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Binding and Reaction Studies with Adenine Nucleotides on Purified Coupling Factor from *Rhodospirillum rubrum**

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Abstract

Equilibrium binding studies with ATPase isolated from *Rhodospirillum rubum* chromotophores have been carried out using gel filtration.

Binding experiments with variable concentrations of [^{14}C]ADP show a biphasic saturation curve. With a parameter fitting computer program the dissociation constants for two distinct binding sites are determined as 7×10^{-6} and 9×10^{-5} M, respectively. The enzyme-bound radioactivity is recovered as ADP (80–90%), and the rest is converted to AMP and ATP. In the free nucleotides a large amount of AMP (about 70%) is found in addition to ADP.

Analogous binding experiments with [^{14}C]ATP are monophasic. Most of the bound radioactivity can be identified as ADP showing a dissociation constant corresponding to the high affinity site. The pattern of the free nucleotides is the same as in the experiments with ADP.

These results indicate three separate binding sites on the enzyme: a low and a high affinity site for ADP, and a site at which ATP hydrolysis takes place. The analysis of the nucleotides suggests for the ADP sites a phosphoryl group transfer to produce ATP and AMP. Various experiments exclude the contamination of the enzyme preparation with adenylate kinase.

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Introduction

Several mechanisms have been proposed for ATP synthesis in oxidative and photosynthetic phosphorylation. Approaches have been made with whole membrane systems as well as with the isolated coupling factor exhibiting ATPase activity. The interaction of the latter enzyme with the *in vivo* substrates may give important indications to mechanistic questions of the process of ATP synthesis. Nucleotide binding experiments have been made with enzymes isolated from different sources, such as spinach chloroplasts [1–3], mitochondria of beef heart [4] and of rat liver [5], and bacterial membranes [6–9].

The coupling factor from chromatophores of the photosynthetic bacterium *Rhodospirillum rubrum* has been isolated and purified with several methods so far. The chromatophores have been treated with acetone [8] or with Mg^{2+}-free buffers of low ionic strength and French pressure cell [9] to solubilize the coupling factor from the membranes. An almost pure form of ATPase is obtained by shaking the membranes with chloroform as first described by Beechey et al.[10] for the preparation of the mitochondrial enzyme, and by Younis et al. [11] for the chloroplast coupling factor. This simple and rapid method is used here for the isolation and purification of the ATPase from *Rhodospirillum rubrum*. With this enzyme equilibrium binding experiments with labeled adenine nucleotides were carried out. The number, types, and nucleotide specificities of the binding sites on the enzyme have been determined, as well as the composition of the reaction products.

Materials and Methods

Preparation of Chromatophores and Isolation of Membrane-Bound Coupling Factor

Rhodospirillum rubrum S–1 was grown photosynthetically in a malate medium [12], harvested after having reached growth saturation, washed three times with 50 mM Tris-HCl pH 8, 200 mM sucrose, and stored at −90°C. Chromatophores were prepared by sonicating a cell suspension containing 1.5 mg DNAse/20 g cells in this buffer, followed by centrifugation for 20 min at 20,000 × *g* to remove unbroken cells. The supernatant was centrifuged for 60 min at 300,000 × *g*. After three washings the chromatophores were suspended at room temperature in a buffer containing 10 mM Tris-SO_4, pH 7.8, and 250 mM sucrose to a final concentration of 0.23 mg BChl/ml. Bacteriochlorophyll was determined using the *in vivo* extinction coefficient reported by Clayton [13].

The coupling factor was solubilized from the chromatophore membranes by treatment with organic solvents, as first described for the

mitochondrial coupling factor by Beechey et al. [10]. Analytical grade chloroform (0.5 vol) was added to the chromatophore suspension and the two phases were vigorously mixed for 2 min on a lab shaker. After centrifugation for 40 min at 12,000 × *g* the aqueous phase was separated from the organic phase, which was discarded. The aqueous phase was further centrifuged for 30 min at 200,000 × *g* to remove membrane particles. The supernatant was concentrated by ultrafiltration using a Diaflo XM 100 A membrane filter. The protein solution was precipitated with ammonium sulfate. The fraction precipitating between 30 and 60% saturation was kept at 4°C over a few weeks without loss in ATPase activity.

Analytical Methods

ATPase activity was measured in an assay containing 100 mM Tris-acetate pH 8, 10 mM $CaCl_2$, and 5 mM ATP partially γ-^{32}P labeled in a final volume of 1 ml. The reaction was terminated after 1-min reaction time at 35°C by adding 0.2 ml of 25% trichloroacetic acid. [^{32}P]Phosphate was determined in an aqueous solution using Cerenkov emission [14]. Protein concentration was estimated by the method of Lowry et al. [15] using bovine serum albumin as reference protein. Polyacrylamide gel electrophoresis was performed by the procedure described by Davis [16].

Detection of Protein and Enzyme Activity on the Gels

Proteins were revealed by staining with Coomassie brilliant blue 0.05% in 50% methanol and 5% acetic acid for 20 min at 60°C, followed by destaining overnight in 10% methanol and 10% acetic acid. To locate directly the position of the ATPase activity the histochemical procedure for the detection of phosphatase in tissues was modified [17]. The gel was incubated in the medium described for the ATPase assay during 15 min at 30°C. The phosphate liberated by the enzyme reaction was detected after immersing the gel in a solution containing 80 mM Tris maleate pH 7, and 3 mM $Pb(NO_3)_2$ for 30 min at 25°C to form $Pb(P0_4)_2$. In a final step in an incubation with 5% $(NH_4)_2S$ the $Pb(PO_4)_2$ is immediately converted into the insoluble PbS, a black band denoting the position of the enzyme activity.

Nucleotide Binding Experiments

After incubating the enzyme with different nucleotides in Tris-HCl 10 mM, pH 8, the protein was separated from free nucleotides on a Sephadex G–50 column (1.5 × 24 cm, void volume 16.7 ml) equilibrated with 10 mM Tris-HCl, pH 8. In order to determine the bound nucleotides the pooled

protein fractions were treated with perchloric acid (final concentration 7%. After neutralization the released nucleotides were loaded on a DEAE-Sephadex-A25 column equilibrated with 100 mM Tris-HCl, pH 8. ATP, ADP, and AMP were separated with a linear gradient from 0 to 300 mM NaCl. Radioactivity was measured in an Isocap 300, Nuclear Chicago scintillation counter with a scintillation medium for ^{14}C [scintillators: 2,5-diphenyloxozol and 1,4-bis-2-(4-methyl-5-phenyloxazyl)benzene; solvents: toluene and ethanol] and in aqueous solution for ^{32}P using Cerenkov emission [14].

Materials

[8-^{14}C]ADP, [U-^{14}C]ADP, and [U-^{14}C]ATP were obtained from the Radiochemical Centre, Amersham. γ-^{32}P-labeled ATP was prepared in our laboratory. Unlabeled nucleotides were purchased from Boehringer, Mannheim. The chemicals used in polyacrylamide gel electrophoresis are products from Serva Feinbiochemica, Heidelberg.

Results

Enzyme Preparation

The chloroform treatment with the bacterial membranes releases specifically the coupling factor from the chromatophore membranes. After purification gel electrophoresis shows a single protein band which contains the full enzymatic activity (Fig. 1). The major advantages of this procedure

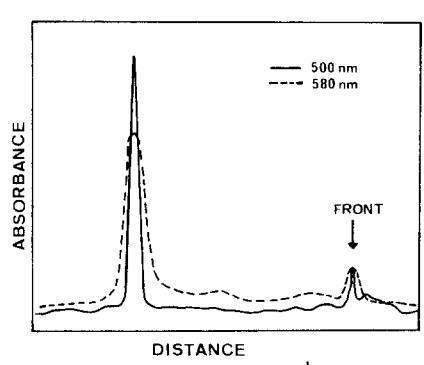

Figure 1. Gel profile of purified coupling factor (see Methods). The 7.5% polyacrylamide gels were loaded with 42 µg protein. The solid line denotes the Coomassie brilliant blue-stained protein, the dashed line the PbS precipitation indicating the ATPase activity. The arrow labels the bromphenol blue front band.

are speed and simplicity. The specific activity ranges from 16 to 27 μmole P_i /min · mg protein. A molecular weight of 360,000 was taken for the calculations [8].

Nucleotide Binding Profiles and Mathematical Treatment of the Data

The binding of ADP to the enzyme is slow; it reaches saturation after about 100 min (Fig. 2, inset). Therefore, a standard incubation time of 2 hr was selected for the present studies. The binding of ^{14}C label as a function of [^{14}C]ADP and [^{14}C]ATP concentrations is shown in Figs. 2 and 3. Equilibrium binding experiments with ADP show a biphasic saturation curve, indicating the existence of two binding sites for ADP. In analogous experiments with ATP, the binding profile of bound label is rather monophasic. Figures 4 and 5 represent the same results plotted as proposed by Scatchard [18]. The biphasic curve results in a hyperbola, which is characteristic for a system with one ligand and two binding groups, while the monophasic curve gives a straight line. In Fig. 5 the values for the equilibrium constant can be deduced directly from the graph. For the determination of the equilibrium parameters in Fig. 4 a mathematical treatment of the hyperbola as proposed by Feldman [19] was

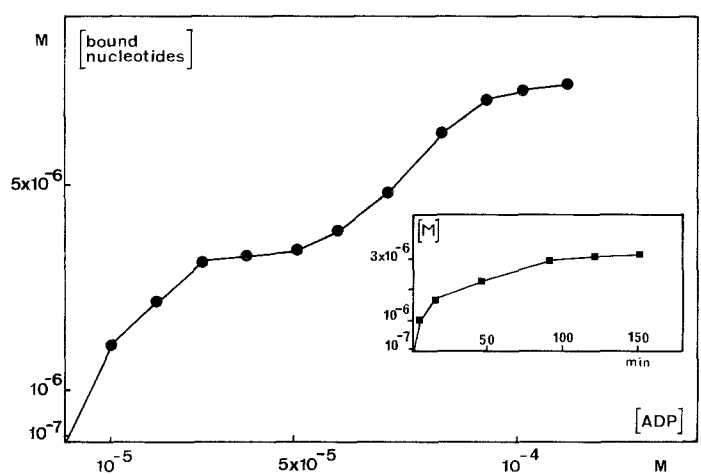

Figure 2. Binding of ADP to purified coupling factor. The reaction mixture contained 10 mM Tris-HCl, pH 8, [8-^{14}C]ADP (specific radioactivity 53 mCi/mmole) in the concentrations indicated in a total volume of 0.5 ml. The protein concentration was 1.837×10^{-6} M. For details see Methods. Inset: Time course of binding determined by incubating the enzyme with [8-^{14}C]ADP (2.09×10^{-4} M) under identical conditions. Abscissa and ordinate are drawn in linear scales.

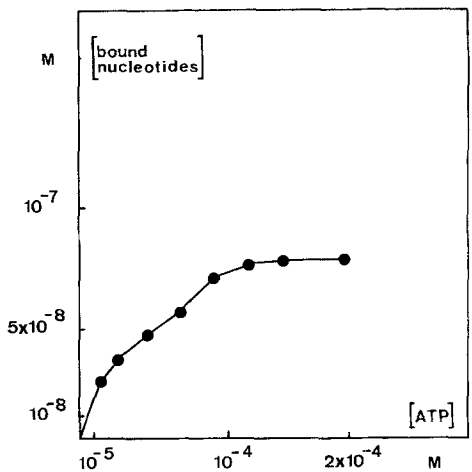

Figure 3. Binding of ATP to purified coupling factor. The reaction mixture contained 10 mM Tris-HCl, pH 8, [U-^{14}C]ATP (specific radioactivity 567 mCi/mmole) in the concentrations indicated in a total volume of 0.5 ml. The protein concentration was 0.76 x 10^{-6} M. For details see Methods. Abscissa and ordinate are drawn in linear scales.

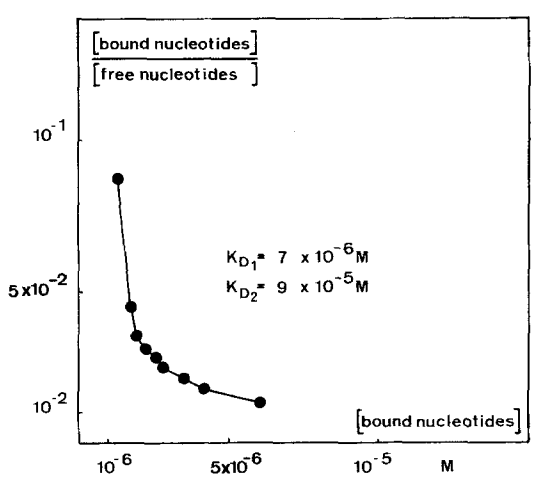

Figure 4. Data of Fig. 2 plotted as proposed by Scatchard [18]. The K_D data represent computed average values of three experiments (see Table I). Abscissa and ordinate are drawn in linear scales.

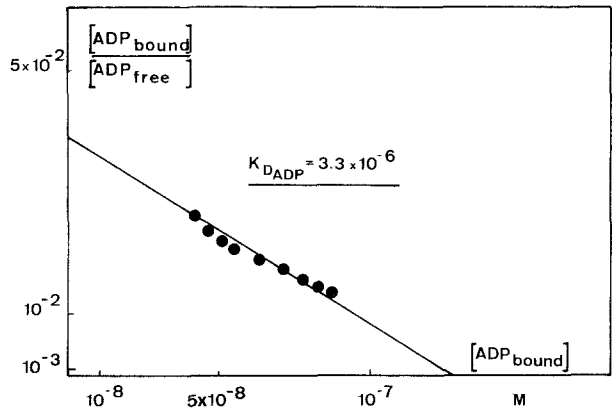

Figure 5. Data of Fig. 3 drawn as Scatchard plot. Abscissa and ordinate are drawn in linear scales.

used. This leads to a quadratic equation for the bound-to-free ratio of the ligand (R):

$$R=\tfrac{1}{2}[K_1(q-b)+K_2(q_2-b)+([K_1(q_1-b)1-K_2(q_2-b)]^2+4K_1K_2q_1q_2)^{\frac{1}{2}}]$$

K_1 and K_2 are the two affinity constants, q_1 and q_2 denote the total concentrations of binding groups, and b is the concentration of bound ligand. The method of parameter fitting with a FORTRAN computer program [20] was used for the resolution of the four parameters (K_1, K_2, q_1, q_2), which define the position of the asymptotes to the hyperbola. The results of three independent experiments with three different enzyme preparations are given in Table I.

TABLE I. Numerical Resolution of Three Experiments with [^{14}C]ADP

Parameter	Experiment			Average	Standard deviation
	1	2	3		
k_1	0.12×10^6	0.22×10^6	0.12×10^6	0.15×10^6	0.58×10^5
k_2	0.15×10^5	0.18×10^5	0.66×10^4	0.13×10^5	0.59×10^4
q_1	0.70×10^{-6}	0.28×10^{-6}	0.36×10^{-6}	0.47×10^{-6}	0.22×10^{-6}
q_2	0.17×10^{-5}	0.28×10^{-5}	0.96×10^{-6}	0.18×10^{-5}	0.92×10^{-6}
Dissociation constants					
K_{D_1}	8.19×10^{-6}	4.46×10^{-6}	8.43×10^{-6}	7.03×10^{-6}	2.23×10^{-6}
K_{D_2}	6.62×10^{-5}	5.44×10^{-5}	1.52×10^{-4}	9.09×10^{-5}	5.33×10^{-5}

[a] For conditions, see Fig. 2.

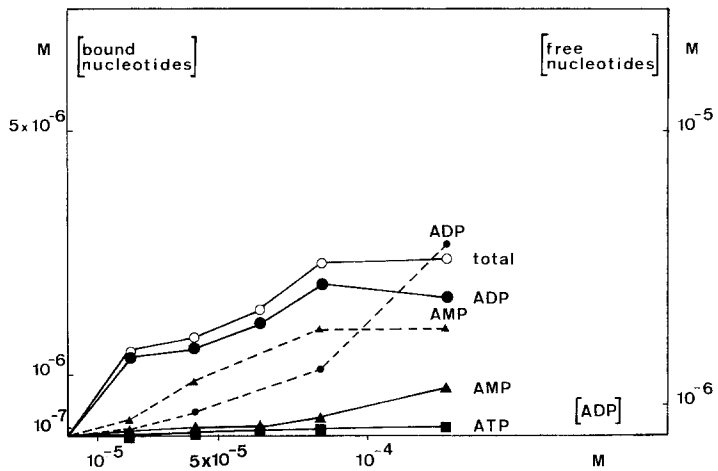

Figure 6. Analysis of enzyme bound(——) and free (- - -) nucleotides after incubation with [U-^{14}C]ADP (specific radioactivity 592 mCi/mmole). The protein concentration was 1.144 $\times 10^{-6}$ M. For details of the incubation assay and conditions see Methods. The values obtained were calculated for 1 mg protein. Abscissa and ordinate are drawn in linear scales.

Nucleotide Analysis

The radioactivity profiles of the material extracted from the coupling factor showed only peaks, which coincide in all cases with ultraviolet absorption of the three marker nucleotides AMP, ADP, and ATP (not shown).

Figure 6 represents a binding experiment with [^{14}C]ADP, analogous to the one in Fig. 2. Between 80 and 90% of the total bound radioactivity was identified as ADP. The rest was recovered as AMP and ATP. Since the original [^{14}C]ADP contained less than 1% contamination with [^{14}C]AMP and no [^{14}C]ATP, bound AMP as well as bound ATP must have been formed on the enzyme from bound ADP. To test for contaminating adenylate kinase activity, the purified ATPase was incubated with equimolar amounts of [γ-^{32}P]ATP and unlabeled AMP. No [β-^{32}P]ADP could be detected after incubation conditions identical to the binding experiments. Figure 7 shows a binding experiment with [^{14}C]ATP, analogous to the one in Fig. 3. Again about 90% of the radioactivity was bound as ADP. AMP and ATP appeared in the same relation as in the former experiment where the binding of ADP was studied. ATP as the substrate of the ATPase is hydrolyzed. Since the rate of hydrolysis of ATP by the coupling factor preparation is rapid compared to the time to reach

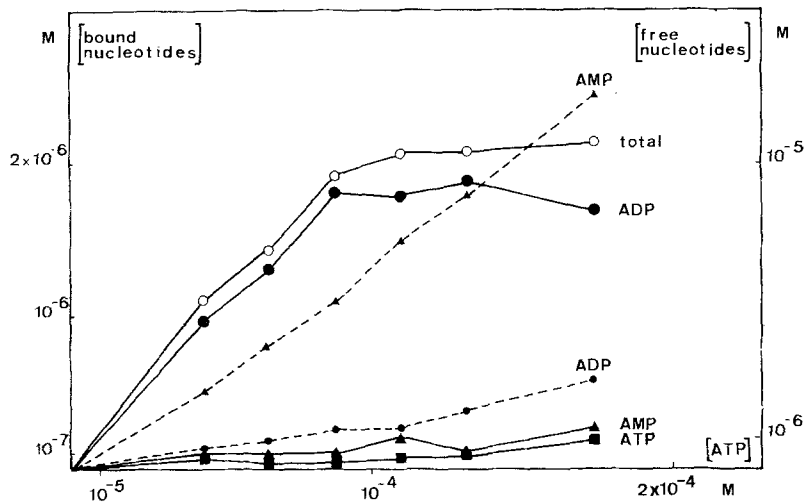

Figure 7. Analysis of enzyme bound(——) and free (- - -) nucleotides after incubation with [U-^{14}C]ATP (specific radioactivity 567 mCi/mmole. The values obtained were calculated for 1 mg protein. Abscissa and ordinate are drawn in linear scales.

equilibrium in the binding studies, the dissociation constant calculated in Fig. 5 refers to ADP as the binding species rather than to ATP, given as substrate. For the analysis of the free nucleotides, the incubation mixture was analyzed on an anion exchanger after removal of the protein. At low ADP concentrations more AMP than ADP was found (Fig. 6). At higher concentrations AMP reached a constant level, whereas ADP showed a sharp increase. In experiments with ATP (Fig. 7) the values for AMP were at any concentration higher (about 80% of the total) than of free ADP. In both experiments no free ATP could be found.

Discussion

The finding of two distinct ADP binding sites on the *Rhodospirillum rubrum* coupling factor stands in good agreement with data obtained with isolated enzymes from spinach chloroplasts [1–3] and rat liver mitochondria [4]. The dissociation constants for the high affinity site range from 0.28 [4] to 68 μM [7], a result obtained with an ATPase from *Mycobacterium phlei*, which exhibits three identical ADP binding sites. For the lower affinity site, dissociation constants between 47 [4] and 300 μM [5] are published. These differences for the dissociation constants may be due to the presence or absence of divalent cations during binding and furthermore to the various

sources for the ATPase. The experimental data were mostly treated with graphical methods separately for each site. Our results, on the other hand (7 and 90 μM), were obtained with a formula specially derived for a system with one ligand and two binding groups. The parameters worked out with a computer program in three independent series of experiments show good agreement for the high affinity site (Table I). However, the greater deviations observed for the second site could be due to some unspecific binding of the nucleotides at high ADP concentrations.

An analysis of the species of the bound nucleotides has only been carried out so far by Roy and Moudrianakis [1]. In agreement with our results, most of the radioactivity bound to the chloroplast coupling factor was identified as ADP. The finding of small amounts of bound AMP and ATP indicates a phosphoryl group transfer between two bound ADP. Further evidence that AMP is formed on the enzyme lies in the fact that free AMP does not bind [1, 5]. In addition, this idea is supported by the analysis of the free nucleotides, which shows that AMP and ATP formed on the enzyme are released. The free ATP, however, is hydrolyzed by the enzyme and the formed ADP is immediately rebound where it may undergo transphosphorylation with a second bound ADP. The rapid hydrolysis of ATP leads to an increasing amount of free ADP while the limiting step for the production of free AMP seems to be the slow binding of ADP and the following transphosphorylation step. Phosphorylation experiments with spinach subchloroplast particles [21] support the postulated transphosphorylation reaction. Short-time illumination in the presence of [^{32}P]phosphate showed a fast labeling of ADP, reaching a constant level of about 1 mole ADP/1 mole coupling factor, paralleled to ATP synthesis exhibiting a short lag before reaching steady state phosphorylation rates.

The data obtained with ADP give rise to the question of whether one of the two binding sites or a separate site is responsible for ATP hydrolysis. An indication for the existence of a separate site was given by Pedersen [22]. He identified ADP as a weak competitive inhibitor ($K_I = 240–300$ μM) of ATP hydrolysis. The much lower affinity of ADP for the ATP hydrolytic site compared to the site involved in binding ADP with high affinity (1–2 μM) would suggest that these two sites are distinct. To avoid ATP hydrolysis the analog AMP–PNP is often used for further elucidation of the problem. In experiments with coupling factor from spinach chloroplasts AMP–PNP was a potent inhibitor for all ADP sites [3]. These results are not consistent with data published by Lee et al. [7], where AMP–PNP exhibits only one binding site per molecule ATPase, this site being different than the one obtained for the binding of ADP. Furthermore, bound AMP–PNP was not displaced by ADP.

In our work the interaction of only physiological substrates was studied

and no analogs with altered binding and enzymatic activities were used. The dissociation constant for bound ADP after incubation with ATP lies in the order of magnitude of the high affinity site for ADP. These findings suggest either a binding of ATP to this site or a second site with the same affinity for ADP or a rebinding of the released ADP to the high affinity site after hydrolysis on a separate catalytic site. The binding of ATP itself to one of the ADP sites involved in transphosphorylation must be excluded, since the same amount of bound AMP and ATP was observed whether ATP or ADP was used as substrate. This indicates that transphosphorylation has to take place in both cases, independent of the simultaneous activity of ATP hydrolysis.

The tentative mechanistical scheme presented in Fig. 8 is based on the following summarized results:

1. The biphasic saturation curve for ADP and the mathematical resolution of the equilibrium parameters suggest two distinct binding sites for ADP.
2. Besides ADP, almost equal amounts of AMP and ATP were identified bound to the enzyme.
3. The reaction products (free nucleotides) after incubation with ADP are composed of AMP and ADP.
4. The saturation curve for ATP is monophasic.
5. The dissociation constant for ADP as the product of hydrolysis lies in the same order of magnitude of the high affinity site for ADP.
6. After incubation with ATP the enzyme-bound nucleotides show the same composition as in the experiments with ADP.
7. The reaction products are AMP and ADP as after incubation with ADP.

The scheme presented also explains the following data obtained by other groups:

1. Enzymes isolated from several different organisms exhibit two distinct binding sites for ADP [1–4].

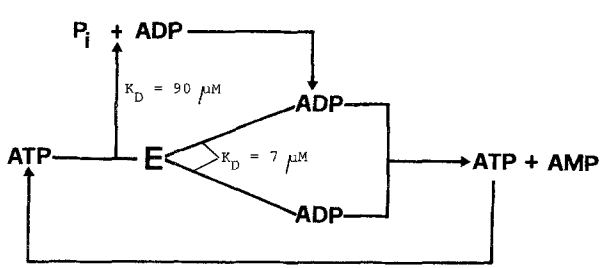

Figure 8. Mechanistic working hypothesis (see text).

2. ADP is a weak competitive inhibitor for the site of ATP hydrolysis [22].
3. AMP–PNP binds to a site different than the one for binding ADP; furthermore AMP–PNP is not displaced by ADP [7].
4. Phosphorylation experiments require in a first step the formation of AMP on the enzyme, which is then esterified with added P_i [23, 21].

The scheme implicates separate sites for ATP hydrolysis and ADP binding with high affinity with a total of three nucleotide binding sites. A phosphoryl group transfer would take place between the two high affinity sites for ADP, resulting in free AMP and ATP. On the third site with high affinity for ATP and low affinity for ADP, hydrolysis of ATP occurs. The ADP thus formed is released and replaced immediately by free ATP.

Following the conclusion of our investigation and prior to publication of the results, a similar work by Moudrianakis et al. [24, 25] appeared which supports our model.

Acknowledgments

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