Molybdenum nitrogenase of *Azotobacter chroococcum*

Tight binding of MgADP to the MoFe protein

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The dye-oxidized or dithionite-reduced forms of the MoFe protein of molybdenum nitrogenase of *Azotobacter chroococcum* were shown to bind 2 mol of MgADP/mol of protein, as determined by column equilibrium techniques. The gel-filtration elution profile of unbound Mg[¹⁴C]ADP was not symmetrical, consistent with a low rate of dissociation from the protein. Symmetrical elution profiles were observed for the oxidized Fe protein of nitrogenase, which bound 2 mol of MgADP/mol of protein. The low rate of dissociation of MgADP from MoFe protein was shown by non-equilibrium column techniques, where 1 mol of MgADP/mol of MoFe protein remained tightly bound during chromatography. Very weak binding of MgATP (< 0.01 mol of MgATP/mol of MoFe protein) to dye-oxidized but not to dithionite-reduced MoFe protein was observed. These results are discussed in terms of their relevance to the catalytic cycle of nitrogenase catalysis.

INTRODUCTION

Extensive studies on the well-characterized molybdenum-containing nitrogenases have shown them to be complexes of two redox proteins: a larger MoFe protein, which contains an iron and molybdenum co-factor (FeMoco), the probable site at which substrates are reduced, and a smaller Fe protein, which acts as a specific electron donor to the MoFe protein. Substrate reduction requires the presence of both proteins and is obligatorily coupled to the hydrolysis of MgATP to MgADP and P_i. A minimum of 16 mol of ATP is hydrolysed/mol of N₂ reduced. MgADP is a potent competitive inhibitor of electron transfer and substrate reduction (for recent reviews see Thorneley & Lowe, 1985; Stephens, 1985; Eady, 1986; Smith *et al.*, 1987, 1988).

The consensus view of the mechanism of nitrogenase function is that the nucleotide-binding site is located on the Fe protein (for reviews see Orme-Johnson, 1985; Mortenson, 1987). The MgADP- or MgATP-induced conformational change and associated decrease in redox potential of the [4Fe-4S] centre that these proteins contain is well documented (see Ashby & Thorneley, 1987). In contrast, there are contradictory reports in the literature as to whether nucleotides do or do not bind to MoFe proteins. No binding of MgADP or MgATP to Av1 could be detected by flow dialysis (Cordewener *et al.*, 1983, 1985), nor to Cp1 by gel-exclusion techniques (Tso & Burris, 1973). However, weak binding of MgATP to Kp1 was detected both by gel-exclusion (Miller *et al.*, 1980) and water-¹H-n.m.r.-relaxation-enhancement techniques (Kimber *et al.*, 1982). A presumed ATP-binding site on the β-subunit of Kp1 has been assigned on

the basis of amino acid sequence comparison with *Escherichia coli* ATPases (Robson, 1984).

We report here that the dithionite-reduced and thionine-oxidized redox states of Ac1 bind 2 molecules of MgADP tightly and that nucleotide dissociation occurs slowly. The significance of these observations for the role of MgATP in nitrogenase function and the reasons for the apparent discrepancy between published data are discussed. A preliminary account of part of this work has been reported previously (Miller & Eady, 1988).

MATERIALS AND METHODS

Purification of protein components

Nitrogenase proteins MoFe protein (Ac1) and Fe protein (Ac2) were purified from extracts of *Azotobacter chroococcum* strain MCD50 by previously described methods (Yates & Planque, 1975). The proteins used in this work were homogeneous by the criteria of Coomassie Blue staining of SDS/polyacrylamide gels after electrophoresis and both had specific activities of 1400 nmol of C₂H₂ reduced/min per mg of protein when assayed under standard conditions (Eady *et al.*, 1972). Protein was assayed with bicinchoninic acid with reagents supplied by Pierce Chemical Co. (Rockford, IL, U.S.A.), with bovine serum albumin used as protein standard with ϵ_{279} 45.36 × 10³ M⁻¹·cm⁻¹ (Smith *et al.*, 1985).

Preparation of protein solutions

All manipulations were carried out in a high-integrity glove-box through which N₂ was circulated, and an O₂ concentration below 1 p.p.m. was maintained. Before binding experiments were carried out, Na₂S₂O₄ was

Abbreviations used: nitrogenase components are abbreviated as follows: Ac1, Av1, Kp1 and Cp1 are the MoFe proteins of molybdenum nitrogenases of *Azotobacter chroococcum*, *Azotobacter vinelandii*, *Klebsiella pneumoniae* and *Clostridium pasteurianum* respectively, and Ac2, Av2 and Kp2 are the Fe proteins of *A. chroococcum*, *A. vinelandii* and *K. pneumoniae* nitrogenase respectively; oxidation state is indicated by subscripts, ox. or red., where necessary.

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removed from solutions of Ac1 by gel filtration in Bio-Gel P6DG in a 0.7 cm × 15 cm column equilibrated with 50 mM-Tris/HCl buffer, pH 8.0, containing 50 mM-NaCl and 10 mM-MgCl₂. The protein was then used immediately or concentrated on a Minicon A25 concentrator (Amicon Co.) to a protein concentration of 50 μM. Ac1 was oxidized with the redox dye Lauth's Violet (thionine). Protein solutions were percolated into a 2 cm layer of Dowex 1 (Cl⁻ form) resin saturated with thionine, located above a 15 cm column of Bio-Gel P6DG. The protein was allowed to remain in contact with the resin-bound thionine for 10 min before elution from the column with the Tris/NaCl/MgCl₂ buffer described above. Ac2 was freed of Na₂S₂O₄ and oxidized in a similar manner except that NaCl was omitted from the elution buffer and Indigocarmine dye replaced thionine as the resin-bound oxidant. After oxidation the proteins retained 85–90 % of their original activity.

Nucleotide-binding studies

For non-equilibrium binding studies, nitrogenase proteins were incubated with a 2–5-fold molar excess of [¹⁴C]ADP or a substoichiometric amount of [α -³²P]-ATP for 10 min and then passed through a column (0.7 cm × 15 cm) of Bio-Gel 6PDG equilibrated with the buffers described above. The column effluent was collected in 0.45 ml fractions, which were analysed for protein concentration and radioactivity by scintillation counting. The chromatographic separation of protein and free ligand was complete in 3 min.

Equilibrium binding measurements were made in a similar manner except that the gel-filtration columns were equilibrated with buffer containing lower concentrations of radioactive nucleotide before application of the protein in buffer lacking the ligand. Protein and ligand concentrations were determined in the fractions containing the protein peak as above, and bound nucleotide in each tube was calculated by subtracting the unbound ligand radioactivity in the buffer. The values for ratios of nucleotide ligand bound to protein, as discussed below, are those obtained for the peak protein fractions unless otherwise specified. The trough area in the elution pattern appearing at the elution volume of free ligand represented the sum of depleted ligand bound to protein and depleted ligand in the volume of the unlabelled buffer applied to the column with the protein. The total amount of ligand bound to the protein chromatographed also could be calculated from the negative area of this trough after correction for the volume of unlabelled protein applied (Hummel & Dreyer, 1962).

RESULTS

Tight binding of MgADP to Ac1

Dithionite-reduced Ac1 from which free dithionite ions had been removed showed significant binding of [¹⁴C]ADP when investigated by the equilibrium gel-filtration column technique of Hummel & Dreyer (1962). When highly purified protein was chromatographed on columns that had been pre-equilibrated with the nucleotide at a concentration near that of the protein, ADP was bound to the protein and appeared in the protein effluent peak at the column void volume as a peak of radioactivity coincident with the protein peak, as shown in Fig. 1. The molar ratio of ADP bound to protein varied over the peak tubes from 1.6:1 to 2.2:1 in repeat experiments,

Fig. 1. Interaction of ADP with dithionite-reduced MoFe protein of *A. chroococcum* (Ac1)

A gel-filtration column (Bio-Gel P6DG) was equilibrated with 67 μM-[¹⁴C]ADP having a specific radioactivity of 2810 c.p.m./nmol as described in the Materials and methods section. Ac1 (8 mg, 76 μM) was applied to the column, and 0.45 ml fractions were collected. Radioactivity (○) and protein concentration (□) 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. Ratios of ADP bound to protein were calculated as reported in the Results section. Data were fitted with cubic spline curves to illustrate congruence of the elution patterns for protein and bound ADP.

being near 1.6:1 at the highest protein concentration. These observations indicate tight binding of MgADP to Ac1, and the stoichiometry of this process precludes the observed binding being due to adventitious proteins contaminating our preparations of Ac1.

However, the post-protein elution profile of the column did not show ideal behaviour, in that the fractions immediately following the protein were also depleted of ADP. Radioactivity in the effluent did not return to the equilibrium level until after the expected trough corresponding to the position of free ADP (6.0 ml) had been eluted completely. This atypical elution pattern was observed consistently and may be attributed to a slow binding and low rates of release of MgADP for the protein relative to the rate of passage of the protein through the gel-filtration column. Thus true equilibrium between ligand and protein was not achieved with Ac1.

To establish that this elution pattern was not due to our experimental conditions, we measured the binding of MgADP to Ac2 by this method. In the case of the oxidized Fe protein, where equilibration of MgADP with the protein binding sites is known to be rapid ($k_2 > 4 \times 10^{10} \text{ M}^{-2}$ for binding to Kp2; Ashby & Thorneley, 1987), a normal elution pattern was observed for both the protein peak at the column void volume and for the ADP trough, with radioactivity of the effluent returning to the equilibrium level between the peak and trough (Fig. 2). This control experiment was carried out under similar conditions to those employed for Fig. 1 and indicated that the methodology employed was free of systematic errors. A value of 1.7 ± 0.5 mol of ADP bound/mol of Ac2 was obtained. Taking into account the volume of the protein sample not containing radioactive ligand that was applied to the column, a value of 2.2 mol of ADP/mol of protein applied was calculated

Fig. 2. Interaction of ADP with Indigocarmine-oxidized Fe protein of *A. chroococcum* (Ac2)

A gel-filtration column was equilibrated with $133 \mu\text{M}$ - $[\text{}^{14}\text{C}]\text{ADP}$ as indicated in the legend to Fig. 1. Ac2 (13.1 mg, $100 \mu\text{M}$) was oxidized as described in the Materials and methods section and applied to the column. ADP radioactivity (○) and protein concentration (□) were determined for each fraction and molar ratios of ligand to protein were calculated.

from the depletion of radioactive ADP from the trough. The results of this experiment established the elution pattern expected from a protein/ligand system in rapid equilibrium relative to the transit time through the column, and gave values for binding ratios in agreement with those determined for other Fe proteins (see Burgess, 1984; Watt *et al.*, 1985). Ac1 clearly did not give this elution pattern in either dye-oxidized or dithionite-reduced oxidation states.

Non-equilibrium binding of MgADP to Ac1

When fractions containing the Ac1 protein-ADP complex obtained in the protein peak from the experiment of Fig. 1 were pooled, concentrated and separated by gel-filtration separation on a Bio-Gel P6DG column in the same buffer lacking MgADP, the bound MgADP did not dissociate completely from the protein since a peak of radioactivity coincident with the protein peak was observed (Fig. 3). Approximately half of the bound MgADP originally bound dissociated and appeared in the extended broad peak that commenced immediately following the complete elution of the protein, suggesting two classes of binding sites with differing rates of dissociation. Again, the behaviour of the free ligand was atypical, as evidenced by the incomplete resolution of the protein and radioactive ligand peaks. Essentially similar results were obtained with thionine-oxidized Ac1 or reduced protein in the presence of dithionite (2 mM). These data indicate that MgADP binds to the protein in both oxidation states in a similar manner, i.e. tight binding with relatively low rates of association/dissociation.

This non-ideal behaviour of the ligand precludes detailed analysis of the data obtained by the 'equilibrium binding' method in terms of determination of binding constants or total number of ligand-binding sites, but provides firm evidence for the presence of tight binding sites of MgADP on the MoFe protein of nitrogenase. One of the classes of nucleotide-binding sites apparently had a lower rate of dissociation of MgADP, indicated by the lack of resolution of MgADP and protein peaks

Fig. 3. Partial resolution of ADP from ADP-MoFe protein complex by gel filtration

The $[\text{}^{14}\text{C}]\text{ADP}$ -MoFe protein complex (6.5 mg) eluted from a preliminary gel-filtration column run under equilibrium binding conditions (Fig. 1) was chromatographed on a second gel-filtration column lacking ADP, in an attempt to remove the ligand. ADP radioactivity (○) and protein concentration (□) were determined in each fraction. Note that approximately half of the bound ligand remained bound to the reduced protein during the second gel-filtration step, which required 3 min to complete.

Fig. 4. Weak binding of $[\text{}^{32}\text{P}]\text{ATP}$ to thionine-oxidized Ac1

$[\text{}^{32}\text{P}]\text{ATP}$ ($15.9 \mu\text{M}$) was incubated with thionine-oxidized Ac1 (3.8 mg, $30 \mu\text{M}$) and the mixture was chromatographed on an unlabelled Bio-Gel P6DG gel-filtration column under non-equilibrium binding conditions. Effluent protein concentration (□) and ADP radioactivity (○) were calculated as for Fig. 1.

in these experiments. This behaviour is similar to that reported for Av2. When dithionite-reduced Av2 was incubated with MgADP at a concentration sufficient to saturate the two binding sites on this protein, and the mixture was then separated on a column of Sephadex G-50 (fine grade), 1 mol of MgADP/mol remained associated with Av2 (Cordewener *et al.*, 1987).

Binding of MgATP to Ac1

The interaction of MgATP with thionine-oxidized Ac1 was much weaker than that of MgADP measured by either equilibrium or non-equilibrium methods. Fig. 4 shows a typical profile for a non-equilibrium column, and under these conditions approx. 0.01 mol of MgATP/mol was calculated to bind to Ac1_{ox} . Under similar conditions with Ac1_{red} , no binding of MgATP

could be detected with AcI_{red} within the experimental error of the method.

DISCUSSION

The interaction of MgATP with MoFe proteins of nitrogenase isolated from different organisms has only been observed with Kp1, where weak binding ($K_d = 600 \pm 100 \mu\text{M}$ at four sites) was reported (Miller *et al.*, 1980; Kimber *et al.*, 1982). Our failure to detect significant binding of MgATP to reduced AcI reported here is consistent with published data for Av1 (Cordewener *et al.*, 1983, 1985) and Cp1 (Tso & Burris, 1973), but the existing discrepancies may reflect species differences between these organisms, resulting in weaker MgATP–MoFe protein interactions in some cases. Although the amino acid sequences of MoFe proteins together with their biochemical uniformity indicate that they comprise a highly conserved family of proteins, differences between them are observed (see Eady, 1986). For example, when MoFe and Fe components isolated from different organisms are recombined, they do not always form a functional nitrogenase, and at least in one instance are unable to hydrolyse MgATP (Smith *et al.*, 1976). A species difference in binding affinity is unlikely to account for the failure of others to detect tight binding of MgADP to MoFe proteins, since such binding is in principle experimentally easier to detect. However, the flow-dialysis technique used to investigate nucleotide binding to Av1 by Cordewener *et al.* (1983, 1985) would not reveal the presence of slowly dissociating very-high-affinity binding sites. In that technique the amount of radioactive nucleotide bound to protein is determined from the appearance of radioactivity in the buffer flowing through the dialysis cell following displacement of the bound radioactive nucleotide by the addition of a large excess of unlabelled nucleotide to the protein solution. This 'chase' method will not detect tightly bound nucleotides that do not exchange rapidly with the added excess of unlabelled compound, and could well account for no interaction between MgADP and Av1 being observed. Although Tso & Burris (1973) reported that Cp1 did not bind MgADP as measured by a gel-exclusion technique, no detailed results were presented, which makes comparison with the present work difficult.

It has been shown that electron transfer between nitrogenase components is not necessary for MgATP hydrolysis (Cordewener *et al.*, 1987), and previously, that redox cycling of isolated Fe protein to which MgATP is bound does not result in ATP hydrolysis (Burris & Orme-Johnson, 1977). Since neither MoFe protein nor Fe protein of nitrogenases separately catalyses MgATP hydrolysis, the data reported here showing tight binding of MgADP to AcI are consistent with our earlier suggestion of a bridging mode of binding of MgATP to nitrogenase (Eady *et al.*, 1980; Kimber *et al.*, 1982). Stoichiometric binding interactions of MgADP and AcI reported above, and with the VFe protein of vanadium nitrogenase of *A. chroococcum* (Miller & Eady, 1988), and also to Av2_{ox} (Cordewener *et al.*, 1987), are unlikely to be due to adventitious non-specific interactions.

Although our data present clear evidence for MgADP-binding sites on Kp1, the presence of sites of different affinity may possibly reflect the presence of catalytically inactive species of this protein that have an altered affinity for nucleotide. The presence of such inactive species is evident from the specific activity of 1400 mol of

C_2H_2 reduced/min per mg of protein for the protein used in this work. Such contamination is a problem that is frequently incurred with nitrogenase MoFe proteins, where for different apparently homogeneous preparations the specific activity ranges from 1400 to 2800 nmol of H_2 evolved/min per mg of protein. A similar situation may arise with Fe proteins, since one of the two ADP-binding sites on Av2 is characterized by a low rate of ADP release (Cordewener *et al.*, 1987), and it has been suggested that preparations of Fe proteins currently available are, at best, only 50% active (Ashby & Thorneley, 1987).

It is not possible to stimulate directly nitrogenase–nucleotide binding interactions that occur under catalytic turnover conditions. The necessity of working with the individual nitrogenase proteins to obtain data for nucleotide binding and the low rate of MgADP release from Kp1 that we observe raises the question of the relevance of these binding interactions to the catalytic cycle of nitrogenase functions. However, the rate of MgADP dissociation from Fe protein is strongly dependent on oxidation state. Pre-steady-state kinetic studies of Kp2 (Ashby & Thorneley, 1987) have shown that, for the nucleotide-binding protein species of altered conformation $\text{Kp2}_{\text{ox}}^*(\text{MgADP})_2$, dissociation of MgADP is too slow ($k < 2 \text{ s}^{-1}$) to occur in turnover, where the rate-limiting step for protein complex dissociation following MgATP hydrolysis and electron transfer has $k = 6.4 \text{ s}^{-1}$ (Thorneley & Lowe, 1983). Since exchange of MgADP for MgATP occurs rapidly (limited by MgADP release, $k = 2 \times 10^2 \text{ s}^{-1}$) in $\text{Kp2}_{\text{red}}(\text{MgADP})_2$ (Thorneley & Cornish-Bowden, 1977), the oxidation level of nitrogenase proteins can clearly have a marked effect on the rate of release of MgADP. In the present case, although slow release of bound MgADP was observed from dithionite-reduced and thionine-oxidized AcI in isolation, the rate might well be faster under turnover, when MgATP hydrolysis and subsequent electron transfer result in the formation of the 'super-reduced' species of AcI when the FeMoco centres of the protein become further reduced, beyond the dithionite-reduced level, during turnover.

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