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Properties of noncatalytic sites of thioredoxin-activated chloroplast coupling factor 1

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Abstract

Nucleotide binding properties of two vacant noncatalytic sites of thioredoxin-activated chloroplast coupling factor 1 (CF_1) were studied. Kinetics of nucleotide binding to noncatalytic sites is described by the first-order equation that allows for two nucleotide binding sites that differ in kinetic features. Dependence of the nucleotide binding rate on nucleotide concentration suggests that tight nucleotide binding is preceded by rapid reversible binding of nucleotides. ADP binding is cooperative. The preincubation of CF_1 with Mg^{2+} produces only slight effect on the rate of ADP binding and decreases the ATP binding rate. The ATP and ADP dissociation from noncatalytic sites is described by the first-order equation for similar sites with dissociation rate constants $k_{-2}(ADP) = 1.5 \times 10^{-1}$ min^{-1} and $k_{-2}(ATP) \cong 10^{-3}$ min^{-1}, respectively. As follows from the study, the noncatalytic sites of CF_1 are not homogeneous. One of them retains the major part of endogenous ADP after CF_1 precipitation with ammonium sulfate. Its other two sites can bind both ADP and ATP but have different kinetic parameters and different affinity for nucleotides.

Keywords: Chloroplast coupling factor 1 (CF_1); ATP synthase; F_1-ATPase; Noncatalytic site

1. Introduction

F_0F_1-ATP synthases of energy transducing membranes of chloroplasts, mitochondria and bacteria couple ATP synthesis and hydrolysis to proton (sodium) electrochemical gradients. They are composed of an integral membrane part (F_0) that mediates ion transport, and a peripheral membrane part, the coupling factor F_1 (CF_1). When separated from F_0, F_1 is an ATPase composed of five types of subunits with stoichiometry of $3\alpha{:}3\beta{:}\gamma{:}\delta{:}\varepsilon$. At the interfaces between α and β subunits, there are three catalytic and three noncatalytic nucleotide binding sites. The catalytic sites are present mostly on β subunits whereas noncatalytic sites are mostly on α subunits [1]. Unlike catalytic sites, noncatalytic sites do not have catalytic activity and retain nucleotides during turnover [2].

F_1-ATPases of different origin have been shown to develop turnover-dependent entrapment of inhibitory MgADP in a catalytic site [3,4]. Preincubation of mitochondrial F_1 with ATP prevents enzyme inactivation [3,5]. It has been postulated that transition from inactive to active state depends on ATP binding to noncatalytic sites, which promotes MgADP dissociation from the affected catalytic site [5,6]. A mutant $\alpha_3\beta_3\gamma$ subcomplex from thermophilic bacterium PS3 was generated in which noncatalytic sites lost their ability to bind nucleotides. The mutant complex was unable to dissociate inhibitory MgADP and recover its active state [7]. Strong inhibition of catalytic activity of mitochondrial F_1 was observed upon affinity labeling of noncatalytic sites [8–11]. It was shown that ATP binding to noncatalytic sites of chloroplast F_1-ATPase (CF_1) is necessary for catalytic activity [12]. The binding of ADP or nonadenine nucleotides inhibited the enzyme [13,14]. Kinetics of ADP and ATP binding to noncatalytic sites and their nucleotide specificity were described in Refs. [14,15]. Since isolated CF_1 has latent ATPase activity, it was heat activated in the presence of ADP and DTT in these studies. However, when activated in this manner, CF_1 loses its ability to reconstitute photophosphorylation when added to thylakoids depleted of the enzyme. An alternative activation procedure that maintains CF_1 ability to

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synthesize ATP after binding to the thylakoid membrane includes 14 h incubation in the presence of DTT and ATP [16]. The presence of ATP is necessary for enzyme stabilization during the long-term incubation. However, this prevents assessment of the affinities of noncatalytic sites for nucleotides. In chloroplasts, activation of ATP synthase is performed by reduced thioredoxin [17]. In this study, we took the advantage of the ability of thioredoxin to accelerate DTT-dependent activation of CF_1 [18]. To obtain an active enzyme in the absence of CF_1-stabilizing nucleotides, the enzyme was activated by a short incubation with thioredoxin in the presence of DTT. A kinetic analysis suggested that rapid reversible binding precedes the tight nucleotide binding to CF_1 noncatalytic sites. The tight binding was shown to occur in two phases. One mole of ATP per mole of CF_1 bound to noncatalytic sites during the initial fast phase, and approximately the same quantity of ATP bound during the slow phase. A short preincubation with magnesium ions significantly retarded the rates of both phases. The binding of ADP had a clearly cooperative pattern. Kinetic and equilibrium constants of ATP and ADP binding/dissociation were calculated.

2. Materials and methods

Spinach chloroplast coupling factor CF_1 isolated according to Binder et al. [19] was purified essentially as described by Ren and Allison [20]. A solution of CF_1 (about 100 mg) in buffer A (50 mM Tris–SO_4, pH 8.0, 1 mM EDTA, 1 mM ATP, and 0.5 mM PMSF) containing 0.45 M $(NH_4)_2SO_4$ was applied to a 2×8 cm column of Fractogel TSK Butyl-650S (Merck) and washed consecutively with 2 volumes of the same buffer, 100 ml of 0.2 M $(NH_4)_2SO_4$ and 100 ml of 0.15 M $(NH_4)_2SO_4$ in the same buffer. Finally, pure CF_1 as assessed by SDS-PAGE was eluted with buffer A. CF_1 was stored in 2 M ammonium sulfate in the presence of 1 mM ATP, 1 mM EDTA, and 50 mM Tris–SO_4, pH 7.8. Nucleotides and ammonium sulfate were removed by forced gel filtration using a fine Sephadex G-50 column equilibrated with 50 mM Tris–HCl, pH 7.8, 1 mM EDTA, and 50 mM KCl. The resulting preparation contained 1.7 mol of ADP and about 0.05 mol of ATP per mole of CF_1; one ADP is bound to a catalytic site [21] and the balance is probably at noncatalytic sites. It was activated in the presence of 2 μM thioredoxin (Promega, *E. coli* strain) and 2 mM dithiothreitol (DTT) at room temperature for 30 min. The protein concentration was determined according to Bradford [22] using a coefficient (1.18) for undersensitivity of this method to CF_1 as compared with that by Lowry et al. [23]. CF_1 molecular weight was assumed to be 400 kDa [24]. Nucleotide to CF_1 binding was performed in 50 μl medium containing [^{3}H]ATP or [^{3}H]ADP, 2 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.8, and 50 mM KCl. To maintain the [^{3}H]ATP concentration, the incubation medium also contained pyruvate kinase and phosphoenolpyruvate. Cessation of binding of labeled nucleotides and their selective dissociation from catalytic sites were performed using the "chase" method [21]. For this purpose, 5 μl of chase solution containing 15 mM ATP and 500 mM $KHSO_3$ were added to the reaction mixture; 20 s later, a 50 μl aliquot was applied onto a Sephadex G-50 (fine) column equilibrated with 50 mM Tris–HCl, pH 7.8, 2 mM $MgCl_2$, and 50 mM KCl. Forced gel filtration lasted for 25–30 s. CF_1 concentration was determined taking into account the presence of pyruvate kinase in the resulting fraction. After protein denaturation at 100 °C for 1.5 min, the fraction was subjected to centrifugation (2 min at $14\,000 \times g$) and HPLC using a 0.5×7.5 cm DEAE 5PW column. The eluent contained 80 mM KH_2PO_4 and 120 mM KCl. The nucleotide content was counted from radioactivity of the obtained fractions. ATPase activity was estimated by the phosphate release rate [25] at 37 °C or according to Pullman et al. [26] at 22 °C. A mathematical analysis of the experimental data was carried out using *Origin 6.0 Program*.

3. Results

The isolated and purified coupling factor has a rather low ATPase activity [27]. Its activation can be achieved by reduction of the disulfide bond of CF_1 γ-subunit during either short incubation of CF_1 in the presence of a minor amount of DTT at an elevated temperature (65 °C) [28] or its long-term incubation (14 h) at high DTT concentrations [16]. Either way implies ATP involvement for the purpose of CF_1 stabilization. As a result, nucleotides bind to noncatalytic sites at the step of ATPase activation, thus making it difficult to study interactions. The current study utilized the ability of thioredoxin to accelerate DTT-induced reduction of the disulfide bond of γ-subunit [18]. In the presence of 2 μM thioredoxin and 2 mM DTT, the maximal activation of CF_1-ATPase is achieved after 30 min, whereas it takes more than 3 h at high DTT concentrations with the commonly used procedure. The shortened procedure of activation in the presence of thioredoxin demands no CF_1-stabilizing nucleotides, and therefore no additional nucleotide binding to noncatalytic sites occurs. It was suggested that noncatalytic sites of DTT-activated CF_1 at room temperature bind nucleotides not as tightly as those of heat-activated CF_1 [12]. Therefore, it was necessary to learn to what extent the noncatalytic site-bound nucleotide quantitation technique developed for heat-activated CF_1 [21] is applicable in the case of thioredoxin-activated CF_1. To answer this question, thioredoxin-activated CF_1 incubated with [^{3}H]ATP and Mg^{2+} for 2 h was subjected to gel filtration: (a) immediately; (b) after addition of 40 μM ATP; (c) after addition of excess unlabeled ATP and sulfite. The sample applied on a column immediately after incubation contained 4 mol of tightly bound nucleotide at catalytic and noncatalytic sites; 3.3 of these were [^{3}H] nucleotides. As follows from Fig. 1, 40 μM ATP produced virtually no effect on the rate of

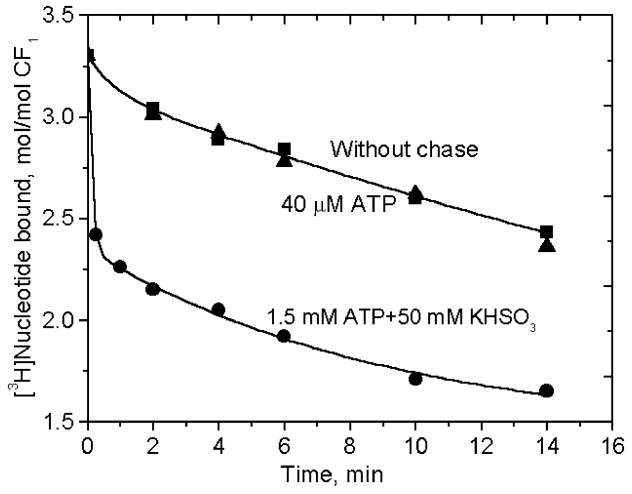

Fig. 1. Effect of CF_1 turnover on dissociation of tightly bound nucleotides. Thioredoxin-activated CF_1 (0.72 mg/ml) was incubated for 40 min in a medium containing 50 mM Tris–HCl, pH 7.8, 50 mM KCl, 30 μM [^{3}H]ATP, 2 mM $MgCl_2$, 1 mM phosphoenolpyruvate, and 0.2 mg/ml pyruvate kinase. Then CF_1 was (■) applied on Sephadex G-50 columns of different heights (in 50 μl aliquots) and gel-filtrated during time intervals indicated in the figure; mixed with 40 μM ATP (▲), or with 1.5 mM ATP, 50 mM $KHSO_3$ (ATP chase) (●), and 50 μl aliquots at indicated intervals were applied on a Sephadex G-50 column. Amount of tightly bound nucleotides was determined as described under Materials and methods.

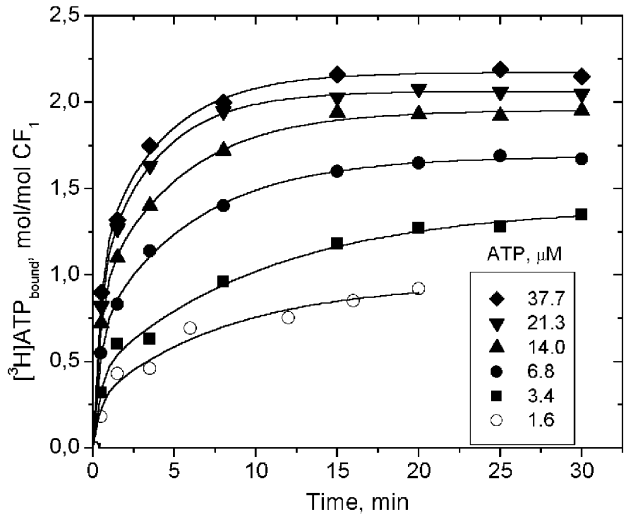

Fig. 2. Binding of [^{3}H]ATP to CF_1. CF_1 (0.07–0.14 mg/ml) was incubated with [^{3}H]ATP as indicated in a medium containing 50 mM Tris–HCl, pH 7.8, 50 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM EDTA, 1 mM phosphoenolpyruvate, and 0.29 mg/ml pyruvate kinase and, after ATP chase, was separated from unbound nucleotide as described under Materials and methods.

nucleotide dissociation. Addition of ATP and sulfite to the reaction medium initiated a decrease in radioactivity caused by exchange of labeled nucleotides for unlabeled ones. In the course of incubation, about 0.9 mol of nucleotide is removed after the first 15 s; then the process slows down: after 4 min, about 2 mol of labeled nucleotides still remain bound to CF_1. In the absence of sulfite that stimulates catalytic activity many fold, the phase of rapid radioactivity decrease was not observed. This suggests that the rapid phase occurs due to displacement of labeled nucleotides from the catalytic sites by unlabeled nucleotides. CF_1 activity measured in the control and amounting to 150 min^{-1} supports the suggestion. For further experiments, it was assumed that 20 s incubation with 1.5 mM ATP and 50 mM sulfite was sufficient for selective removal of nucleotides from CF_1 catalytic sites.

3.1. ATP and ADP binding to noncatalytic sites

Within the ATP concentration range of 1.6–37.7 μM, noncatalytic sites bind about 2 mol of ATP per mole of the enzyme (Fig. 2). In spite of a considerable excess of pyruvate kinase in the incubation medium, not only ATP but 0.2–0.3 mol of ADP becomes bound (not shown). There are two phases in the process of ATP binding to noncatalytic sites. About 1 mol of ATP/mol of CF_1 is bound during 1 min. The next ATP molecule binds to noncatalytic site significantly slower. As shown in Fig. 2, nucleotide binding is time- and concentration-dependent. At a given nucleotide concentration, Eq. (1) describes time dependence of tight nucleotide binding to two sites different in kinetic properties (*Origin 6 Program*).

$$y = N_1(1 - e^{-k(\mathrm{app1})t}) + N_2(1 - e^{-k(\mathrm{app2})t}) \quad (1)$$

The first-order equation usage is allowed by more than 20-fold excess of nucleotides over the CF_1 concentration. Here y is the number of noncatalytic sites filled after the time t; N_1 and N_2 are the numbers of sites involved in the rapid and slow phases of nucleotide binding; k_{app1} and k_{app2} are apparent rate constants of the rapid and slow phases, respectively.

Fig. 3 shows dependence of these constants on ATP concentration. The both plots are described by the Michae-

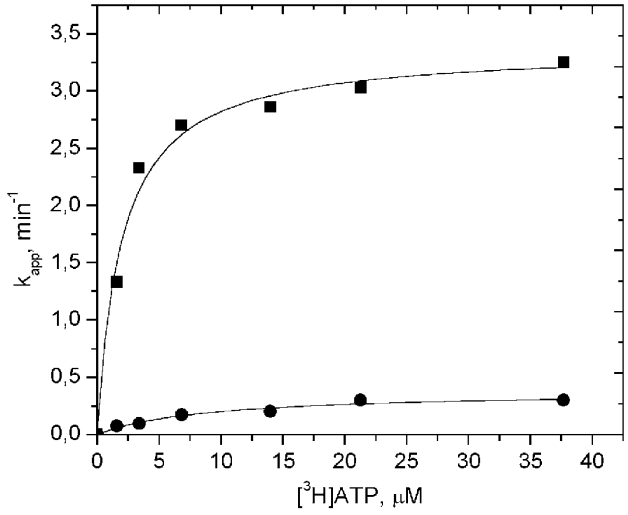

Fig. 3. The dependence of k_{app} values on ATP concentration for the fast (■) and slow (●) phase of [^{3}H]ATP binding to CF_1.

Table 1
Rate and dissociation constants of ATP and ADP binding and release from CF_1 noncatalytic sites

Nucleotide	Site	K_1 (μM)	k_{+2} (min^{-1})	k_{-2} (min^{-1})
ATP	1	1.3	3.2	$\approx 1 \times 10^{-3}$
ATP	2	9.0	0.4	$\approx 1 \times 10^{-3}$
ADP	1	8.0	6.7	1.5×10^{-1}
ADP	2	5.0	0.13	1.5×10^{-1}

lis–Menten function. This denotes that the tight binding is preceded by rapid equilibrium binding of nucleotides to the noncatalytic sites. The dissociation constants of initial nucleotide binding (K_1) and rate constants (k_{+2}) of tight binding calculated with the use of Origin 6.0 Program are given in Table 1.

Like ATP binding, ADP binding to CF_1 noncatalytic sites is a two-phase process (Fig. 4). For the studied 1.6–26.5 μM concentrations, the rapid binding is about 2-min long. In this phase, the amount of enzyme-binding nucleotide and the binding rate increase dramatically with increasing ADP concentration, which is probably indicative of the cooperative nature of binding. The maximal ADP binding amounts to about 2 mol per mole of CF_1. Within the given range of ADP concentrations, kinetics of nucleotide binding to noncatalytic sites is described by Eq. (1) provided the ratio between sites involved in the rapid (N_1) and slow (N_2) phases of binding increases with increasing nucleotide concentration. This is consistent with the suggested cooperativity of ADP binding. However, the rate constants and dissociation constants calculated in this manner should be regarded as tentative (Table 1).

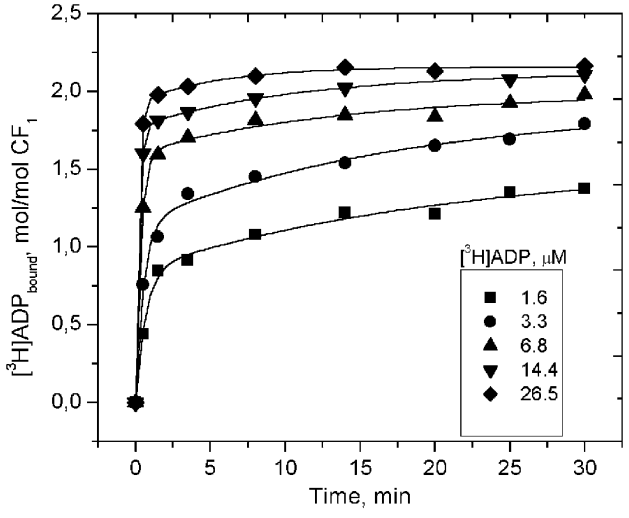

Fig. 4. Binding of [^{3}H]ADP to CF_1. CF_1 (0.08–0.14 mg/ml) was incubated with [^{3}H]ADP as indicated in a medium containing 50 mM Tris–HCl, pH 7.8, 50 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM EDTA, and after ATP chase was separated from unbound nucleotide as described under Materials and methods.

3.2. Nucleotide binding as affected by preincubation of CF_1 with magnesium ions

A short-term incubation of CF_1 with magnesium ions resulted in a considerable decrease of the rate of ATP binding to noncatalytic sites (Fig. 5). At an ATP concentration of 3.5 μM in the incubation mixture, the calculated apparent rate constants of the rapid and slow binding phases from Eq. (1) decreased 5- and 10-fold, respectively. The preincubation of CF_1 with Mg^{2+} produced nearly no effect on ADP binding kinetics (not shown).

3.3. Nucleotide dissociation from noncatalytic sites

To study kinetics of nucleotide dissociation from noncatalytic sites, CF_1 was incubated with 26 μM labeled ATP or ADP for 30 min before addition of 1.5 mM ATP and 50 mM sulfite to the incubation medium. At indicated intervals, aliquots were subjected to gel filtration to separate free nucleotides. Kinetics of ADP and ATP release from noncatalytic sites was fitted to a function of Eq. (2) (Fig. 6):

$$y = Ne^{-kt} \quad (2)$$

ATP dissociation was about 0.1 mol/mol CF_1 in 1 h, which corresponds to the dissociation rate constant $k_{-2}(ATP) = 1 \times 10^{-3}$ min^{-1}. During the same time, ADP dissociation amounted to 1.9 mol with the dissociation

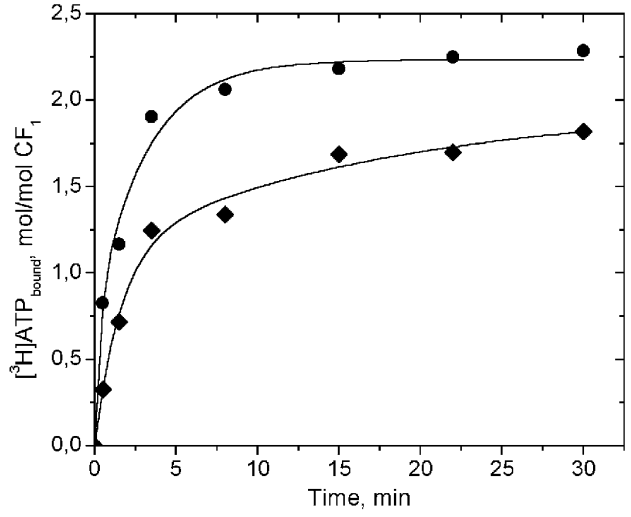

Fig. 5. Effect of CF_1 preincubation with $MgCl_2$ on [^{3}H]ATP binding. Control CF_1 (●) or CF_1 preincubated with 2 mM $MgCl_2$ for 2 min (♦) was incubated for the indicated times in a medium containing 3.4 μM [^{3}H]ATP, 50 mM Tris–HCl, pH 7.8, 50 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM EDTA, 1 mM phosphoenolpyruvate, and 0.29 mg/ml pyruvate kinase and after ATP chase was separated from unbound nucleotides using columns equilibrated with the buffer containing 50 mM Tris–HCl, pH 7.8, 50 mM KCl, 2.0 mM $MgCl_2$, and 0.1 mM EDTA.

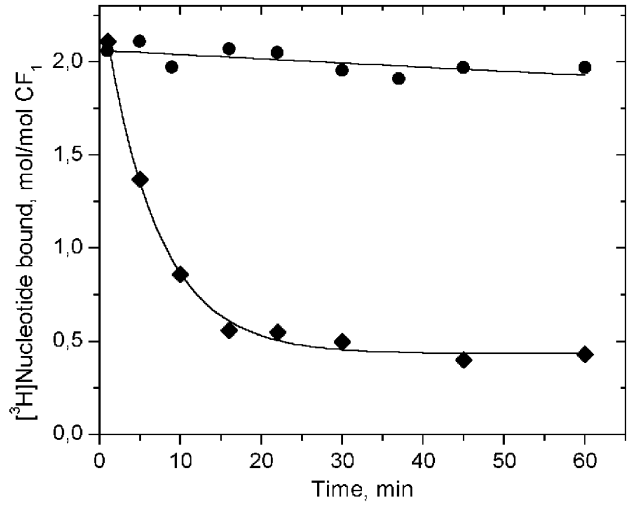

Fig. 6. Dissociation of nucleotides from CF_1 noncatalytic sites. CF_1 (0.14 mg/ml) was incubated for 30 min in a medium containing (●) 32 μM of [^{3}H]ATP, 50 mM Tris–HCl, pH 7.8, 50 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM EDTA, 1 mM phosphoenolpyruvate, and 0.29 mg/ml pyruvate kinase or (♦) 28 μM of [^{3}H]ADP, 50 mM Tris–HCl, pH 7.8, 50 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM EDTA; then 1.5 mM ATP and 50 mM K_2SO_3 was added. After indicated times, unbound nucleotides were separated as in the legend to Fig. 5.

rate constant $k_{-2}(\mathrm{ADP}) = 1.5 \times 10^{-1}$ min^{-1}, and about 0.4 mol ADP remained tightly bound.

4. Discussion

The concentration dependence of the apparent rate constants of ATP binding observed in this study suggests that the tight nucleotide binding to noncatalytic sites is preceded by a rapid and reversible nucleotide binding as described below (Fig. 3):

$$\mathrm{ATP} + \mathrm{CF_1} \overset{(1)}{\leftrightarrow} \mathrm{ATP} \cdot \mathrm{CF_1} \overset{(2)}{\leftrightarrow} \mathrm{ATP} > \mathrm{CF1}$$

Kinetics of subsequent formation and dissociation of tight complexes is indicative of considerable differences in ATP- and ADP interactions with CF_1 noncatalytic sites. The fraction of sites characterized by a high rate of tight complex formation increases with increasing ADP concentration (Fig. 4). Unlike ATP, interaction of ADP with two noncatalytic sites results in formation of less stable complexes (Fig. 6, Table 1). The rate of tight ATP binding decreases significantly as a result of preincubation of CF_1 with magnesium ions. As shown previously, such preincubation causes reversible inactivation of CF_1 ATPase activity due to formation of a tight complex between MgADP and one of the CF_1 catalytic sites [4,29]. The two effects are probably connected. This suggestion is supported by the effect of different noncatalytic site-bound ligands and modification of these sites on kinetics and the degree of reversible inactivation of F_1-ATPases of different origin [5,10,12,15,30].

Our results demonstrate considerable differences in specificity and affinity of the three noncatalytic sites of CF_1 for nucleotides. After separation from ammonium sulfate and ATP with subsequent thioredoxin activation, the preparation of coupling factor contained about 1.7 mol of ADP and hardly 0.05 mol of ATP per mole of CF_1. Of which, according to Ref. [21], 1 mol of ADP is bound to catalytic sites, and hence, the balance is probably at noncatalytic sites. The highest possible insertion of labeled ADP and ATP in noncatalytic sites observed after CF_1 incubation with nucleotides amounts to 2.2–2.4 mol/mol (Figs. 2 and 4). The coherent explanation for the lacking 0.6–0.8 mol/mol would be the suggestion that ADP retained by the noncatalytic site after CF_1 isolation and activation is incapable of exchanging with nucleotides of the incubation medium. The same noncatalytic site most probably retained about 0.3–0.4 mol of labeled ADP after CF_1 incubation for 60 min with sulfite and surplus ATP (Fig. 6). The other two noncatalytic sites can bind both ADP and ATP. These sites have different dissociation constants for steps (1) and (2) and different nucleotide binding rate constants (Table 1). Heterogeneity of noncatalytic sites was earlier observed in studies of mitochondrial [31] and heat-activated chloroplast coupling factor [15]. Unlike thioredoxin-activated CF_1, noncatalytic sites of MF_1 and heat-activated CF_1 bind 3 mol of labeled ATP per mole of the enzyme. It may be suggested that accessibility of the third site is determined by dissociation of endogenous ADP tightly bound to this site. In case of heat-activated CF_1, the dissociation is caused by changes in the enzyme structure occurring at an elevated temperature of activation. A report on the effects of ATP and ADP bound to noncatalytic sites on ATPase activity of CF_1 [2] suggests the possibility that noncatalytic sites might have a role in regulation of photophosphorylation. However, this possibility appears to be ruled out by the above results that show extremely slow dissociation of 2 mol of ATP and 1 mol of ADP from the noncatalytic sites. However, there are considerable structural differences between isolated and membrane-bound CF_1 [32,33]. The membrane energization also causes changes in the enzyme structure [34,35]. These changes may produce a significant effect upon the properties of nucleotide–noncatalytic sites complexes. Specifically, unlike the described above isolated CF_1, the ATP synthase complex CF_oCF_1 isolated by Possmayer et al. [36] contained about 2 mol ATP/mol CF_1. Thus, to shed light upon the possible role of noncatalytic sites in regulation of ATP synthase activity, one has to clarify the effect of the membrane and its energization on nucleotide binding properties of these sites.

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