Energy transduction by nitrogenase: binding of MgADP to the MoFe protein is dependent on the oxidation state of the iron–sulphur ‘P’ clusters

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Hydrolysis of MgATP to MgADP is essential for nitrogenase action. There is good evidence for binding of both nucleotides to the Fe protein of nitrogenase, but data indicating their binding to the MoFe protein have been controversial [see Miller and Eady (1989) *Biochem. J.* 263, 725–729]. The binding of MgADP to the MoFe protein of nitrogenase of *Klebsiella pneumoniae* was investigated by non-equilibrium gel-filtration column methods. No binding of MgADP to the dithionite-reduced protein could be detected. Treatment of the MoFe protein with phenosafranine [midpoint potential (E_m) – 270 mV] did not affect the activity, and oxidized the ‘P’ clusters but not the iron–molybdenum cofactor (FeMoco) centres. This oxidized species bound 3.9 mol

of MgADP with a binding pattern characteristic of low rates of ligand dissociation. These observations suggest that the variability in published data on nucleotide binding to the MoFe protein is related to poor control of the protein oxidation level. Our data, coupled with the observation that ‘P’ clusters become oxidized during reduction of N_2 [Lowe, Fisher and Thorneley (1993) *Biochem. J.*, in the press], led us to propose that the ADP binding sites are transiently filled during enzyme turnover by hydrolysis of ATP originally bound to the Fe protein, and that hydrolysis occurs on a bridging site on the MoFe–Fe-protein complex.

INTRODUCTION

Despite many years of intensive effort, the role(s) of ATP in nitrogenase function remain unclear (see [1,2]). In Mo-containing nitrogenases the electron transfer from the Fe-protein to the MoFe-protein and subsequent reduction of substrates is coupled to the hydrolysis of MgATP to MgADP. During enzyme turnover, a minimum of two ATP molecules are hydrolysed for every electron transferred to substrate. The protein–protein electron transfer results in the reduction of the iron–molybdenum cofactor (FeMoco) centres, the putative active sites, of the MoFe protein [3]. These centres are ~ 1.9 nm (~ 19 Å) [4] from the other type of redox centre in MoFe proteins, the ‘P’ clusters, which are unique linked pairs of Fe_4S_4 clusters [5] and have been implicated as having a capacitor role of electron storage and discharge during N_2 reduction [6,7]. A detailed model of sequential electron transfers and generation of enzyme intermediates involved in dinitrogen reduction has been developed (reviewed in [8]).

The interaction of MgATP and MgADP with the Fe protein alone is well documented [1,3,9]; nucleotide binding results in a conformation change and decrease in redox potential of the [4Fe–4S] centre. Sequence analysis [10] and the three-dimensional X-ray crystal structure of the Fe protein [11] has revealed a potential nucleotide-binding site on each subunit. However, the hydrolysis of MgATP by nitrogenase requires both the MoFe protein and the Fe protein, although protein–protein electron transfer is not necessarily required, since hydrolysis is catalysed by dye-oxidized proteins [12]. Thus the MgATP-hydrolysing site is generated when the MoFe protein and the Fe proteins form a complex, which has been physically demonstrated by ultracentrifugation studies [13]. Either complex-formation between

the MoFe protein and the Fe protein induces ATP hydrolysis activity in the ATP-binding site on the Fe protein, or hydrolysis occurs while MgATP is held in a bridging configuration between the two proteins. This would imply that MgATP-binding site(s) exist on the MoFe protein and, further, that such site(s) bind to a part of the nucleotide–magnesium complex molecule distinct from that which is already bound to the Fe protein. Weak binding of MgATP to both isolated dithionite-reduced and thionine-oxidized MoFe proteins has been reported [14–17]. In the case of the dithionite-reduced MoFe protein of *Klebsiella pneumoniae* nitrogenase (Kp1), four sites with a K_d of ~ 700 μ M were found [15]. The weak binding of MgATP to these proteins thus appears to be independent of their oxidation state.

We have previously reported the binding of 2 mol of MgADP to the dye-oxidized form of the MoFe protein of *Azotobacter chroococcum* Mo-nitrogenase (Ac1^{Mo}) and the VFe protein of the V-nitrogenase of this organism [14] and also to dithionite-reduced Ac1^{Mo} [15]. However, in subsequent work the number of binding sites was found to vary. The work reported here shows that MgADP binding depends on the oxidation state of the ‘P’ centre clusters of the MoFe proteins; thus the earlier observations of variability in the number of binding sites were almost certainly a consequence of inadequate control of the redox state of the protein. These findings, and their relevance to the mechanism of interaction of nucleotides with nitrogenase, are discussed.

MATERIALS AND METHODS

Purification and assay of nitrogenase

Nitrogenase components of *K. pneumoniae* were purified and assayed as described previously [18,19]; the specific activity of

Abbreviations used: E_m , midpoint potential; FeMoco, iron–molybdenum cofactor; Kp1, *Klebsiella pneumoniae* nitrogenase; Ac1^{Mo}, Mo-nitrogenase of *Azotobacter chroococcum*.

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the Kp1 (M_r 224000) used was 1900 nmol of H_2 produced/min per mg of protein

Nucleotide-binding experiments

All manipulations were carried out under N_2 in an anaerobic box operating at 1 p.p.m. of O_2 or less. Sodium dithionite was removed from protein samples (24 mg of Kp1 in 0.78 ml) by gel filtration on a column (0.8 cm $\times$ 16 cm) of Bio-Gel P6DG equilibrated with 50 mM Tris/HCl buffer, pH 7.8, containing 50 mM NaCl and 10 mM $MgCl_2$. Effluent fractions containing undiluted Kp1 were pooled, and dithionite-free Kp1 was oxidized by the addition of 10 μ l of a solution of 4.4 mg of phenosafranine [midpoint potential (E_m) -270 mV] in 1 ml of ethanol. After 15 min incubation the redox potential of the solution was measured directly in the anaerobic chamber using calomel and platinum electrodes and a high-impedance millivoltmeter. A sample of Kp1 was removed for subsequent e.p.r. spectroscopy to determine the oxidation state of the FeMoco centres, and the remainder applied to a column (0.8 cm $\times$ 16 cm) of Bio-Gel P6DG pre-equilibrated with buffer containing [8- ^{14}C]ADP (165 μ M) and flowing at 1.2 ml/min. Fractions were collected in Eppendorf tubes and removed from the chamber for measurement of protein concentration, Kp1 activity and radioactivity; the column development was complete within 15 min. The same procedure, but omitting the oxidation step, was used for measurement of ADP binding to reduced dithionite-free Kp1.

RESULTS AND DISCUSSION

Equilibrium binding of MgADP to dithionite-reduced Kp1

Dithionite-reduced Kp1 from which free dithionite and bisulphite ions had been removed by gel filtration did not bind [^{14}C]ADP when investigated by the equilibrium gel-filtration column technique, since no increase of radioactivity above the background was associated with the protein elution peak (Figure 1a). The dip in the level of radioactivity of the ADP elution profile following the elution peak of Kp1 is due to dilution, since the protein was loaded on to the column in buffer lacking [^{14}C]ADP. This finding contrasts with our observations for the MoFe protein of the Mo-nitrogenase of *A. chroococcum* [15]. In this case, dithionite-reduced $Ac1^{Mo}$ bound 2.0 ± 0.2 mol of MgADP/mol of protein, and the binding profile indicated slow release of the bound nucleotide. Considering the high degree of conservation of the amino acid sequences of these proteins and their functional interchangeability in forming active nitrogenases (see [3] for review), this variability in ADP binding is surprising.

Equilibrium binding of MgADP to 'P'-centre-oxidized Kp1

When dithionite-free Kp1 was oxidized with phenosafranine to a measured potential of -200 mV, the low-temperature e.p.r. spectrum of the protein indicated that the FeMoco centres were still reduced, but the linewidth was broader than that of the dithionite-reduced protein. In Kp1 with the 'P' centres oxidized, the change in linewidth of the FeMoco e.p.r. signal is interpreted as being due to an altered magnetic interaction between these two centres (D. J. Lowe, personal communication). Previous Mössbauer studies of Kp1 [7] have shown that oxidation of the 'P' centres with an E_m of ~ 340 mV at pH 8.7 occurs at a lower potential than the FeMoco centres, which have an E_m of -216 mV at pH 8.7 ($E_m = -160$ mV at pH 7.5) [20]. Fur-

thermore, re-reduction of oxidized 'P' centres by dithionite was slow (~ 1 h) [21].

When the 'P'-centre-oxidized Kp1 was used in binding experiments, 3.9 mol of MgADP bound per mol of protein (Figure 1b). ADP was bound to the protein and appeared in the protein effluent peak as a coincident peak of radioactivity. The molar ratio varied over the peak in the three different fractions containing protein, but integrated (total nmol of ADP bound/nmol of Kp1) to give an average of 3.94 ADP/mol of protein. At the peak the ratio was 4.4 ± 0.6 . Since later fractions over the peak did not show an increase in the amount of ADP bound to protein, equilibration between ligand and protein was considered to be complete, and therefore no systematic study of equilibration was undertaken. However, the post-protein elution profile did not show ideal behaviour, since fractions were also depleted of ADP. The dip is more protracted in Figure 1(b), since the non-equilibrium nature of the binding results in continued binding occurring during separation of Kp1 from the unlabelled loading buffer. These observations are very similar to our previously obtained data [15] for dithionite-free reduced $Ac1^{Mo}$. This atypical pattern was shown in this case to arise from low rates of release of nucleotide relative to the rate of passage of the protein through the gel-filtration column [15]. We attribute the difference between the data reported previously and the data reported here as being due to inadvertent oxidation of the 'P' centres in dithionite-containing solutions in the earlier experiments. MoFe proteins are O_2 -sensitive and are therefore routinely isolated under N_2 in buffers containing 1–2 mM $Na_2S_2O_4$ to protect them

Figure 1 Interaction of ADP with MoFe protein of *K. pneumoniae* (Kp1)

A gel-filtration column (Bio-Gel P6DG) was equilibrated with 165 μ M [^{14}C]ADP having a specific radioactivity of 418 c.p.m./nmol in buffer, as described in the Materials and methods section. Kp1 (140 μ M) was applied to the column and eluted with the equilibration buffer, and 0.2–0.4 ml fractions were collected. Radioactivity ($\square$), volume and protein concentrations ($\blacksquare$) were determined in each effluent fraction. Net radioactivity (c.p.m.) was calculated by subtraction of the equilibrium level of radioactivity from total radioactivity (c.p.m.) found in a 0.05 ml portion of each fraction; 8.5 mg of Kp1 was recovered in the three protein-containing tubes. (a) Dithionite-reduced Kp1; (b), 'P'-centre-oxidized Kp1. Note the differences in radioactivity scales of (a) and (b), which overemphasize the post-protein trough in (a).

from inactivation by O_2 during purification. Variable inadvertent exposure to air results in variable concentrations of bisulphite being formed; as a consequence the redox potential of the solution rises owing to perturbation of the $S_2O_4^{2-}/HSO_3^-$ equilibrium, and the redox state of solutions of nitrogenase components as isolated is uncertain. The increase in potential in the presence of bisulphite could result in some of the 'P' centres of the protein being oxidized and, therefore, variable numbers of nucleotide-binding sites being observed in our experiments. Although the Kp1 would be exposed to fresh dithionite during column chromatography, the re-reduction of the 'P' clusters would be slow [21] and they would not be re-reduced in the time (~ 30 min) taken to complete the experiment.

The binding of MgADP to 'P'-centre-oxidized Kp1 may be significant in the mechanism of N_2 reduction by nitrogenase. During dithionite-dependent turnover, oxidation of 'P' centres occurs when the MoFe protein has been reduced by sequential addition of four electrons to a state capable of binding N_2 , and of protonating it to the hydrazido(2^-)-level. At this point the enzyme is irreversibly committed to further reduction of this intermediate to form NH_3 via the intermediates of the Lowe-Thorneley mechanistic scheme of nitrogenase turnover [6,8]. We show here that a 'P'-centre-oxidized species of isolated Kp1 is capable of binding 4 mol of MgADP/mol of protein. This finding is significant for nitrogenase-nucleotide interactions and, in conjunction with the data summarized below, supports the suggestion that MgATP binds to the nitrogenase complex during turnover in a bridging mode between the two proteins. During enzyme turnover the Fe protein acts as a single electron donor to the MoFe protein, and for each electron transferred to substrate, two ATP molecules are hydrolysed, yielding a minimum value of 4 for the ATP/2e ratio with protons as the reducible substrate. This stoichiometry can be rationalized in terms of our binding data as follows.

(a) The FeMoco centres in the $\alpha_2\beta_2$ tetramer are 7 nm (70 Å) apart [4] and act independently [8]; therefore the MoFe protein is considered to have two binding sites for Fe protein.

(b) Two ATP molecules are bound to each of the two Fe protein molecules.

(c) We find four ADP-binding sites of a 'P'-centre-oxidized form of the MoFe protein related to that generated transiently during turnover.

(d) We therefore propose that the ADP-binding sites that we have detected on the MoFe protein are transiently filled during turnover by hydrolysis of ATP molecules which are originally bound to Fe protein molecules and which form a bridge between the two proteins in the Fe-protein-MoFe-protein complex.

The observed slow release (minutes) of MgADP from 'P'-centre-oxidized Kp1 is much slower than the rate-limiting step of

protein-protein dissociation following electron transfer (6.5 s^{-1}) [8]. We propose that the rapid release of tightly bound MgADP occurs when the 'P' clusters are re-reduced by the reduced Fe-protein-MgATP complex, a reaction which is known to be fast and enables the recharging of the electron capacitor. This is consistent with our observations above that MgADP does not bind to MoFe protein having reduced 'P' centres. We further suggest that the tight binding of MgADP to the 'P'-cluster-oxidized MoFe protein at a critical stage during turnover is necessary to prevent the reversal of electron transfer from the substrate binding site to the 'P' clusters.

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REFERENCES

- Thorneley, R. N. F. (1992) *Philos. Trans. R. Soc. London B* **336**, 73–82
- Miller, R. W. (1991) in *Biology and Biochemistry of Nitrogen Fixation* (Dilworth, M. J. and Glenn, A. R., eds.), pp. 9–36, Elsevier, New York
- Smith, B. E. and Eady, R. R. (1992) *Eur. J. Biochem.* **205**, 1–15
- Bolin, J. T., Ronco, A. E., Mortenson, L. E., Morgan, T. V., Williamson, M. and Xuong, N.-L. (1990) in *Nitrogen Fixation: Achievements and Objectives* (Gresshoff, P. M., Roth, L. E., Stacey, G. and Newton, W. E., eds.), pp. 117–124, Chapman and Hall, New York and London
- Kim, J. and Rees, D. C. (1992) *Science* **257**, 1677–1682
- Lowe, D. J., Fisher, K. and Thorneley, R. N. F. (1993) *Biochem. J.*, in the press
- Smith, B. E., O'Donnell, M. J., Lang, G. and Spartalian, K. (1980) *Biochem. J.* **191**, 449–455
- Thorneley, R. N. F. and Lowe, D. J. (1985) in *Molybdoenzymes* (Spiro, T. G., ed.), pp. 221–284, Wiley and Sons, New York
- Burgess, B. K. (1984) in *Advances in Nitrogen Fixation Research* (Veeger, C. and Newton, W. E., eds.), pp. 103–114, Nijhoff/Junk/Pudoc, Wageningen
- Robson, R. L. (1984) *FEBS Lett.* **173**, 394–398
- Georgiadis, M. M., Kamiya, H., Chakrabarti, P., Woo, D., Kornuc, J. J. and Rees, D. C. (1992) *Science* **257**, 1653–1659
- Cordewener, J., Asbroek, A., Wassink, H., Eady, R. R., Haaker, H. and Veeger, K. (1987) *Eur. J. Biochem.* **162**, 265–270
- Eady, R. R. (1973) *Biochem. J.* **135**, 531–535
- Miller, R. W. and Eady, R. R. (1988) in *Nitrogen Fixation: Hundred Years After* (Bothe, H., de Bruijn, F. J. and Newton, W. E., eds.), p. 90, Gustave Fischer, Stuttgart and New York
- Miller, R. W. and Eady, R. R. (1989) *Biochem. J.* **263**, 725–729
- Miller, R. W., Robson, R. L., Yates, M. G. and Eady, R. R. (1980) *Can. J. Biochem.* **58**, 542–548
- Kimber, S. J., Bishop, E. O. and Smith, B. E. (1982) *Biochim. Biophys. Acta* **705**, 385–395
- Eady, R. R., Smith, B. E., Cook, K. A. and Postgate, J. R. (1972) *Biochem. J.* **128**, 655–675
- Smith, B. E., Thorneley, R. N. F., Yates, M. G., Eady, R. R. and Postgate, J. R. (1976) in *Proc. Int. Symp. Nitrogen Fixation* (Newton, W. E. and Nyman, C. J., eds.), pp. 150–176, Washington State University Press, Pullman
- O'Donnell, M. J. and Smith, B. E. (1978) *Biochem. J.* **173**, 831–839
- Smith, B. E. and Lang, G. (1974) *Biochem. J.* **137**, 169–180