5. Dependence of the steady-state rate of H_2 evolution by *K. pneumoniae* nitrogenase on protein concentration and the effect of MgADP

Concentrations in the assays were: $MgCl_2$, 10 mM; Hepes/NaOH, 25 mM; pH 7.4; 23°C. $\circ$, 10 mM-ATP, 0.8 mM-ADP; Δ , 1.0 mM-ATP, 0.8 mM-ADP. The arrows indicate the concentrations of Kp protein used for the H_2 -evolution data presented in Figs. 3 and 4.

which were obtained at high protein concentrations, with the steady-state data for H_2 evolution obtained at low protein concentrations. In both cases the protein concentrations were high enough, even in the presence of MgADP, for the component proteins to be fully complexed.

The interpretation of steady-state kinetic data for such a complex multicomponent system as nitrogenase is extremely difficult, particularly since it is known from e.p.r. studies (Smith *et al.*, 1973) that the Fe-protein is only partially oxidized (approx. 50%) in the steady state. However, nitrogenase does have some advantages that facilitate the interpretation of steady-state data. The long turnover time of approx. 2 s at 23°C for H_2 evolution means that a rapid-equilibrium assumption with respect to substrate and inhibitor binding and protein-conformation changes is probably valid. The pre-steady-state data discussed above support this assumption for MgATP and MgADP binding to Kp2 protein.

The data shown in Fig. 4 closely resemble a theoretical Hill plot given by Segel (1975; Fig. VIII-3 therein) and it is therefore useful to examine his explanation of such behaviour. He suggests that if binding of a single substrate molecule at one site on any enzyme prevents binding of inhibitor at all of several inhibitor-binding sites, and vice versa, Hill coefficients greater than 1.0 will be observed. We agree with this conclusion, and indeed the models that we consider below (Scheme 2, and Scheme 3 with $\alpha = 0$) are essentially of this type. However, we do not agree that 'this does not indicate co-operativity in the usual sense' (Segel, 1975, p. 472). In our view, if binding of a single small molecule such as MgATP prevents binding of MgADP at both of two sites, whereas binding of one molecule of MgADP at one site has no effect on binding of a second molecule at the other site, then site-site interactions must be involved.

The dimeric Fe-protein undergoes conformation changes induced by MgATP and by MgADP (Smith *et al.*, 1973; Orme-Johnson *et al.*, 1972;

Scheme 2. *Symmetry model for MgADP inhibition of MgATP-dependent H_2 evolution*

The T and R conformations are represented by circles and squares respectively. S represents MgATP (substrate) and I represents MgADP (inhibitor).

Scheme 3. *Sequential model for MgADP inhibition of MgATP-dependent H_2 evolution*

The A, B and C conformations are represented by circles, squares and triangles respectively. S represents MgATP (substrate) and I represents MgADP (inhibitor).

Zumft *et al.*, 1972, 1974). This suggested that the data could perhaps be explained in terms of models of the type proposed by Monod *et al.* (1965) and Koshland *et al.* (1966). Scheme 2 is based on the symmetry model of Monod *et al.* (1965) for the special case of a dimer, and postulates that each protomer can exist in either of two conformational states T and R, and that both protomers of a molecule must exist in the same state at any time, i.e. states T_2 and R_2 are allowed but TR is not. We assume that the

Fig. 6. *Dependence of the rate of H_2 evolution on MgATP concentration in the absence of MgADP*

The line is a computer fit according to eqn. (2) for Scheme 2. The parameters used, $V = 7.68 \pm 0.24$ nmol of H_2 /min, $K_s = 0.136 \pm 0.023$ mM, $K_1 = 72.2 \pm 3.54$ μ M, $L = 3.7 \pm 1.1$, were obtained by fitting all the data presented in Fig. 3. The curvature predicted by eqn. (2) indicating positive co-operativity in the absence of MgADP due to the occurrence of T_2 as a free species is seen to be so slight that deviations from linearity are easily accommodated in the scatter of the data. Pronounced curvature only becomes apparent in the presence of MgADP; see insert to Fig. 3.

substrate MgATP binds only to the R conformation and that the inhibitor MgADP binds only to the T conformation. In principle this model predicts that the binding of MgATP should show positive co-operativity even in the absence of MgADP, because of the occurrence of T_2 as a free species. But this co-operativity may well be undetectable if a significant amount of the free enzyme exists as R_2 (Fig. 6).

Scheme 3 is based loosely on the sequential model of Koshland *et al.* (1966) and Kirtley & Koshland (1967). It postulates three conformational states A, B and C for each subunit, but unlike Scheme 2 it admits the possibility of mixed conformational states AB, AC or BC for the dimer. To account for the absence of co-operativity in the absence of MgADP we propose that the change in subunit interactions on changing from AA to AB is the same as that on changing from AB to BB, i.e. $K_{AB}^2 = K_{BB}$ in the symbolism of Koshland *et al.* (1966). As shown by those workers the absence of co-operativity does not imply the absence of subunit interactions. We make a similar assumption for the MgADP-induced conformational change, i.e. $K_{AC}^2 = K_{CC}$.

Scheme 2 requires a rate equation of the following form:

$$v = \frac{V \left(\frac{[\text{MgATP}]}{K_s} \right) \left(1 + \frac{[\text{MgATP}]}{K_s} \right)}{L \left(1 + \frac{[\text{MgADP}]}{K_i} \right)^2 + \left(1 + \frac{[\text{MgATP}]}{K_s} \right)^2} \quad (2)$$

in which the initial velocity v is expressed in terms of the maximum velocity V , the concentrations of MgATP and MgADP, the site dissociation constant K_s for release of MgATP, the site dissociation constant K_i for release of MgADP, and an 'allosteric constant' L , defined as $[\text{T}_2]/[\text{R}_2]$, the ratio of concentrations of the two states of the free enzyme.

After fitting the data to eqn. (2) we obtained $V = 7.68 \pm 0.24$ nmol of H_2/min , $K_s = 0.136 \pm 0.023$ mM, $K_i = 72.2 \pm 3.54$ μM , $L = 3.7 \pm 1.1$, for the parameter values and their standard errors. The sum of squares was 1.77, defined as $\sum(1 - v/\hat{v})^2$, where v and $\hat{v}$ are the observed and calculated rates respectively. This indicates an experimental standard error for v of $\pm 13\%$. The lines drawn through the data points in Fig. 3 were calculated according to eqn. (2) by using these values.

Scheme 3 requires an equation of the following form:

$$v = \frac{V \left(\frac{[\text{MgATP}]}{K_s} \right) \left\{ 1 + \left(\frac{[\text{MgATP}]}{K_s} \right) + \left(\frac{\alpha[\text{MgADP}]}{K_i} \right) \right\}}{\left(1 + \frac{[\text{MgADP}]}{K_i} \right)^2 + \left(1 + \frac{[\text{MgATP}]}{K_s} \right)^2 + \left(\frac{\alpha[\text{MgADP}][\text{MgATP}]}{K_i K_s} \right) - 1} \quad (3)$$

in which v and V are defined as in eqn. (2); K_s and K_i are the apparent site dissociation constants for release of MgATP and MgADP respectively and include subunit-interaction terms as defined by Koshland *et al.* (1966) and Kirtley & Koshland (1967); and α is a parameter that expresses the stability of the mixed conformation BC relative to the other conformations, i.e. $\alpha = K_{\text{BC}}/K_{\text{AB}}K_{\text{AC}}$, where K_{AB} , K_{BC} and K_{AC} are subunit-interaction terms.

After fitting the data to eqn. (3) we obtained $V = 8.45 \pm 0.34$ nmol of H_2/min , $K_s = 0.383 \pm 0.034$ mM, $K_i = 90.6 \pm 5.5$ μM , $\alpha = 0.0008 \pm ?$. The program failed to define a standard error for α , because the calculated variance was found to be large and negative: this is usually an indication that the parameter in question is ill-defined. The sum of squares (defined as above) was 2.40, with a corresponding estimate of $\pm 15\%$ for the experimental standard error for v . This result led us to consider a modified Scheme 3 from which the mixed species ABIS was omitted, with an equation the same as eqn. (3) with the terms in α omitted. The best fit of the data in this case, gave, to within 0.1%, the same values as above

for V , K_s , K_i , their standard errors, and the sum of squares, with essentially the same estimate of the experimental standard error.

The data fit Scheme 2 rather better than Scheme 3, though the difference is not great. There is evidence that binding of MgADP at either site prevents binding of MgATP at either site, and vice versa, as the fit of Scheme 3 was not improved by allowing a mixed complex. Nonetheless, both models fit the data appreciably less well than one would hope from the reproducibility of replicate observations. These allowed an estimate of the 'pure error sum of squares' (see Draper & Smith, 1966), independent of the correctness of the model fitted, of 0.330 with 59 degrees of freedom, indicating the true experimental standard error to be of the order of 7.5%, about half of the value obtained with either model. The significance of this result is probably exaggerated by the experimental design required by practical considerations: it was necessary to make the observations (112 in all) over a period of several weeks, and the replicates are unlikely to have been strictly independent. Nonetheless it does seem likely that a better model than Scheme 2 exists, though we have not been able to get much improvement in fit by means of more complicated models. For example, we have tried an eight-parameter model in which ES, ES₂,

EIS, EI and EI₂ all exist with no relationships between dissociation constants, and in which ES, ES₂ and EIS all yield products with unrelated rate constants: this model gave an estimate of 10% for the experimental standard error, a fairly modest improvement on Scheme 2, and parameter values that did not suggest any meaningful simple model.

For the present we prefer to regard Scheme 2 as a working hypothesis for the enzyme. There have been four reports that indicate that different conformations of the Fe-protein are stabilized by MgADP and MgATP as required by Scheme 2: (1) iron-extraction studies with $\alpha\alpha'$ -bipyridine showed that MgATP but not MgADP enhanced the rate of transfer of iron from Cp2 protein to $\alpha\alpha'$ -bipyridine (G. A. Walker & Mortenson, 1974); (2) the change in symmetry of the $g = 1.94$ e.p.r. signal of Kp2 protein, although similar, is not the same for MgADP as for MgATP (B. E. Smith & D. J. Lowe, personal communication); (3) the accessibilities of the thiol groups of Kp2 protein to 5,5'-dithiobis-(2-nitrobenzoic acid) were different in the presence of MgADP and MgATP (Thorneley & Eady, 1973); (4) MgADP

but not MgATP inhibited the rate of reduction by dithionite of oxidized Fe-protein of *A. chroococcum* (Yates *et al.*, 1975). Unfortunately these studies do not provide any values of K_s and K_i for comparison with those obtained for Scheme 2 above from H_2 -evolution data. The pre-steady-state section above has shown that a complex of reduced Fe-protein+semi-reduced Mo-Fe-protein has dissociation constants for MgATP and MgADP different from those determined from the steady-state kinetics of H_2 evolution. No allosteric effects between MgADP and MgATP are evident from these pre-steady-state data. We conclude that the control of H_2 evolution by the MgATP/MgADP ratio is not at the level of electron transfer between the two proteins. Since the sites for MgATP and MgADP on reduced Kp2 protein are responsible for the control of electron transfer between the two proteins (Fig. 2), and do not show allosteric effects, the control of substrate reduction (H_2) could involve different MgATP-MgADP sites, possibly on the Mo-Fe-protein. Two roles for MgATP have been proposed by Smith *et al.* (1976b), one concerned with electron transfer between the component proteins, the other with substrate reduction. Thus Schemes 2 and 3 may refer to two identical subunits of either the Mo-Fe- or Fe-protein or of a complex of both proteins in an oxidation state or form (i.e. phosphorylated, adenylylated) which is only present during turnover.

The tight binding of MgADP relative to MgATP and the value of the 'allosteric constant', L , ensures rapid inhibition of substrate reduction for small decreases in the MgATP/MgADP ratio. The weak binding of MgATP presumably facilitates the selective 'switch-off' of the expensive (in terms of ATP requirement) nitrogenase while allowing other ATP-dependent enzymes to continue functioning under conditions of maintenance rather than growth of the organism.

We hope that further work may provide more definite experimental evidence for a kinetically significant conformation change, as well as data that permit the establishment of a more refined model for the steady-state kinetics.

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