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THE BINDING OF ATP AND ADP BY NITROGENASE COMPONENTS FROM *CLOSTRIDIUM PASTEURIANUM*

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SUMMARY

A simple gel equilibration method differing from gel filtration was used for the measurement of ATP binding by purified nitrogenase components from *Clostridium pasteurianum*. It was clearly established that the binding site is on the iron protein of nitrogenase (Fe protein). Mg^{2+} is required for the binding. The Fe protein also binds MgADP. The number of binding sites and the dissociation constants of the MgATP- and MgADP–Fe protein complexes were measured. Inhibition studies indicate that MgADP inhibits MgATP binding by occupying one of the two MgATP sites; MgADP appears to increase slightly the binding of MgATP at its second site.

INTRODUCTION

The binding of ATP by the nitrogenase components has been studied by a number of investigators[1–4]. ATPase contamination in the individual nitrogenase components will give misleading positive binding and therefore, it is important to work with purified proteins, each of which by itself is completely free from ATPase activity.

Another difficulty encountered in this type of work is enzyme inactivation. The standard procedures, such as equilibrium dialysis and gel filtration, ordinarily last for several hours. They are not suitable for the extremely O_2-labile nitrogenase proteins which upon inactivation may give misleading negative binding. A rapid gel method[5] which takes only 10 min for equilibration has been modified to fit the required anaerobic conditions and has been used in this study.

MATERIALS AND METHODS

Nitrogenase of Clostridium pasteurianum

The two protein components were prepared and both the catalytic activity

Abbreviations: MoFe protein, the molybdenum–iron protein of nitrogenase; Fe protein, the iron protein of nitrogenase; MES, 2-(*N*-morpholino)ethanesulfonic acid.

and protein purity were the same as described earlier[6]. There was no N_2 fixation or ATP hydrolysis associated with the individual Fe protein or the molybdenum-iron protein of nitrogenase (MoFe protein)[7]. The μM concentrations of the nitrogenase components were calculated based on our unpublished molecular weight values of 210 000 and 56 000 for the MoFe and Fe proteins, respectively. Protein content was measured by the microbiuret method of Goa[8].

Chemicals

Sodium salts of ATP, ADP and 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer were purchased from Sigma Chemical Company. Sodium [8-^{14}C]ATP and [8-^{14}C]ADP, which contained 49.8 Ci/mole and 53.6 Ci/mole, respectively, were obtained from the New England Nuclear Company. High-purity N_2 was purified by passing it over BTS catalyst (BASF Colors and Chemicals Inc., 866 Third Avenue, New York City) heated to about 125 °C.

Binding experiments

50 mg of washed and dried Sephadex G-50 (coarse) gel was added to a 5-ml serum bottle and a calculated amount (350–550 μl) of 20 mM MES buffer, pH 6.6, was added. After swelling for 4 h or more at room temperature, ^{14}C-labeled ligand in 20 mM MES buffer, pH 6.6, was added to the gel suspension. The bottles containing all the reaction components except the nitrogenase were stoppered with serum stoppers and were evacuated and gassed to achieve anaerobic conditions. The O_2-sensitive nitrogenase protein constituent(s) was injected with a microsyringe. Unless indicated otherwise, the final volume was 600 μl and the $MgCl_2$ concentration was 1 mM in excess of the ATP and ADP concentrations; the ATP or ADP contained approximately 0.02 μCi of ^{14}C. After 10-min incubation at 25 °C with continuous shaking, 60–100 μl of the solution outside the gel was withdrawn with a microsyringe and injected into a vial containing 10 ml of Bray solution[9] with 10 mM $HClO_4$. The radioactivity was measured at 0 °C in a liquid scintillation counter (Packard Model 2018). The background count obtained by substituting an equal volume of buffer for the assay mixture contributed less than 1% of the observed count. Control experiments showed that $MgCl_2$ and varying concentrations of total ATP and ADP did not affect counting rates. No correction was applied for quenching by protein, as the effect was negligible under the experimental conditions used. The measurements of radioactivity on duplicate samples agreed to within 1%.

A test of reversibility[10] was done to insure that the 10-min incubation was long enough for equilibration and that we were measuring equilibrium binding.

The nitrogenase proteins had the same optimal specific activities for acetylene reduction[6] before and after the 10-min incubation in the binding experiment. This shows the validity of the present method and eliminates the possibility that lack of binding arose from enzyme inactivation.

RESULTS

Protein requirement for [^{14}C]ATP and [^{14}C]ADP binding

The MoFe protein, Fe protein and a combination of the two proteins were tested for binding as described. The results in Table I show that the ATP binding site

TABLE I

PROTEIN REQUIREMENT FOR ATP BINDING

The incubation mixture contained: 50 mg Sephadex G-50, 0.1 mM [^{14}C]ATP, 1.1 mM $MgCl_2$, 6 mg creatine phosphate, 0.2 mg creatine kinase, 20 mM MES buffer, pH 6.6.

Nitrogenase protein	*[^{14}C]ATP outside the gel after equilibration* (counts in 5 min)*	*% increase in radioactivity due to binding*
No protein as control	15594	0
20 μM Fe protein	18153	16.4
10 μM MoFe protein	15447	−0.9
20 μM Fe protein + 10 μM MoFe protein	18201	16.7

* This represents the sum of bound ATP and free ATP.

is on the Fe protein. The MoFe protein does not affect this binding. Because there was a trace amount of dithionite in the Fe protein preparation which would support ATP hydrolysis in the presence of both nitrogenase proteins and because this could complicate the binding conditions, an ATP-generating system was included to maintain a constant level of ATP.

ADP, the well-known inhibitor of nitrogenase-mediated ATP hydrolysis, electron transport and substrate reduction, was tested for binding to the nitrogenase protein in the same way as ATP. Again, only the Fe protein was required for ADP binding.

Mg^{2+} requirement for binding

To tell whether the free form of ATP and ADP or their Mg^{2+} complexes bound to the Fe protein, binding with and without added $MgCl_2$ was compared. Table II shows that additional Mg^{2+} is required both for optimal ATP and ADP binding. For all subsequent binding experiments, 1 mM Mg^{2+} in excess of the adenine nucleotide concentration was added to the assay mixture.

TABLE II

Mg^{2+} REQUIREMENT FOR BINDING

The reaction mixture contained 20 mM MES buffer, pH 6.6, [^{14}C]ATP or [^{14}C]ADP, $MgCl_2$ and Fe protein concentrations as indicated.

Adenine nucleotide	*Fe protein (μM)*	*$MgCl_2$ (mM)*	*Radioactivity outside the gel (counts in 5 min)*	*% increase in radioactivity due to binding*
50 μM [^{14}C]ATP	0	0	11150	
50 μM [^{14}C]ATP	0	1.050	11350	
50 μM [^{14}C]ATP	10	1.050	14386	26.7
50 μM [^{14}C]ATP	10	0	13340	19.6
25 μM [^{14}C]ADP	0	0	15400	
25 μM [^{14}C]ADP	0	1.025	15500	
25 μM [^{14}C]ADP	15	1.025	23850	53.9
25 μM [^{14}C]ADP	15	0	19423	26.1

Determination of the dissociation constants, K, and the number of binding sites, N, for MgATP- and MgADP–Fe protein complexes

Qualitatively, we know both MgATP and MgADP bind to Fe protein. To answer the question of how tightly they bind and how many molecules of ATP or ADP can bind to a molecule of protein, measurements over a range of MgATP and MgADP concentrations were made. The protein and ligand concentrations were chosen to give at least 10–15% increase in outside radioactivity over the control as a result of binding. In the simplest possible situation, where all N of the binding sites are identical and have no effect upon one another, the following equation[11] can be used to determine the dissociation constant, K, between the ligand and Fe protein, and N, the maximum number of ligand molecules bound per Fe protein molecule.

$$\frac{1}{R} = \frac{K}{N}\,\frac{1}{[L_{0(\text{free})}]} + \frac{1}{N} \tag{1}$$

The free ligand concentration outside the gel, $[L_{0(\text{free})}]$, and the amount of ligand bound per protein molecule under experimental conditions, R, can be calculated from Eqns 2 and 3, respectively, which were derived in ref. 5 for this gel equilibration method.

$$[L_{0(\text{free})}] = [L]\,\frac{\beta'(\alpha - \beta)}{\alpha - \beta'} \tag{2}$$

$$R = \frac{[L]}{[P]}\,\frac{\beta - \beta'}{\alpha - \beta'} \tag{3}$$

(Notice there is an obvious mistake in Eqn 8 of ref. 5; the right hand side of the equation should be multiplied by $[L]$, the ligand concentration added to the assay mixture). $[P]$ is the μM concentration of protein.

α and β' are constants when the weight of the dried gel and the volume of the solution added to the gel are kept constant. α, the ratio of the total volume and the volume outsise the gel, was determined experimentally as described in ref. 5 and was equal to 2.00. β' is defined as the ratio of the concentration of the ligand outside the

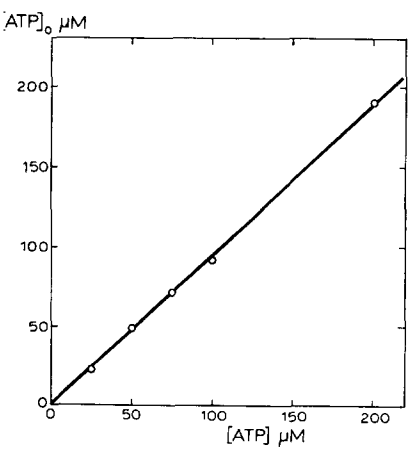
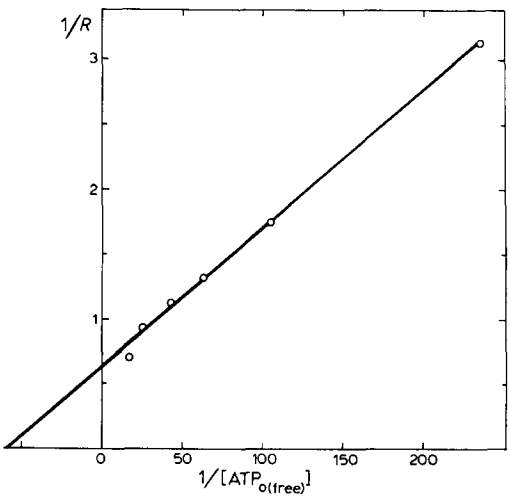


Fig. 1. The linear relationship between the concentration of [^{14}C]ATP (X axis) added to the gel and that of [^{14}C]ATP outside the gel (Y axis).

gel to that of the solution of the ligand added in the absence of binding protein. Fig. 1 shows the plot of the concentration of [^{14}C]ATP outside the gel, $[ATP]_o$, *vs* that of ATP added, [ATP]. The value of β', 0.94, was obtained from the slope of the line.

β, the ratio of the concentration of the ligand outside the gel to that of the solution of the ligand added, is varied by the binding of the ligand by the protein; it depends on the values of $[L]$ and $[P]$. By substituting 2.00 for α, 0.94 for β' and calculating the β value for each $[L]$ and $[P]$, we calculated $[L_{o(free)}]$ and R from Eqns 2 and 3, respectively.



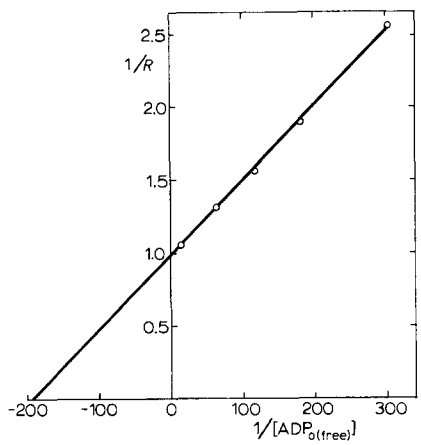


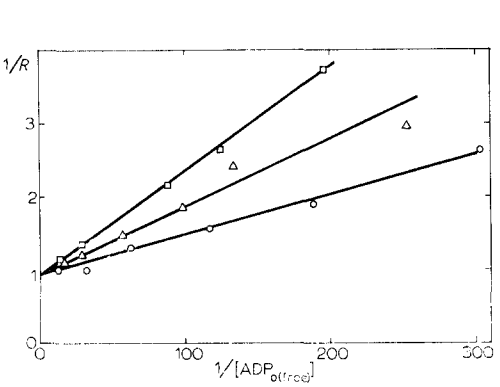
Fig. 2. Plot of $1/R$ *vs* $1/[ATP_{0(free)}]$. The units of the X-axes in Figs 2–5 are mM^{-1}. The conditions were as described in the text. The range of [^{14}C]ATP concentrations was 10–90 μM. 17.1 μM Fe protein was added to each bottle.

Fig. 3. Plot of $1/R$ *vs* $1/[ADP_{0(free)}]$. Same conditions as in the legend for Fig. 2 except ADP was used instead of ATP.

Fig. 2 shows the reciprocal plot of bound ATP per Fe protein molecule *vs* the free ATP concentration outside the gel. As calculated from the intercept of the line on the ordinate and the abscissa, $N = 1.7$ and $K = 16.7$ μM. The same type of plot for ADP (Fig. 3) gives values of $N = 1.01$ and $K = 5.2$ μM. These data indicated that the Fe protein can bind two MgATP, but only one MgADP; the MgADP is more strongly bound. This treatment assumes that the preparation contains no inactivated Fe-protein.

Inhibition of ATP binding by ADP and vice versa

What are the interactions in the binding of MgATP and MgADP? Is the site for the MgADP the same as one of the two sites for the MgATP? These interactions were studied by observing the inhibition of ATP binding by ADP and *vice versa*. Unlabeled ATP was used as an inhibitor of binding of [^{14}C]ADP. The inhibition pattern is competitive as shown in Fig. 4, and this suggests that the ADP binding site is the same as one of the two ATP sites. In Fig. 5, unlabeled ADP was used to



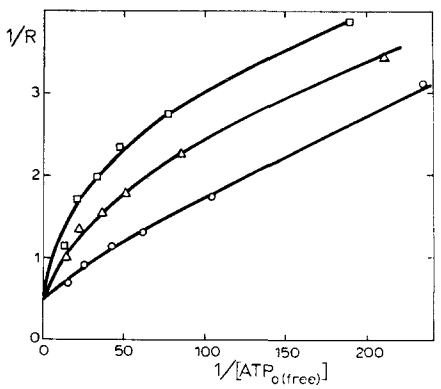


Fig. 4. ATP Inhibition of ADP Binding. [^{14}C]ADP binding in the presence of: ○—○, no ATP; △—△, 15 μM unlabeled ATP; □—□, 30 μM unlabeled ATP.

Fig. 5. ADP Inhibition of ATP Binding. [^{14}C]ATP binding in the presence of: ○—○, no ADP; △—△, 10 μM unlabeled ADP; □—□, 20 μM unlabeled ADP.

inhibit the binding of [^{14}C]ATP. The small curvature of the $1/R$ *vs* $1/[ATP_{o(free)}]$ plot at zero ADP concentration (bottom line) may indicate the heterogenity of the two ATP sites, but their dissociation constants differ two fold at most. The same situation exists in the plot of Fig. 2 with respect to this curvature which tends to increase the value of N for ATP binding. In fact, it is hard to say whether this slight curvature results from heterogenity of the two ATP sites, from a small cooperative effect between them, or from experimental error; the error may be appreciable because the control level of [ATP] is high and the percent increase in radioactivity due to binding is lower. However, in the presence of ADP, the plots obviously deviate from straight lines, and this indicates that the ADP differentiates between the two ATP-binding sites.

DISCUSSION

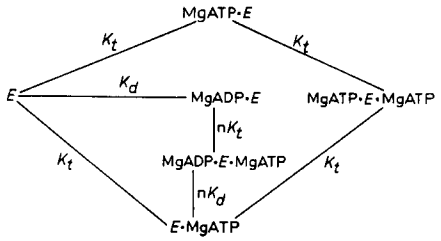
The rapid gel equilibration method used here permits binding studies with the labile nitrogenase proteins. The observed lack of binding of ATP to the MoFe protein is not due to inactivation of the MoFe protein but to a real property of the protein. As ATPase enzymes are common contaminants in nitrogenase preparations, we made sure to work with nitrogenase components free of contaminating ATPase. The positive binding of MgATP and MgADP to the Fe protein therefore is specific and relevant to N_2 reduction. Only three reactants, Mg^{2+}, ATP and Fe protein, are involved in the binding, and addition of other reactants involved in N_2 reduction does not enhance this binding; this agrees with the work of Bui and Mortenson[2] in relation to the protein and Mg^{2+} requirements for ATP binding.

Our results on the number of binding sites are pertinent only to those sites which are measurable with the present method. Sites with excessively high dissociation constants would require measurement at higher ligand concentrations; they could not be detected by this gel equilibration method because they would induce a very small percent increase in radioactivity outside the gel relative to the control. The

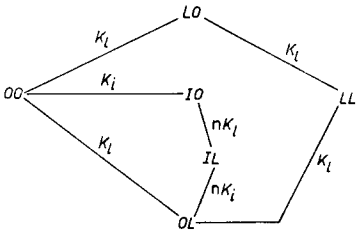
present data indicate that the Fe protein has two MgATP-binding sites with a dissociation constant of 17 μM and one MgADP site with a dissociation constant of 5 μM.

The unique ADP inhibition of substrate reduction, ATP hydrolysis and electron transport in the nitrogenase reaction is understandable because it binds more strongly than does ATP to the Fe protein and because it inhibits ATP binding by occupying one of the two ATP sites.

Based on the nonlinear inhibition pattern of ADP on ATP binding and the available information on the binding of ADP and ATP, a model which describes the molecular events in the binding process follows:



The free Fe protein form, E, has two independent identical sites for MgATP and one site for MgADP, which is one of the two ATP sites; cooperativity may exist between ATP and ADP. When ATP binding is measured in the presence of the inhibitor, ADP, as illustrated in Fig. 5, the above model with the appropriate dissociation constants, K_l and K_i, for the ligand and inhibitor, respectively, can be rewritten as follows in which O represents an open site:



The forms, LO, OL, IL and LL contribute to the observed bound ligand, R. It can be shown that, at equilibrium, Eqn 4 holds.

$$\frac{1}{R} = \frac{1 + K_l\left(2 + \frac{I}{nK_i}\right)\frac{1}{L} + K_l^2\left(1 + \frac{I}{K_i}\right)\frac{1}{L^2}}{2\left[1 + K_l\left(1 + \frac{1}{2n}\frac{I}{K_i}\right)\frac{1}{L}\right]} \tag{4}$$

The asymptotic slope of the curve described by Eqn 4 is equal to:

$$\frac{K_l}{2}\,\frac{K_i + I}{K_i + \frac{I}{2n}}$$

When $n \geqslant 1$, the asymptotes in Fig. 5 would diverge. The fact that they show no divergent tendency rules out the possibilities of zero and negative cooperativity. When $n = 0.5$, the asymptotes are parallel, much like the curves observed. When n is smaller than 0.5, the asymptotes converge. This analysis indicates that the binding of ADP at one site increases the affinity of the Fe protein for MgATP about 2-fold at the second site.

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