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Nitrogenase of Klebsiella pneumoniae: An MgATP Hydrolysing Energy Transduction System with Similarities to Actomyosin and p21ras [and Discussion]

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Nitrogenase of *Klebsiella pneumoniae*: an MgATP hydrolysing energy transduction system with similarities to actomyosin and p21ras

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SUMMARY

The mechanism of ATP hydrolysis by nitrogenase shows some similarity to that proposed for actomyosin and for GTP hydrolysis by p21ras. All three systems involve the formation of an active complex from two component proteins, nucleotide-induced changes in protein conformation, energy transduction that in the case of nitrogenase involves a decrease in redox potential of metal centres, and a slow dissociation of the protein complex. Metal ion activation (Mg^{2+} or Ca^{2+}) and in-line displacement of ADP by H_2O without enzyme phosphorylation are also common features. At 5°C, stopped-flow calorimetry shows that the kinetic and thermodynamic parameters for endothermic, reversible on-enzyme cleavage of MgATP by nitrogenase and myosin subfragment 1 are remarkably similar. [$^{18}O_4$]P_i–water exchange studies also show that ATP cleavage on nitrogenase and myosin are reversible.

1. INTRODUCTION

Nitrogenases, which are only present in certain prokaryotes (diazotrophs such as *Klebsiella pneumoniae*, *Azotobacter vinelandii* or *Clostridium pasteurianum*) catalyse the reduction of dinitrogen to ammonia in a reaction that is coupled to the evolution of H_2 and hydrolysis of ATP. Under optimum conditions *in vitro*, the stoichiometry for the reaction is given by equation 1.

$$N_2 + 8H^+ + 8e^- + 16\ \text{MgATP} \xrightarrow{\text{nitrogenase}} 2\ NH_3 + H_2 + 16\ \text{MgADP} + 16\ P_i. \quad (1)$$

Thus for each electron transferred from reductant (flavodoxin or ferrodoxin *in vivo*, usually dithionite ion *in vitro*) through the enzyme to the reducible substrate (H^+ and N_2), a minimum of two equivalents of MgATP are hydrolysed. The ATP requirement for nitrogenase activity is intriguing not least because the reduction of N_2 to $2NH_4^+$ under physiological conditions is exergonic (Bayliss 1956), equation 2.

$$N_2 + 0.25\ \text{glucose} + 2H^+ + 1.5\ H_2O \rightarrow 2NH_4^+ + 1.5\ CO_2 \qquad \Delta G' = -117\ \text{kJ per } N_2 \text{ reduced.} \quad (2)$$

The aim of this article is to discuss our current understanding of the roles ATP and the hydrolysis products, ADP and P_i, play in the mechanism of nitrogenase action. Where appropriate comparisons will be made with other, perhaps better understood, energy transduction or signalling transduction proteins such as muscle actomyosin and p21ras. This review will be necessarily selective and will concentrate on recent results from my own laboratory where transient kinetic and spectroscopic techniques have been used to study the partial reactions that comprise the catalytic cycle of nitrogenase. Although these studies utilized the molybdenum-containing nitrogenase isolated from the facultative anaerobe, *K. pneumoniae*, all nitrogenases including the recently discovered vanadium systems from the aerobes *A. vinelandii* and *A. chroococcum* are thought to have a common mechanism.

For more detailed background information, the reviews by Orme-Johnson (1985), Thorneley & Lowe (1985), Smith *et al.* (1987), and by Eady (1991) can be consulted.

2. STRUCTURES OF THE TWO PROTEINS THAT COMPRISE NITROGENASE

Nitrogenase comprises two metallo-proteins, the MoFe-protein and the Fe-protein. Both proteins are required for activity. Throughout this article the nitrogenase components of the various organisms are denoted by a capital letter indicating the genus and a lower case indicating the species; the number 1 indicates the MoFe-protein and the number 2 the Fe-protein: Kp, *Klebsiella pneumoniae* (oxytoca); Av, *Azotobacter vinelandii*; Ac, *Azotobacter chroococcum*; Rr, *Rhodospirillum rubrum*.

(*a*) *The Fe-protein*

The Fe-proteins of all nitrogenases are homodimers with a subunit M_r in the range 28 000–36 000. Their sequences show a high degree of homology (see the

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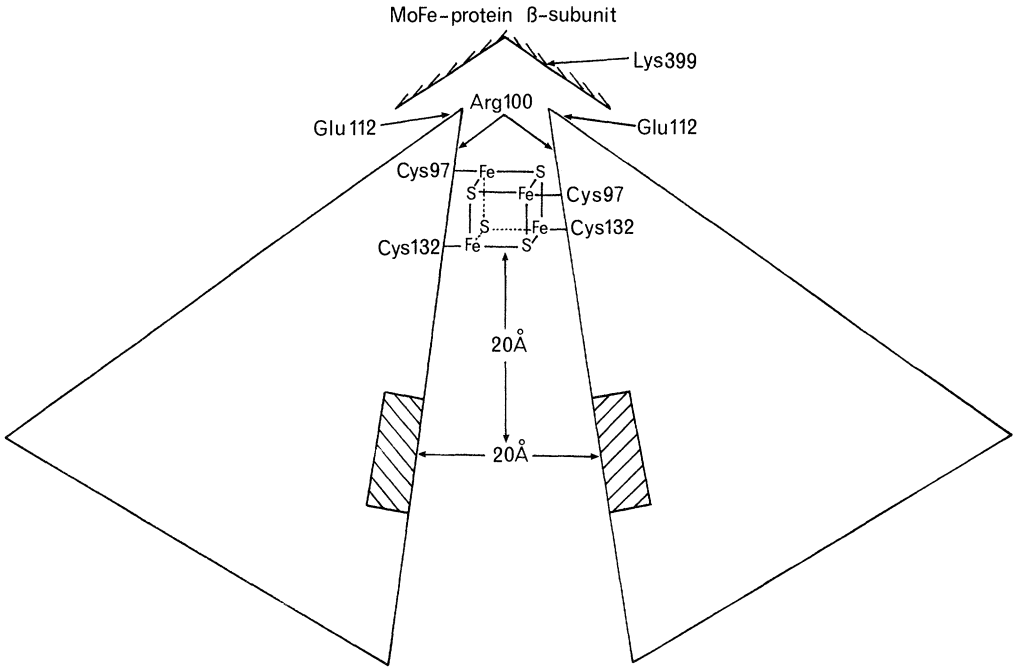

Figure 1. Representation of nitrogenase Fe-protein (Av2) showing the 4Fe–4S cluster bridging the two subunits. Two nucleotide binding sites are shown (shaded blocks) as are the residues at the top of the molecule that interact with the β-subunit of the MoFe-protein (Av1) on complex formation (after Georgiadis *et al.* 1990).

review by Eady (1986) and references therein) with five conserved cysteine residues. Chemical modification (Hausinger & Howard 1983) and site directed mutagenesis (Howard *et al.* 1988) of Av2 suggested that the single 4Fe–4S cubane cluster is held between the two subunits by coordination of each of the four Fe atoms to either a Cys-97 or a Cys-132 located in each subunit. This has been conformed by the X-ray structure of Av2 at 3 Å† resolution from Rees' laboratory (Geordiadis *et al.* 1990). A cartoon of this structure is shown in figure 1.

Two βαβ nucleotide binding folds containing the consensus sequence Gly X Gly XX Gly beginning at position 9, that were originally recognized by sequence comparison (Robson 1984), have now been confirmed in the X-ray structure. They are shown schematically in figure 1 on each side of the cleft that separates the two subunits. Although the position of the adenine-binding site has not been precisely located, a molybdate derivative indicates that the terminal phosphate group of ATP is probably located close to the above consensus sequence. This places the ATP-binding sites at *ca.* 20 Å from the 4Fe–4S cluster. The two molybdate sites are also 20 Å distant from each other. These data are entirely consistent with the results of nuclear magnetic resonance (NMR) (Meyer *et al.* 1988), electron paramagnetic resonance (EPR)-stimulated spin echo (Morgan *et al.* 1990) and electron nuclear deouble resonance (ENDOR) experiments (D. J. Lowe & B. D. Howes, unpublished results) that failed to detect any interaction between the paramagnetic, reduced 4Fe–4S cluster and ^{31}P of bound MgATP, MgADP or ^{19}F of Mgp[CHF]ppA (adenosine 5′-(βγ-fluoromethylene) triphosphate). The significance of this is that the changed properties of the 4Fe–4S cluster that occur on nucleotide binding are not the result of a direct interaction but must be mediated by a change in protein structure. The 4Fe–4S clusters of native Fe-proteins have E_m values in the range −200 mV to −393 mV (NHE) depending on source, exhibit a rhombic $S=\frac{1}{2}$, $g_{av}=1.94$ and a $S=\frac{3}{2}$, $g_{av}\approx 4.0$ EPR signal and react slowly with iron chelating agents. When two equivalents of MgATP are bound, the E_m values shift by *ca.* −120 mV, the EPR signal becomes more axial and the 4Fe–4S cluster is rapidly destoyed by iron chelating agents. However pulsed EPR studies show that in both the native and MgATP states, there is rapid (less than 15 s) exchange of D_2O with H_2O near the 4Fe–4S cluster and that the MgATP-induced conformation change does not alter the unusual crystal field parameters of the cluster (Morgan *et al.* 1990).

Although the Fe-protein binds MgATP, hydrolysis only occurs in the presence of the MoFe-protein (Imam & Eady 1980; Thorneley *et al.* 1991). Protein complex formation therefore either induces a further conformation change in the Fe-protein that triggers MgATP hydrolysis or residues on the MoFe-protein that are required to complete the MgATP hydrolysis active site move into place when the protein complex forms.

The X-ray structure of Av2 (figure 1) shows that Arg-100 is on the surface of the protein close to the 4Fe–4S cluster. This residue is reversibly ADP-ribosylated in Rr2 (and several other Fe proteins) as part of a regulatory mechanism in response to ammonia levels (Ludden & Roberts 1989). Replacing Arg 100 by His in Kp2 produces an inactive nitrogenase (Lowery *et al.* 1989) as does ADP-ribosylation. These data suggest that the surface of the Fe-protein in the vicinity of the 4Fe–4S cluster docks with the MoFe-

† 1 Å = 10^{-10} m = 10^{-1} nm.

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protein. This is not surprising as in the catalytic cycle, electron transfer has to occur from the 4Fe–4S cluster of the Fe-protein to an as yet unidentified site on the MoFe-protein. It is not known how extensive this contact area on the Fe-protein is or whether there are additional contacts between the proteins, for instance in the vicinity of the MgATP binding sites. It may not be be a coincidence that the *ca.* 20 Å distance between the MgATP binding sites and the 4Fe–4S cluster of the Fe-protein matches that between the 'P' and 'M' centres of the FeMo protein (see § 2*b* and the review by Mortenson (1987)). Cross linking studies have shown that Glu-112 on Av2 interacts with Lys-399 on the β-subunit of Av1 protein as is shown in figure 1 (Willing *et al.* 1989; Willing & Howard 1990).

(*b*) *The MoFe Protein*

All MoFe-proteins are $\alpha_2\beta_2$ tetramers, M_r in the range 200–240K, with subunits of M_r *ca.* 55K and 60K. Fully active MoFe-proteins contain 2 Mo atoms, about 30 Fe atoms and 30 S^{2-} ions. A schematic description of the arrangement of these inorganic components is shown in figure 2. This is based on the 5 Å resolution structure of Cp1 obtained by X-ray anomalous scattering (Bolin *et al.* 1990).

Sixteen of the Fe atoms form two 'P' centres each of which probably has two 4Fe–4S clusters. There is currently much debate as to the precise nature of these centres, in particular whether each 'P' centre should be considered to be a single 8F3–8S cluster (Hagen *et al.* 1987). The rest of the Fe atoms and the two Mo atoms comprise two FeMo cofactors or 'M' centres which have poorly defined stoichiometry of $MoFe_{7\pm1}S_{6\pm2}$. Each FeMo-cofactor also contains a homocitrate ligand (Hoover *et al.* 1989). The X-ray data of Bolin *et al.* (1990) locate the 'M' centres *ca.* 70 Å apart from each other and *ca.* 19 Å from the nearest 'P' cluster. The 'M' centres, which are located in the α subunits, are the site of substrate reduction. The role of the 'P' clusters is unknown but it is reasonable to assume that at some stage in the catalytic cycle they are involved in electron transfer to the 'M' centres.

In the context of this article, the interactions of the MoFe-protein with MgATP and MgADP are more relevant than a more detailed description of the metal

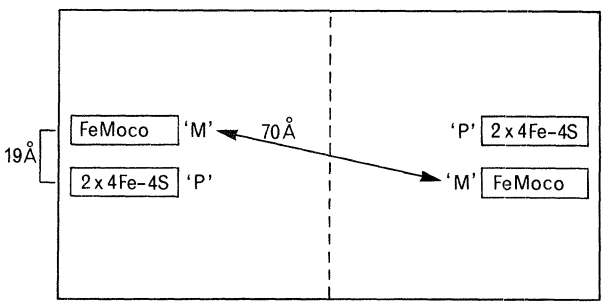

Figure 2. Representation of nitrogenase MoFe-protein (Cp1) showing the relative positions of the FeMo-cofactors ('M' centres) and eight 4Fe–4S clusters ('P' centres) (after Bolin *et al.* 1990).

centres (see review by Eady (1991)). Reduced Kp1 binds four equivalents of MgATP in a non-cooperative manner with a $K_D \approx 600$ μM (Miller *et al.* 1980). The bound MgATP is not hydrolysed. Although Miller *et al.* (1980) and Miller & Eady (1989) could not demonstrate MgADP binding to reduced Kp1 and Ac1, tight binding was observed after the 'P' centres had been selectively oxidized using a dye (R. R. Eady, R. W. Miller & B. E. Smith, personal communication). These experiments establish for the first time a link between redox state of the MoFe-protein and affinity for nucleotides. The location of the nucleotide binding sites on the MoFe-protein is not known. They may have a function independent of the sites on the Fe-protein or may be complementary and allow MgATP to bridge across the interface of the two proteins. This latter possibility is consistent with each MoFe-protein binding two Fe-proteins and with ATP hydrolysis being a property of the protein complex not of the individual proteins (see §§ 5 and 6).

3. AN OXIDATION–REDUCTION CYCLE FOR Fe-PROTEIN INVOLVING MgATP HYDROLYSIS

Scheme 1 is an expanded version of the Fe-protein cycle originally described by Thorneley & Lowe (1983). It comprises two coupled and competing cycles designated *a* and *b*. Cycle *a* has been resolved into six partial reactions that result in the transfer of a single electron from $Kp2(MgATP)_2$ to Kp1 and the hydrolysis of two equivalents of MgATP. This cycle is repeated eight times to effect the reduction of N_2 with concomitant H_2 evolution (equation 1). Cycle *b* of scheme 1, which has not been resolved into partial reactions, only involves MgATP hydrolysis with no electron transfer. The competition between cycles *a* and *b* accounts for the uncoupling of MgATP hydrolysis from electron transfer under conditions of low electron flux. In both cycles MgATP hydrolysis is associated with the Kp2-Kp1 protein complex. Neither protein has ATPase activity on its own (Iman & Eady 1980).

(*a*) *Complex formation*

The first step in cycle *a* is the rapid, reversible formation of a protein complex involving reduced $Kp2(MgATP)_2$ and $Kp1(k = 5 \times 10^7\ M^{-1}\ s^{-1}$ at 23 °C, Lowe & Thorneley 1984*b*). It is not known whether MgATP binding to Kp2 induces protein association or whether assembly of the $Kp2(MgATP)_2Kp1$ complex is random. Lowe & Thorneley (1984*a*) have argued that the close to diffusion controlled rate of protein complex assembly is an essential part of the mechanism that minimizes wasteful H_2 evolution that occurs prior to N_2 binding and reduction.

(*b*) *ATP cleavage and electron transfer*

The second and third reactions of cycle *a* are MgATP cleavage to yield $MgADP + P_i$ bound to the complex followed by electron transfer from Kp2 to

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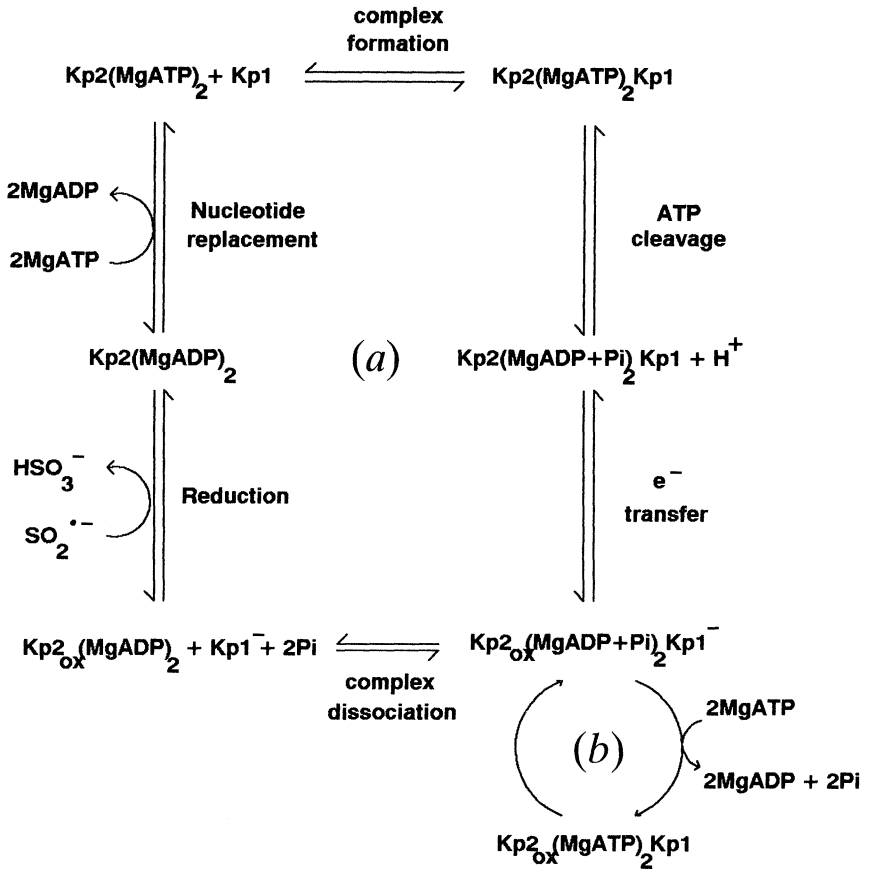

Scheme 1.

The Fe-protein cycle. (*a*) Reductant-dependent ATPase cycle; (*b*) reductant-independent ATPase cycle.

Kp1. These reactions have recently been resolved at 6°C using stopped-flow calorimetry to monitor the cleavage step ($k_{obs} = 9\ s^{-1}$) and stopped-flow spectrophotometry to observe the electron transfer reaction ($k_{obs} = 3\ s^{-1}$, Thorneley *et al.* 1989). At 10°C, Eady *et al.* (1978) showed that electron transfer and P_i release on acid quenching occurred with the same rate ($k_{obs} = 24\ s^{-1}$). At 23°C, the temperature usually used in this laboratory to study nitrogenase kinetics, electron transfer occurs with $k = 200\ s^{-1}$. MgATP cleavage occurs too rapidly at 23°C to be monitored by rapid chemical quench or using the calorimeter currently available (Millar *et al.* 1987). These steps of the cycle are discussed in greater detail in §4 where a comparison is made with the myosin subfragment 1 system.

(*c*) *Complex dissociation*

The fourth step in cycle 1 is the slow dissociation of $Kp2_{ox}(MgADP)_2$ from reduced Kp1. This reaction is the rate-limiting step for nitrogenase activity when all components are at saturating concentrations (Thorneley & Lowe 1983). At 23°C, the rate of complex dissociation ($k = 6.4\ s^{-1}$), which was measured after mixing $Kp2_{ox}(MgADP)_2$ with Kp1 in the absence of P_i, predicts the measured rate of substrate reduction (i.e. electron flux). This indicates that P_i release, which necessarily follows ATP cleavage, does not limit the rate of protein complex dissociation in a system that is hydrolysing MgATP. Data obtained at 6°C, when ATP hydrolysis is uncoupled from electron transfer show that dissociation of $Kp2_{ox}(MgADP)_2$ is not required for the replacement of $MgADP + P_i$ by MgATP. This is shown in cycle *b* of scheme 1 which competes with cycle *a* via the common intermediate $Kp2_{ox}(MgADP)_2Kp1$. In the absence of $Na_2S_2O_4$, it is this protein complex that accounts for 'reductant independent' ATPase activity (see §5). How far the ATP:$2e^-$ ratio (a measure of nitrogenase efficiency) deviates from the optimum value of 4 (two turns of cycle *a*) depends on the steady-state level of this intermediate. This is determined by the pH, temperature, relative and absolute concentrations of the Fe- and MoFe-proteins and the concentration and nature of the reductant ($Na_2S_2O_4$ or flavodoxin).

(*d*) *Reduction of oxidized Fe-protein and replacement of MgADP by MgATP*

Ashby & Thorneley (1987) showed that reduction of $Kp2_{ox}(MgADP)_2$ by $SO_2^{\bullet -}$ ($k = 3 \times 10^6\ M^{-1}\ s^{-1}$, step 5 in scheme 1, cycle *a*) precedes the release of 2MgADP and subsequent binding of 2MgATP. This sequence is due to the tight binding of 2MgADP to $Kp2_{ox}$ (composite $K > 4 \times 10^{10}\ M^{-2}$) and its slow release, which is probably limited by a conformation change in the protein ($k < 2\ s^{-1}$). Loss of 2MgADP

from reduced Kp2(MgADP)$_2$ is rapid ($k \approx 2 \times 10^2$ s^{-1}, Thorneley & Cornish-Bowden 1977) as is the binding of 2MgATP ($k > 2 \times 10^6$ M^{-1} s^{-1}, Thorneley, 1975). The tighter binding of MgADP and MgATP to Kp2$_{ox}$ is consistent with the *ca.* 150 mV lower mid-point potentials of the nucleotide bound protein (Thorneley & Deistung 1988). Kp2$_{ox}$ is reduced by $SO_2^{\bullet -}$ ($k > 10^8$ M^{-1} s^{-1}) much more rapidly than is KP2$_{ox}$(MgADP)$_2$ ($k = 3 \times 10^6$ M^{-1} s^{-1}, Ashby & Thorneley 1987). The decrease in rate of reduction and the lowering of the mid-point potential are a consequence of the change in protein conformation, not the binding of MgADP *per se*.

(*e*) *The reduction level of the MoFe-protein does not affect the kinetics of the Fe-protein cycle*

Under optimum conditions, the completion of one Fe-protein cycle results in the transfer of one electron from the Fe-protein to the MoFe-protein and the hydrolysis of two equivalents of MgATP, i.e. cycle *a*, with a negligible contribution from cycle *b* in scheme 1. Lowe & Thorneley (1984*a*, *b*) and Thorneley & Lowe (1984*a*, *b*) showed how eight of these Fe-protein cycles could be coupled together to give one MoFe-protein cycle which describes the binding and reduction of substrates (H^+ and N_2) and the product release sequence (H_2 then $2NH_3$). A complete description of the MoFe-protein cycle is not appropriate in this article. However a key assumption of the Lowe–Thorneley scheme that is relevant, is that the rate constants of the Fe-protein cycle are independent of the reduction level of the MoFe-protein. Fisher *et al.* (1991) recently verified this assumption for the first two Fe-protein cycles by demonstrating that the rates of complex formation ($k = 5 \times 10^7$ M^{-1} s^{-1}), electron transfer ($k_{obs} = 140$ s^{-1}) and oxidized-complex dissociation ($k = 6.4$ s^{-1}) are the same for Kp1 in states E_o (first Fe-protein cycle) and E_1H (second Fe-protein cycle). This suggests that on reduction there is no change in protein conformation or charge density at the surface of Kp1 that docks with Kp2. It is also consistent with proton transfer being coupled to electron transfer from Kp2 such that the additional negative charge generated on Kp1 is neutralized by formation of a metal-hydride at the FeMo-cofactor. Such intermediates not only account for competing H_2 evolution reactions but also suggest a mechanism for N_2 binding by H_2 displacement that explains the limiting stoichiometry of one H_2 evolved for each N_2 reduced (equation 1) (see Thorneley & Lowe (1985) for a review of the mechanism of H_2 evolution and N_2 reduction).

4. SOME SIMILARITIES BETWEEN THE MECHANISMS OF MgATP HYDROLYSIS BY NITROGENASE AND ACTOMYOSIN

Mortenson *et al.* (1985) used ^{31}P-NMR to characterize the stereochemistry of the thiophosphate released from nitrogenase when it hydrolyses chiral ATP-γ-S labelled in the γ-phosphate with ^{18}O, ^{17}O and ^{16}O. They concluded that ATP-γ-S, and by inference ATP, hydrolysis occurs by in-line displacement of ADP by water oxygen with no phospho-enzyme intermediate. McKenna *et al.* (1989) used ^{18}O-labelled ATP to show that it is the $P_{(\gamma)}$—$^{18}OP_{(\beta)}$ bond that is cleaved in this reaction. A similar one step mechanism for ATP hydrolysis is thought to occur on p21ras (Feuerstein *et al.* 1989) and with actomyosin (see Hibberd & Trentham (1986) for a review).

Kinetic and thermodynamic similarities for the on-enzyme ATP-cleavage reaction occurring on myosin subfragment-1 and the nitrogenase protein complex have been shown by stopped-flow calorimetry (Thorneley *et al.* 1989). The apparatus described by Howarth *et al.* (1987) was originally used to study the partial reactions of myosin subfragment 1 ATPase activity at 5°C, pH7 (Millar *et al.* 1987). The same reaction conditions were used by Thorneley *et al.* (1989) to study the on-enzyme hydrolysis of MgATP by nitrogenase (step 2, scheme 1).

Figure 3 shows the similarity of the calorimetric traces recorded after mixing either myosin (*a*) or

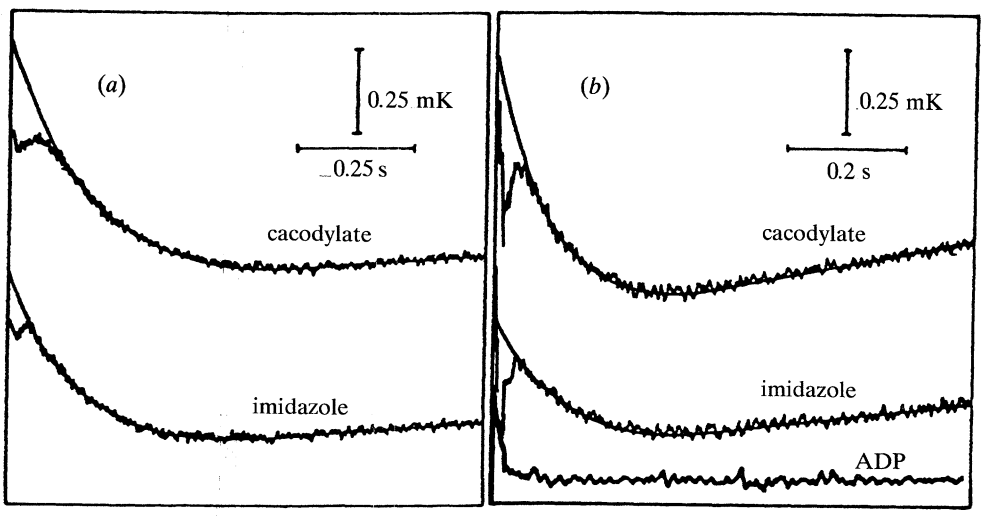

Figure 3. Stopped-flow calorimetry traces showing the endothermic, on enzyme cleavage of MgATP. (*a*) Myosin; (*b*) Kp-nitrogenase (after Thorneley *et al.* 1989).

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Table 1. *Kinetic and thermodynamic parameters for MgATP cleavage on nitrogenase and myosin (data from Thorneley* et al. *1989)*

$$\text{E.MgATP} \underset{k_{obs}\Delta H_{obs}}{\overset{K}{\rightleftharpoons}} \text{E(MgADP} + \text{P}_i) + \text{H}^+$$

	nitrogenase	myosin	T °C
k_{obs}/s^{-1}	9	7	5
k_{obs}/s^{-1}	>140[a]	160	23
$\Delta H_{obs}/(kJ\ mol\ ATP^{-1})$	$+36$ (72)[b]	$+32$ (64)	5
H^+ release (observed)	0.5 (1.0)	0.20 (0.4)	5
equilibrium constant K	≈ 1	1	5
	$\geqslant 10$	9	23

[a] Assuming ATP cleavage precedes electron transfer.
[b] Numbers in parentheses corrected for K represent complete cleavage.

nitrogenase (*b*) with MgATP in two different buffers. A comparison of the amplitudes of the effects in two buffers with different enthalphies of ionization (36 kJ mol^{-1} for imidazole, zero for cacodylate), allows any associated proton release or uptake to be measured. Table 1 summarizes the kinetic and thermodynamic parameters for the two systems. The endothermic reaction that results in a large temperature dependence for the rate constant shows the similarity of the ATP-cleavage reaction of nitrogenase and myosin subfragment 1. The endothermic process observed for myosin was correlated with the on-enzyme cleavage of ATP since the rate was similar to that of the 'phosphate burst' reported from rapid quench experiments (Sleep & Taylor 1976). MgADP, which is a competitive inhibitor of MgATP-dependent nitrogenase activity (Thorneley & Cornish-Bowden 1977) and which also induces a conformation change in Kp2 (see § 1a), did not produce any enthalpy change measurable in the stopped-flow calorimeter. This observation supports the assignment of the effects shown in figure 3*b* to MgATP cleavage and not to a change in protein conformation.

At 5°C, the cleavage of MgATP on myosin is reversible with an equilibrium constant of unity (table 1). Thorneley *et al.* (1989) concluded that a similar equilibrium probably occurs on nitrogenase at 5°C because the amplitude of the absorbance change associated with electron transfer (step 3 in scheme 1) was *ca.* 50% of that observed at 23°C. In addition, because electron transfer occurred with $k_{obs} = 3\ s^{-1}$, *ca.* one third as quickly as the MgATP cleavage step ($k_{obs} = 9\ s^{-1}$), they concluded that MgATP hydrolysis precedes electron transfer as is shown in steps 2 and 3 of scheme 1. However, a simple coupled equilibrium may be a simplification since neither the calorimetry data (step 2) nor the spectrophotometric data for electron transfer (step 3) were biphasic. This may be explained in the case of the calorimetric data by compensating exothermic reactions, possibly associated with the electron transfer reaction. However, further evidence for reversible cleavage of ATP on nitrogenase is provided by the MgADP catalysed $[^{18}O_4]P_i$–water oxygen exchange data discussed in § 6.

In the actomyosin system, P_i release is exothermic, rate determining and associated with a protein association reaction (myosin–actin) which is the energy coupling step in muscle contraction (Hibberd & Trentham 1986). It is possible that with nitrogenase, some of the free energy available after MgATP cleavage to yield bound MgADP + P_i but before or concomitant with P_i release, is used to lower the potential of the already reduced substrate binding site on the MoFe-protein. This energy transduction step may also trigger the dissociation of the Fe-protein – MoFe-protein complex that is obligate for substrate binding to MoFe-protein and reduction of oxidized Fe-protein. The key to understanding the mechanism of energy transduction by nitrogenase is probably the P_i release step which will have to be studied without the perturbations associated with rapid quenching methods.

5. MgATP HYDROLYSIS IN THE ABSENCE OF ELECTRON TRANSFER – REDUCTANT-INDEPENDENT ATPase ACTIVITY

Studying nitrogenase–nucleotide interactions under conditions which only allow reductant-independent ATPase activity ($Kp2_{ox}$ with Kp1 in the absence of $Na_2S_2O_4$) has the advantage that complications due to the exhaustion of $Na_2S_2O_4$ and a change from reductant-dependent (cycle *a*, scheme 1) to reductant-independent (cycle *b*, scheme 1) ATPase activities can be avoided.

Figure 4 shows linear timecourses obtained by Thorneley *et al.* (1991) for the release of P_i during reductant-independent ATP hydrolysis. No P_i is formed when either Kp1 or $Kp2_{ox}$ alone are present in the assay, showing that ATPase activity is a property of the $Kp2_{ox}$Kp1 protein complex. MgADP is a competitive inhibitor ($K_i^{MgADP} = 145\ \mu M$) of reductant-independent ATP hydrolysis ($K_i^{MgATP} = 400\ \mu M$). This value of (K_m^{MgATP} is identical to that previously determined for MgATP binding to the reduced Kp2Kp1 complex by monitoring the MgATP dependence of the electron transfer reaction by stopped-flow

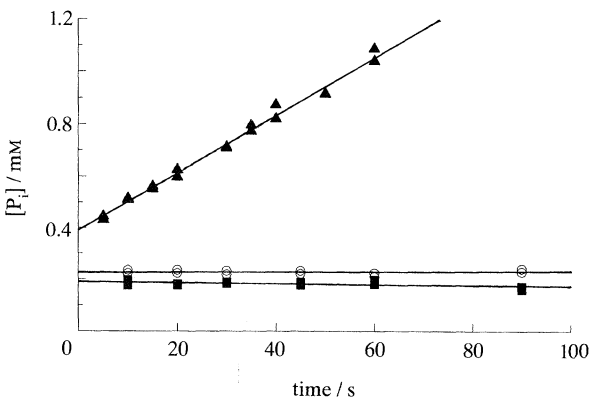

Figure 4. Reductant-independent ATPase activity is a property of the $Kp2_{ox}$ Kp1 protein complex (closed triangles) not of the isolated proteins, Kp1 (closed squares), $Kp2_{ox}$ (open circles) (after Thorneley *et al.* 1991).

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spectrophotometry (Thorneley 1975). The oxidation level of the Fe-protein does not therefore affect the affinity of the Kp2Kp1 complex for MgATP. However, as was discussed above, free $Kp2_{ox}$ binds MgADP ($K_D^{MgADP} < 10$ μM, Ashby & Thorneley 1987) much more tightly than does the $Kp2_{ox}Kp1$ complex ($K_i^{MgADP} = 145$ μM). This suggests a mechanism by which $Kp2_{ox}$ and Kp1 are induced to dissociate after the cleavage of MgATP in the catalytic cycle. The driving force for this reaction is the tighter binding of MgADP to free $Kp2_{ox}$ relative to its binding to the $Kp2_{ox}Kp1$ complex, i.e. $Kp2_{ox}(MgADP)_2$ must relax to a more stable conformation as it dissociates from Kp1.

6. THE ATP CLEAVAGE REACTION ON NITROGENASE IS REVERSIBLE

The on-enzyme MgATP cleavage step of the reductant-independent ATPase cycle (step 8 in cycle *b* of scheme 1) was shown to be reversible. Evidence for this was obtained by Thorneley *et al.* (1991) using the $[^{18}O_4]P_i$–water exchange technique developed by Hackney *et al.* (1980) and applied to muscle fibres by Webb *et al.* (1986). The technique is based on P_i being able to bind to the $Kp2_{ox}(MgADP)_2Kp1$ complex and if ATP cleavage is reversible, then an ^{18}O atom will be lost from $[^{18}O_4]P_i$ as $H_2\,^{18}O$ on formation of the $\beta\gamma$-phosphate bond (^{16}O-bridge originating from the β-phosphate) to yield protein bound MgATP. The bound MgATP will then be hydrolysed and P_i containing one oxygen from water (^{16}O) will be released. ADP and the protein complex therefore catalyse the exchange of oxygen atoms in the P_i. The key results of Thorneley *et al.* (1991) are shown in table 2 and figure 5.

The catalysis by MgADP was only significant when Kp1 and $Kp2_{ox}$ were present together in the assay. The extent of ^{18}O-exchange depends on the rate of formation and breakdown of bound ATP from and to P_i and ADP and on whether the phosphate group loses more than one oxygen atom while it remains bound to the protein. In the case of myosin subfragment 1, each phosphate group loses all its ^{18}O before it is released from the protein (Webb *et al.* 1978).

Table 2. *^{18}O-exchange from $[^{18}O_4]P_i$ catalysed by Kp nitrogenase in the presence and absence of ADP and p[CH_2]ppA at 23 °C, pH7.4 (data from Thorneley* et al. *1991)*

components[a]					velocity of exchange[b] (μM s^{-1})
$Kp2_{ox}$	+	Kp1	+	ADP	48.1, 48.9
$Kp2_{ox}$		—	+	ADP	12.7, 15.5
—		Kp1	+	ADP	5.8, 6.3
$Kp2_{ox}$	+	Kp1	+	p[CH_2]ppA	15.3, 16.0
$Kp2_{ox}$		—	+	p[CH_2]ppA	8.8, 8.7
—		Kp1	+	p[CH_2]ppA	4.5, 4.5
$Kp2_{ox}$	+	Kp1		—	25.3, 23.4
$Kp2_{ox}$		—		—	13.5, 13.2
—		Kp1		—	4.7, 4.9

[a] $[Kp2_{ox}] = 100$ μM; $[Kp1] = 33$ μM; $[ADP] = 250$ μM; p[CH_2] ppA = 1 mM; $[^{18}O]P_i = 5$ mM; $[MgCl_2] = 10$ mM.
[b] Replicate determinations.

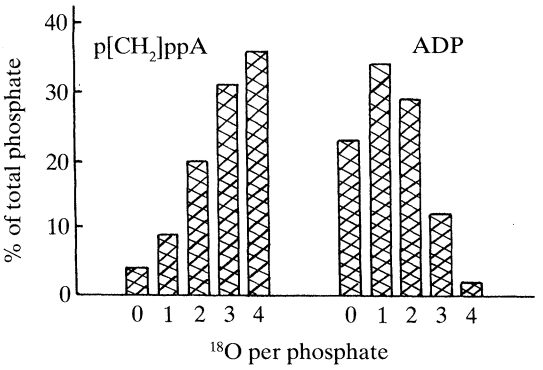

Figure 5. Distribution of ^{18}O in P_i after ADP-catalysed P_i–water oxygen exchange occurring on the $Kp2_{ox}$ Kp1 complex. The isotope distribution pattern for the ADP catalysed reaction shows that P_i release is rapid compared with the rate of ATP formation. The control using p[CH_2]ppA shows a small 'background exchange' with the major product unchanged $[^{18}O_4]P_i$ (after Thorneley *et al.* 1991).

The data for nitrogenase show that the ratio of the rate constants (Rp) for release of phosphate from E.MgADP.P_i and the formation of E.MgATP is greater than 2.5, i.e. there is little loss of a second ^{18}O atom before phosphate is released. For the phosphate to lose all four ^{18}O atoms, it must bind and dissociate at least four times. The large Rp value for nitrogenase is consistent with the positional isotope exchange (PIX) data reported by McKenna *et al.* (1989). In their study, ATP labelled with ^{18}O in the $\beta\gamma$-bridge position was recovered unchanged within the (5%) limit of the ^{31}P-NMR method used. They concluded that the forward reaction (ATP cleavage) must be significantly faster than the back reaction (reaction of ADP with P_i and MgATP release) unless rotation of the $P_{(\alpha)}$—O—$P_{(\beta)}$ bond is unusually restricted in the bound MgADP.

Thorneley *et al.* (1991) interpreted their $[^{18}O_4]P_i$–water oxygen exchange data in terms of a site formed or activated when $Kp2_{ox}$ and Kp1 form a complex. The same site is responsible for reductant-independent ATPase activity. This site has a group X that catalyses a relatively low rate of ^{18}O exchange from $[^{18}OP_i]$ in the absence of ADP. Group X may be located on either Kp1 or $Kp2_{ox}$ but it does not act as a nucleophile since hydrolysis of MgATP does not involve a phosphoenzyme intermediate (see §5). Group X may be analogous to the carbonyl group of Thr-35 in H-ras p21 which hydrogen bonds to water 175 in the structure of Pai *et al.* (1990). This water has been suggested as the one that performs the in-line nucleophilic attack on the γ-phosphate of GTP (Pai *et al.* 1990). The reversible nature of the MgATP cleavage reaction on nitrogenase is proposed to account for the ADP induced enhancement of ^{18}O exchange from $[^{18}O_4]P_i$. Such a mechanism does not require MgATP to be released from the protein

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complex, but only the transient reversible formation of ATP on the enzyme complex.

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REFERENCES

Ashby, G.A. & Thorneley, R.N.F. 1987 Nitrogenase of *Klebsiella pneumoniae*: kinetic studies on the Fe-protein involving reduction by sodium dithionite, the binding of MgADP and a conformation change that alters the reactivity of the 4Fe-4S centre. *Biochem. J.* **246**, 455–465.

Bayliss, N.S. 1956 The thermochemistry of biological nitrogen fixation. *Aust. J. biol. Sci.* **9**, 364–370.

Bolin, J.T., Ronco, A.E., Mortenson, L.E., Morgan, T.V., Williamson, M. & Xuong, N.-h. 1990 Structure of the nitrogenase MoFe-protein: spatial distribution of the intrinsic metal atoms determined by X-ray anomalous scattering. In *Nitrogen fixation: achievements and objectives* (ed. P. M. Gresshoff, L. Roth & G. Stacey), pp. 117–124. New York: Chapman Hall.

Eady R.R., Lowe, D.J. & Thorneley, R.N.F. 1978 Nitrogenase of *Klebsiella pneumoniae*: a pre-steady-state burst of ATP hydrolysis is coupled to electron transfer between the component proteins. *FEBS Lett* **95**, 211–213.

Eady, R.R. 1986 Enzymology in free-living diazotrophs. In *Nitrogen fixation*, vol. 4 (*Molecular Biology*) (ed. W. J. Broughton & A. Pühler), pp. 1–49. Oxford: Clarendon Press.

Eady, R.R. 1991 The Mo-, V-, and Fe-based nitrogenase system of *Azotobacter*. *Adv. inorg. Chem.* **36**, 77–102.

Feuerstein, J., Goody, R.S. & Webb, M.R. 1989 The mechanism of guanosine nucleotide hydrolysis by p21 c-Ha-*ras*. The stereochemical course of the GTPase reaction. *J. biol. Chem.* **264**, 6188–6190.

Fisher, K., Lowe, D.J. & Thorneley, R.N.F. 1991 *Klebsiella pneumoniae* nitrogenase. The pre-steady-state kinetics of MoFe-protein reduction and hydrogen evolution under conditions of limiting electron flux show that the rates of association with Fe-protein and electron transfer are independent of the oxidation level of the MoFe-protein. *Biochem. J.* **279**, 81–85.

Georgiadis, M.M., Chakrabarti, P. & Rees, D.C. 1990 Crystal structure of the nitrogenase iron protein from *Azotobacter vinelandii*. In *Nitrogen fixation: achievements and objectives* (ed. P. M. Gresshoff, L. Roth & G. Stacey), pp. 111–116. New York: Chapman & Hall.

Hackney, D.D., Stempel, K.E. & Boyer, P.D. 1980 Oxygen-18 probes of enzymic reactions of phosphate compounds. *Methods Enzymol.* **64**, 60–83.

Hagen, W.R., Wassink, H., Eady, R.R., Smith, B.E. & Haaker, H. 1987 $S=\frac{7}{2}$ epr of P-clusters in thionine oxidised MoFe-proteins of nitrogenase. *Recuil des Travaux des Pays-Bas* **106**, 229.

Hausinger, R.P. & Howard, J.B. 1983 Thiol reactivity of the nitrogenase Fe-protein from *Azotobacter vinelandii*. *J. biol. Chem.* **258**, 13 486–13 492.

Hibberd, M.G. & Trentham, D.R. 1986 Relationship between chemical and mechanical events during muscular contraction. *A. Rev. Biophys. Chem.* **15**, 119–161.

Hoover, T.R., Imperial, J., Ludden, P.W. & Shah, V.K. 1989 Homocitrate is a component of the iron-molybdenum cofactor of nitrogenase. *Biochemistry* **28**, 2768–2771.

Howard, J.B., Davis, R., Moldenhauer, B., Cash, V.L. & Dean, D. 1989 Fe:S cluster ligands are the only cysteines required for nitrogenase Fe-protein activities. *J. biol. Chem.* **264**, 11 270–11 274.

Howarth, J.V., Millar, N.C. & Gutfreund, H. 1987 A stopped-flow calorimeter for biochemical applications. *Biochem. J.* **248**, 677–682.

Imam, S. & Eady, R.R. 1980 Nitrogenase of *Klebsiella pneumoniae*: reductant-independent ATP hydrolysis and the effect of pH on the efficiency of coupling of ATP hydrolysis to substrate reduction. *FEBS Lett.* **110**, 35–38.

Lowe, D.J. & Thorneley, R.N.F. 1984*a* The mechanism of *Klebsiella pneumoniae* nitrogenase action. Pre-steady state kinetics of H_2 formation. *Biochem. J.* **224**, 877–886.

Lowe, D.J. & Thorneley, R.N.F. 1984*b* The mechanism of *Klebsiella pneumoniae* nitrogenase action. The determination of rate constants required for the simulation of the kinetics of N_2 reduction and H_2 evolution. *Biochem. J.* **224**, 895–901.

Lowery, R., Chang, C., Davis, L., McKenna, M.C., Stephens, P. & Ludden, P. 1989 Substitution of histidine for arginine-101 of dinitrogenase reductase disrupts electron transfer to dinitrogenase. *Biochemistry* **28**, 1206–1212.

Ludden, P.W. & Roberts, G.P. 1989 Regulation of nitrogenase activity by reversible ADP ribosylation. *Curr. Top. cell. Regul* **30**, 23–56.

McKenna, C.E., Gutheil, W.G., Kenyon, G.L. & Matsunaga, T.O. 1989 Mass spectrometric analysis of phosphate from β,γ-[^{18}O]ATP hydrolysed by *Azotobacter vinelandii* nitrogenase: direct evidence for P_γ-OP_β bond cleavage. *Bioorg. Chem.* **17**, 377–384.

Meyer, J., Gaillard, J. & Moulis, J.-M. 1988 Hydrogen-1 nuclear magnetic resonance of the nitrogenase iron protein (Cp2) from *Clostridium pasteurianum*. *Biochemistry* **27**, 6150–6156.

Millar, N.C., Howarth, J.V. & Gutfreund, H. 1987 A transient kinetic study of the enthalpy changes during the reaction of myosin subfragment-1 with ATP. *Biochem. J.* **248**, 683–690.

Miller, R.W. & Eady, R.R. 1989 Molybdenum nitrogenase of *Azotobacter chroococcum*. Tight binding of MgADP to the MoFe-protein. *Biochem. J.* **263**, 725–729.

Miller, R.W., Robson, R.L., Yates, M.G. & Eady, R.R. 1980 Catalysis of exchange of terminal phosphate groups of ATP and ADP by purified nitrogenase proteins. *Can. J. Biochem.* **58**, 542–547.

Morgan, T.V., McCracken, J., Orme-Johnson, W.H., Mimms, W.B., Mortenson, L.E. & Peisach, J. 1990 Pulsed electron paramagnetic resonance studies of the interaction of MgATP and D_2O with the iron protein of nitrogenase. *Biochemistry* **29**, 3077–3082.

Mortenson, L.E., Webb, M.R., Bare, R., Cramer, S.P. & Morgan, T.V. 1985 Studies on the structure and function of the MoFe protein of nitrogenase. In *Nitrogen fixation research progress* (ed. H. J. Evans, P. J. Bottomley & W. E. Newton), pp. 577–583. Boston: Martinus Nijhoff.

Mortenson, L.E. 1987 ATP and nitrogen fixation. In *Inorganic nitrogen metabolism* (ed. W. R. Ullrich, P. J. Aparicio, P. J. Syrrett & F. Castillo), pp. 165–172. Berlin: Springer Verlag.

Orme-Johnson, W. H. 1985 Molecular basis of biological nitrogen fixation. *A. Rev. Biophys. biophys. Chem.* **14**, 419–459.

Pai, E., Krengel, U., Petsko, G.A., Goody, R.S., Kabsch, W. & Wittinghofer, A. 1990 Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 Å resolution. Implications for the mechanism of GTP hydrolysis. *EMBO J.* **9**, 2351–2359.

Robson, R.L. 1984 Identification of possible adenine nucleotide-binding sites in nitrogenase proteins by amino acid sequence comparison. *FEBS Lett.* **173**, 394–398.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Sleep, J.A. & Taylor, E.W. 1976 Intermediate states of actomyosin adenosine triphosphatase. *Biochemistry* **15**, 5813–5817.

Smith, B.E., Campbell, F., Eady, R.R., Eldridge, M., Ford, C.M., Hill, S., Kavanagh, E.P., Lowe, D.J., Miller, R.W., Richardson, T.H., Robson, R.L., Thorneley, R.N.F. & Yates, M.G. 1987 Biochemistry of nitrogenase and the physiology of related metabolism. *Phil. Trans. R. Soc. Lond.* B **317**, 131–146.

Thorneley, R.N.F. 1975 Nitrogenase of *Klebsiella pneumoniae*, a stopped-flow study of magnesium-adenosine triphosphate-induced electron transfer between component proteins. *Biochem. J.* **145**, 391–396.

Thorneley, R.N.F. & Cornish-Bowden, A. 1977 Kinetics of nitrogenase of *Klebsiella pneumoniae*. Heterotropic interactions between magnesium-adenosine 5′-diphosphate and magnesium-adenosine 5′-triphosphate. *Biochem. J.* **165**, 255–262.

Thorneley, R.N.F. & Lowe, D.J. 1983 Nitrogenase of *Klebsiella pneumoniae*. Kinetics of the dissociation of oxidised iron protein from molybdenum-iron protein: identification of the rate-limiting step for substrate reduction. *Biochem. J.* **215**, 393–403.

Thorneley, R.N.F. & Lowe, D.J. 1984*a* The mechanism of *Klebsiella pneumoniae* nitrogenase action. Pre-steady state kinetics of an enzyme-bound intermediate in N_2 reduction and NH_3 formation. *Biochem. J.* **224**, 887–894.

Thorneley, R.N.F. & Lowe, D.J. 1984*b* The mechanism of *Klebsiella pneumoniae* nitrogenase action. Simulation of the dependence of H_2 evolution rate on component-protein concentration and ratio and sodium dithionite concentration. *Biochem. J.* **224**, 903–909.

Thorneley, R.N.F. & Lowe, D.J. 1985 Kinetics and mechanism of the nitrogenase enzyme system. In *Molybdenum enzymes* (ed. T. G. Spiro), pp. 221–284. New York: J. Wiley & Sons.

Thorneley, R.N.F. & Deistung, J. 1988 Electron-transfer studies involving flavodoxin and a natural redox partner, the iron protein of nitrogenase. Conformational constraints on protein-protein interactions and the kinetics of electron transfer within the protein complex. *Biochem. J.* **253**, 587–595.

Thorneley, R.N.F., Ashby, G., Howarth, J.V., Millar, N.C. & Gutfreund, H. 1989 A transient-kinetic study of the nitrogenase of *Klebsiella pneumoniae* by stopped-flow calorimetry. Comparison with myosin ATPase *Biochem. J.* **264**, 657–661.

Thorneley, R.N.F., Ashby, G.A., Julius, C., Hunter, J.L. & Webb, M.R. 1991 Nitrogenase of *Klebsiella pneumoniae*. Reversibility of the reductant-independent MgATP-cleavage reaction is shown by MgADP-catalysed phosphate/water oxygen exchange. *Biochem. J.* **277**, 735–741.

Webb, M.R., Hibberd, M.G., Goldman, Y.E. & Trentham, D.R. 1986 Oxygen exchange between P_i in the medium and water during ATP hydrolysis mediated by skinned fibres from rabbit skeletal muscle. Evidence for P_i binding to a force-generating state. *J. biol. Chem.* **261**, 15 557–15 564.

Webb, M.R., McDonald, G.G. & Trentham, D.R. 1978 Kinetics of oxygen-18 exchange between inorganic phosphate and water catalysed by myosin subfragment-1, using the ^{18}O shift in ^{31}P-NMR. *J. biol. Chem.* **253**, 2908–2911.

Willing, A., Georgiadis, M.M., Rees, D.C. & Howard, J.B. 1989 Cross-linking of nitrogenase components. *J. biol. Chem.* **264**, 8499–8503.

Willing, A. & Howard, J.B. 1990 Cross-linking site in *Azotobacter vinelandii* complex. *J. biol. Chem.* **265**, 6596–6599.

Discussion

C. Cremo (*Washington State University, Pullman, U.S.A.*). Is there any information as to which part of the Fe-protein interacts with the MoFe-protein?

R. N. F. Thorneley. There is evidence that suggests contact between the MoFe-protein and the top of the Fe-protein in the vicinity of the 4Fe–4S cluster. This is perhaps not surprising since electron transfer has to occur from the 4Fe–4S cluster to the MoFe-protein. Howard and co-workers have shown that Glu-112 on the Fe-protein (Av2) cross-links to Lys-339 on the β-subunit of the MoFe-protein (Av1). ADP-ribosylation of Arg-100, which is also located close to the 4Fe–4S cluster, prevents electron transfer, presumably by interfering with complex formation with the MoFe-protein. The Rees structure of Av2 shows us that the 4Fe–4S cluster is *ca.* 19 Å distant from the proposed MgATP binding sites. This distance matches that between the 'P' and 'M' centres of the MoFe-protein on the Bolin structure for MoFe-protein (Cp1). This may be a coincidence or it may indicate an extensive area of interaction involving the 4Fe–4S cluster and MgATP sites of the Fe-protein.

H. Gutfreund (*University of Bristol, Bristol, U.K.*). Dr Thorneley told us that Kp2 will not hydrolyse ATP in the absence of Kp1. Bearing in mind the extremely slow rates of ATP hydrolysis by p21ras in the absence of GAP, has he looked for really slow ATP hydrolysis by Kp2 protein?

R. N. F. Thorneley. We have run reductant-independent ATPase assays with Kp2 protein alone for 60 min and not detected any P_i release. A difficulty is ensuring that the Kp2 protein is free of traces of contaminating Kp1 protein. I believe other groups working with Av2 protein that has been crystallized have failed to detect any ATPase activity.

D. Hackney (*Carnegie Mellon University, Pittsburgh, U.S.A.*). When Dr Thorneley was doing the experiments with [^{18}O]P_i did he also run those with the label in the terminal phosphate of ATP rather than having it in the phosphate pool of the medium?

R. N. F. Thorneley. We have not done those experiments but I think Martin Webb has with Len Mortenson using Cp nitrogenase.

M. R. Webb (*National Institute for Medical Research, London, U.K.*). Yes, and we got very little exchange in the product P_i.

D. Hackney. Would that result match the result you got with the label in the phosphate?

M. R. Webb. A direct comparison is difficult because the experiments with Mortenson involved Cp nitrogenase and those with Thorneley, Kp nitrogenase under different reaction conditions. However I think the two sets of data are compatible. In the experi-

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ments with Kp nitrogenase, each time [^{18}O]P_i bound to the $Kp2_{ox}(MgADP)_2Kp1$ complex, at most only one ^{18}O was lost. In both cases P_i release is faster than formation of bound ATP.

R. N. F. Thorneley. The ratio of the rate constant for P_i release to that for on-enzyme ATP formation from bound ADP and P_i (Rp) is >2.5. Also Charles McKenna failed to observe any Positional Isotope Exchange (PIX) with β,γ-[^{18}O]ATP with Av nitrogenase.

D. R. Trentham (*National Institute for Medical Research, London, U.K.*). Does Dr Thorneley have any probes on the steps involving nucleoside diphosphate release? Nearly all the investigations of other systems being discussed at this meeting pay attention to these steps which tell us a great deal about the mechanism.

R. N. F. Thorneley. We know that the replacement of MgADP by MgATP on the reduced Fe-protein is fast, $k>200\ s^{-1}$ with slow exchange occurring on the oxidized protein $k<2\ s^{-1}$. The tighter binding of MgADP to the oxidized than to the reduced Fe-protein is consistent with the negative shift in redox potential that occurs on binding. A -120 mV shift requires the binding of MgADP to be two orders of magnitude tighter to the oxidized protein. We have shown that the K_i^{MgADP} for reductant-independent ATPase activity involving oxidized Fe-protein bound to MoFe-protein is 145 μM, while MgADP binds to free oxidized Fe-protein with $K_D^{MgADP}<10$ μM. The tighter binding of MgADP to the free oxidized Fe-protein could be the driving force for dissociation of the protein complex. We are obviously very interested in using the mant-ATP and ADP derivatives in fluorescent studies with nitrogenase in a similar way to which the GTP analogues have been used with p21ras by Dr Eccleston and coworkers (Eccleston *et al.* 1991; Moore *et al.* this symposium).

Reference

Eccleston, J.F., Moore, K.J.M., Brownbridge, G.G., Webb, M.R. & Lowe, P.N. 1991 Fluorescence approaches to the study of p21ras GTPase mechanism. *Biochem. Soc. Trans.* **19**, 432–437.

L. Dutton (*Johnson Foundation, University of Pennsylvania, Philadelphia, U.S.A.*). Dr Thorneley is saying that ATP is hydrolysed to move the redox state of the system below that of the hydrogen electrode, and that before the enzyme can evolve H_2 it starts to reduce N_2. Is ATP needed for H_2 evolution?

R. N. F. Thorneley. Yes, all substrate reductions are ATP dependent.

L. Dutton. Is this happening because everything has to be in contact with water, in which case potentials below -420 mV are a problem, or is it designed not to be in contact with water, in which case one can get very low potentials without worrying about H_2 evolution?

R. N. F. Thorneley. I think the problem is related to the need for protons to have access to the active site. The model systems of Chatt and others suggest that a good way to bind N_2 is to displace the H_2 from a metal site. Indeed, H_2 is a competitive inhibitor of N_2 reduction and the stoichiometry of the reaction shows one equivalent of H_2 to be evolved for each N_2 reduced. Therefore you need to have protons at the active site not only to generate the metal-hydride intermediate that is thought to bind N_2 but also to effect the stepwise reduction and protonation of bound N_2 to $2NH_3$. The kinetics of nitrogenase have evolved to minimize H_2 evolution and optimize N_2 reduction. The slow rate-limiting dissociation of $Kp2(MgADP)_2$ from reduced MoFe-protein ($k=6.4\ s^{-1}$) means that it takes *ca.* 2 s to complete the eight turns of the Fe-protein cycle required to reduce N_2 to $2NH_3$ with concomitant evolution of H_2. To compensate for this low rate, the bacteria synthesizes up to 15% of its cytoplasmic protein as nitrogenase. It manages this under conditions when it is starved of fixed nitrogen. If it could get away with synthesizing ten times less nitrogenase, it would have a tremendous selective advantage under N_2-fixing conditions. Nitrogenase is a slow enzyme that functions most efficiently at high concentrations because it has to minimize wasteful H_2 evolution. It does this by maintaining the proteins largely in the complexed state. My colleague David Lowe and I believe that reducible substrates bind and their products leave free, uncomplexed MoFe-protein. Therefore, reduced MoFe-protein, provided it is complexed to either reduced or oxidized Fe-protein, cannot evolve H_2. This is why we have argued that the rate of $Kp2(MgATP)_2$ binding to Kp1 is close to the diffusion limit ($k=5\times10^7\ M^{-1}\ s^{-1}$) and the rate of dissociation of $Kp2_{ox}(MgADP)_2$ from reduced Kp1 is slow ($k=6.4\ s^{-1}$). To allow the cycle to run forwards, the 'back' rate constant for $Kp2_{ox}(MgADP)_2$ binding to reduced Kp1 is optimized at $k=5\times10^6\ M^{-1}\ s^{-1}$. These protein association and dissociation rates ensure the enzyme functions as a nitrogenase, not as a hydrogenase.

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