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Kinetics of nucleotide exchange and of ATP hydrolysis by isolated chloroplast coupling factor CF_1 in the presence of inhibitors

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Isolated chloroplast coupling factor CF_1 contains a tightly bound ADP which is exchangeable upon addition of medium ADP or ATP. In the presence of $CaCl_2$ and ATP, the activated CF_1 catalyzes ATP hydrolysis during substitution of tightly bound ADP by medium ATP. It was found that exchange of bound nucleotides is fast enough to be a catalytic intermediate during ATP hydrolysis (Feldman, R.I. and Boyer, P.D. (1985) J. Biol. Chem. 260, 13088–13094). In this paper, the influence of various substances on binding of nucleotides to the tight site and on hydrolysis of medium ATP was investigated. Under normal conditions, the exchange of bound nucleotides was found to be faster than hydrolysis of 10 μM ATP. After addition of thiosulfate, however, exchange of tightly bound ADP was nearly completely inhibited, while hydrolysis of ATP was slightly activated. It was observed that preincubation of CF_1 with MgATP inhibited the exchange reaction, while ATP hydrolysis under the same conditions was only slightly inhibited. It is concluded that exchange of tightly bound ADP for medium ATP, and CaATP hydrolysis by activated CF_1 are independent reactions, at least at low medium ATP concentrations.

Introduction

Freshly isolated chloroplast coupling factor contains two tightly bound adenine nucleotides [1–3]: one ADP on an exchangeable site (site 1 or A; Refs. 4 and 5) and one MgATP on a non-exchangeable site (site 2; Ref. 4). During storage under ammonium sulfate (in the presence of EDTA and ATP) the tightly bound ATP dissociates slowly, and only the ADP molecule on site 1 remains bound [3,4]. In addition to this tightly bound ADP, two [4,6] or more [5] additional sites for reversible nucleotide binding were found. Presumably, one of these reversible sites was the site of tight MgATP binding; it is reoccupied by medium ATP during incubation in the presence of Mg^{2+}. This nucleotide undergoes no further exchange. The bound ADP on site 1 can be replaced by labeled ADP or ATP [3,4,6]. It might be that this replacement leads to different enzyme forms, since back exchange is biphasic, and a small amount of nucleotides remain irreversibly bound [17].

Upon addition of inorganic phosphate [7], ATP is formed by isolated CF_1 presumably from bound ADP on site 1; addition of phosphate together with $MgCl_2$, ADP and methanol enhances the amount of enzyme-bound ATP [8] which might be

Abbreviations: AdN, adenine nucleotide(s); CF_1, chloroplast coupling factor 1; DCCD, dicyclohexylcarbodiimide; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetate; RB 2, Reactive Blue 2 (Cibacron Blue 3 G-A; C.I. 61211); TNBS, trinitrobenzene sulfonate.

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bound to site 2. Both sites were assumed to be catalytic in ATP synthesis. Hydrolytic activity was assigned to different sites of activated CF_1; since the exchange of tightly bound MgATP on site 2 is too slow during ATP hydrolysis, this site is inactive under these conditions. Site 3 (Ref. 4) and additional sites other than sites 1, 2 or 3 (Ref. 5) were assumed to be the main catalytic site(s); even site 1 – in cooperation with other sites – was thought to be involved in the catalytic turnover [9]. In the latter hypothesis, the 'alternating site' or 'binding change' mechanism [10,11], the ADP molecule on site 1 is thought to be transitorily bound while a second (open) site releases product ADP and binds substrate ATP. In support of this model, the exchange of tightly bound ADP was found to be faster than hydrolysis of CaATP by activated CF_1 [9,12,13].

In this paper, inhibitors of nucleotide binding as well as of ATP hydrolysis were found and used for discrimination of the two nucleotide-dependent processes; it was found that some anions like thiosulfate effectively inhibit nucleotide exchange but have nearly no effect on ATP hydrolysis under the same conditions.

Materials and Methods

Chloroplast coupling factor CF_1 was isolated from spinach leaves as described [14] and was stored in 50 mM Tris (pH 8), 2 mM EDTA, 1 mM ATP under ammonium sulfate (50% saturation) at 4 °C. Prior to use, an aliquot was centrifuged, the sediment was dissolved in 50 mM Tris (pH 8), 2 mM EDTA and desalted on a Sephadex G-50 column equilibrated with the Tris-EDTA buffer. This CF_1 has one ADP bound per enzyme.

Activation of isolated latent CF_1 was done by incubation with dithiothreitol (25 mM DTT, overnight). Tight binding of nucleotides to the isolated CF_1 was studied by incubation of desalted enzyme (final concentration, 1 μM) at room temperature in Tris-EDTA buffer with [8-^{14}C]adenine nucleotides (2.1 GBq/mmol; Amersham-Buchler); special incubation conditions were indicated in the legends of figures and tables. Excess free nucleotides were removed by centrifugation of the sample through one or two 1-ml Sephadex G-50 columns as described [13]. Data obtained after two centrifugation columns were close to the data after the first centrifugation indicating that removal of medium nucleotides at the given low concentrations is effective during the first separation. ATP hydrolysis was determined with [^{14}C]ATP; after addition of perchloric acid (final concentration, 0.6 M) and centrifugation, the supernatant was neutralized with KOH, centrifuged and chromatographed on PEI-Cellulose plates as described [13]. From the relative amounts of the nucleotides in the samples, the amount of hydrolysed ATP was calculated. Radioactivity was measured in liquid scintillation cocktail (Beckman Optifluor); protein was determined according to Lowry et al. [15] with bovine serum albumin as standard. An extinction coefficient of 0.483 cm^2/mg at 277 nm [4] and a molecular mass of 400 kDa [16] were used for CF_1. Nitrofen and Reactive Blue 2 were kind gifts by Dr. Bernhard Huchzermeyer.

Results

During incubation of isolated latent CF_1 with [^{14}C]ADP or [^{14}C]ATP, incorporation of labeled nucleotides is observed. In the absence of magnesium ions, the final stoichiometry is 1 ^{14}C-nucleotide per CF_1; the formerly bound ADP on site 1 is exchanged during this process [3,4,17].

In the first experiment, [^{14}C]ADP was added to latent CF_1 together with various substances. Prior to the binding assay, CF_1 (1 μM) was preincubated with the substances for 30 min in order to allow complete equilibration with reversibly binding probes and sufficient time for irreversible binding in the case of trinitrobenzenesulfonic acid (TNBS) and dicyclohexylcarbodiimide (DCCD). [^{14}C]ADP was then added (final concentration, 10 μM) and incubated for 60 min; free nucleotides were removed by passing the mixture through a Sephadex centrifugation column. From the resulting amount of radioactivity and the concentration of protein, the stoichiometry of bound labeled nucleotides was determined. The results are listed in Table I.

Most of the substances had no effect on the binding reaction. The reversible inhibitors of Ca-dependent ATP hydrolysis by activated CF_1, Reactive Blue 2 [18] and ammonium ions, and the covalent inhibitor DCCD [19] showed a slight

TABLE I

EFFECT OF VARIOUS SUBSTANCES ON NUCLEOTIDE BINDING TO ISOLATED LATENT CF_1

Latent CF_1 (1 μM) was preincubated for 30 min with the substances (Na^+ or Cl^- salts) at the concentrations given in the table. [^{14}C]ADP was then added to a final concentration of 10 μM. After 1 h at room temperature, the assays were passed through Sephadex centrifugation columns, and the eluates were subjected to the determination of protein and radioactive ADP.

Addition	Concentration (mM)	Bound [^{14}C]ADP per CF_1	% of control
No addition		0.53	
Phloridzin	1	0.57	
Quercetin	1	0.50	
Nitrofen	0.05	0.51	
Reactive Blue 2	1	0.39	73
TNBS	0.1	0.50	
DCCD	0.5	0.42	79
Ammonium	10	0.34	64
Methylammonium	1	0.52	
Azide	10	0.53	
Perchlorate	10	0.52	
Phosphate	10	0.55	
Pyrophosphate	10	0.54	
Thiosulfate	10	0.52	
Carbonate	10	0.67	126
Malonate	10	0.68	128
Citrate	10	0.74	140

inhibition of [^{14}C]ADP binding; carbonate and organic acids showed a slight acceleration of the binding process. Ammonium chloride and Reactive Blue were therefore chosen for the kinetic experiments shown in Fig. 2 (see below).

It was described that pyrophosphate binds to isolated CF_1 [20] and inhibits the interactions of nucleotides with reversible binding sites [5]; it is further known that phosphate and thiosulfate inhibit binding of ADP to the membrane-bound CF_1 [21–24]. These anions had no effect on the incorporation of [^{14}C]ADP in the above described experiment; since binding of pyrophosphate depends on the presence of divalent cations [5,20], a more detailed binding experiment was carried out with pyrophosphate and thiosulfate in the presence of $CaCl_2$.

The incubation assay contained latent CF_1 and [^{14}C]ADP or [^{14}C]ATP $\pm$ $CaCl_2$. Pyrophosphate or thiosulfate were added to the assay when indicated. Separations by Sephadex centrifugation were done after 30 s and after 30 min. The data of this experiment are given in Table II. In the absence of Ca ions, the amount of bound nucleotides was nearly not affected. If $CaCl_2$ is added, pyrophosphate and thiosulfate inhibited binding to about 50% of the control within 30 s; a less inhibitory effect was observed after 30 min in the assay with [^{14}C]ATP and $CaCl_2$. Nearly no inhibition was seen after 30 min in the presence of [^{14}C]ADP + $CaCl_2$ (Table II). The lower inhibition after 30 min is in accordance with the observation that during the incubation with nucleotides the initial amount of bound pyrophosphate is decreased [5,20].

TABLE II

EFFECT OF PYROPHOSPHATE AND THIOSULFATE ON BINDING OF [^{14}C]ADP AND [^{14}C]ATP TO LATENT CF_1

Latent CF_1 (1 μM) was incubated with [^{14}C]ADP or [^{14}C]ATP (10 μM) $\pm$ $CaCl_2$ (5 mM). Pyrophosphate (1 mM) or thiosulfate (1 mM) were added when indicated. Aliquots were centrifuged through Sephadex columns after 30 s and 30 min.

	Bound ^{14}C nucleotides per CF_1	
	after 30 s	after 30 min
[^{14}C]ADP without $CaCl_2$	0.18	0.43
+ pyrophosphate	0.18	0.46
+ thiosulfate	0.09	0.44
[^{14}C]ADP with $CaCl_2$	0.34	0.65
+ pyrophosphate	0.17	0.54
+ thiosulfate	0.16	0.68
[^{14}C]ATP without $CaCl_2$	0.06	0.27
+ pyrophosphate	0.07	0.36
+ thiosulfate	0.04	0.26
[^{14}C]ATP with $CaCl_2$	0.49	0.94
+ pyrophosphate	0.25	0.68
+ thiosulfate	0.22	0.73

In a third binding experiment,some of the above used substances were incubated with activated CF_1 in the presence of [^{14}C]ATP and $CaCl_2$. Under these conditions, binding and ATP hydrolysis occur simultaneously. For the activation of the latent Ca-ATPase activity, the enzyme was preincubated with dithiothreitol overnight. [^{14}C]ATP was then added together with $CaCl_2$. After a constant time (1 min at room temperature), an aliquot was deproteinized with perchloric acid, and another aliquot was passed through a Sep-

TABLE III

EFFECTS OF VARIOUS SUBSTANCES ON ATP HYDROLYSIS AND AdN BINDING BY ACTIVATED CF_1

Preactivated CF_1 (1 μM) was added to the assay medium containing [^{14}C]ATP (10 μM), $CaCl_2$ (5 mM), and the substances as indicated. After 60 s, an aliquot of the assay was centrifuged through a Sephadex column, while another aliquot was deproteinized with perchloric acid for the determination of ATP hydrolysis. Control values (1.8 ATP hydrolysed per CF_1 and 0.59 AdN bound per CF_1) were set to 100%.

Addition		ATP hydrolysed per CF_1 (%)	Nucleotides bound per CF_1 (%)
No addition		100	100
Quercetin	(1 mM)	82	107
Phloridzin	(1 mM)	77	98
Ammonium	(10 mM)	35	114
Azide	(10 mM)	18	80
Citrate	(10 mM)	96	53
Phosphate	(10 mM)	42	31
Phosphate	(1 mM)	69	60
Arsenate	(10 mM)	90	36
Arsenate	(1 mM)	86	62
Sulfate	(1 mM)	97	57
Thiosulfate	(10 mM)	143	19
Thiosulfate	(1 mM)	150	29
Pyrophosphate	(10 mM)	63	3
Pyrophosphate	(1 mM)	55	29

hadex column. The amount of hydrolysed [^{14}C]ATP was calculated after determination of the relative amounts of ATP and ADP by thin-layer chromatography of the samples. The effect of various inhibitors on ATP hydrolysis and on concomitant nucleotide binding is shown in Table III.

Compounds like ammonium and phloridzin inhibited ATP hydrolysis, others like citrate, sulfate and thiosulfate inhibited mainly nucleotide binding. Phosphate and pyrophosphate inhibited both reactions. The most interesting effect was observed with thiosulfate; addition of this anion increased ATP hydrolysis to about 150% of the control, but decreased the amount of bound AdN to 29%.

The effects of two anions, phosphate and thiosulfate, on ATP hydrolysis and nucleotide binding were studied in dependence of their concentration. In Fig. 1, the result of this experiment is shown.

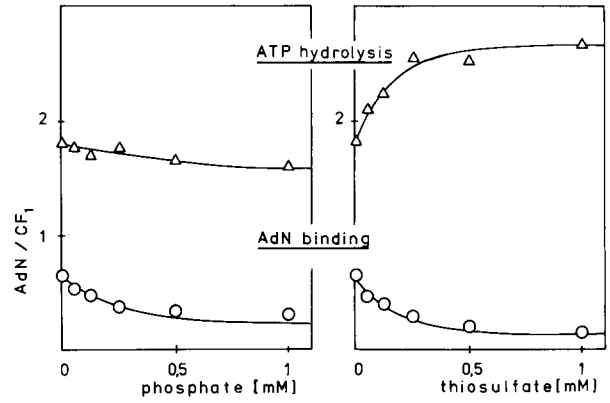

Fig. 1. Effect of phosphate and thiosulfate on ATP hydrolysis and ATP binding. Preactivated CF_1 (1 μM) was added to a medium containing 10 μM [^{14}C]ATP, 5 mM $CaCl_2$, and phosphate or thiosulfate at the concentrations indicated. After 30 s, aliquots were centrifuged through Sephadex columns (determination of bound nucleotides) or deproteinized (determination of hydrolysed ATP).

Both phosphate and thiosulfate had an inhibitory effect on the incorporation of labeled nucleotides into the tight binding site of CF_1; their I_{50} values were 0.15 mM for phosphate and 0.09 mM for thiosulfate. ATP hydrolysis was inhibited at much higher amounts of phosphate (more than 1 mM). Thiosulfate activated ATP hydrolysis; the half-maximal effect was obtained with about 0.12 mM $S_2O_3^{2-}$. The reason for this activation is not known; thiosulfate showed similar effects on DTT- or ethanol-activated ATPase activity under normal assay conditions (i.e., at low CF_1 concentration at 37 °C; data not shown). Since phosphate and

TABLE IV

ATP HYDROLYSIS AND NUCLEOTIDE BINDING AFTER PREINCUBATION OF CF_1 WITH MgATP

CF_1 was preincubated with 25 mM DTT, 3 mM $MgCl_2$ and 50 μM unlabeled ATP overnight. After removing unlabeled ATP and $MgCl_2$ by gel filtration, the activated enzyme (final concentration, 1 μM) was incubated with 10 μM [^{14}C]ATP, 5 mM $CaCl_2$ and 1 mM thiosulfate, when indicated.

	After 30 s	After 60 s
ATP hydrolysed per CF_1	1.3	1.77
+ thiosulfate	1.4	2.16
[^{14}C]AdN bound per CF_1	0.04	0.04
+ thiosulfate	0.03	0.03

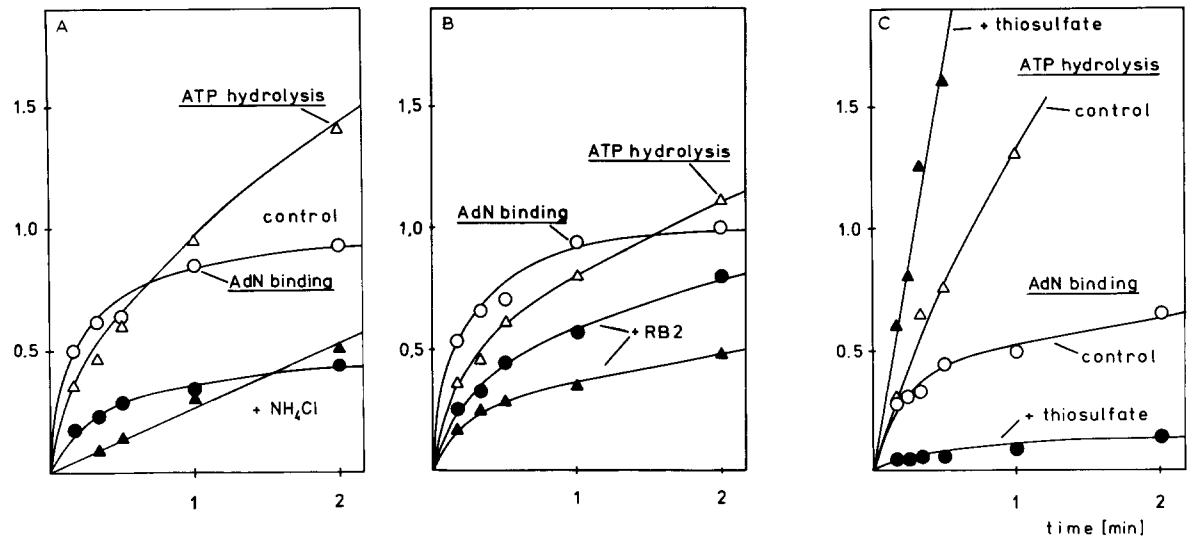

Fig. 2. Effect of inhibitors on ATP hydrolysis and AdN binding. Preactivated CF_1 (1 μM) was incubated with 10 μM [^{14}C]ATP, 5 mM $CaCl_2$, and without (open symbols) or with (closed symbols) inhibitors added. Samples were taken at the times indicated and analyzed for nucleotide binding (○, ●) and ATP hydrolysis (△, ▲). (A) Addition of ammonium chloride (10 mM). (B) Addition of Reactive Blue 2 (1 mM). (C) Addition of sodium thiosulfate (1 mM).

thiosulfate affect nucleotide binding and ATP hydrolysis in different ways, it is concluded that no simple mechanism underlies their actions.

For three of these substances, ammonium chloride, Reactive Blue 2 and thiosulfate, kinetics of ATP hydrolysis as well as of nucleotide binding were measured with higher time resolution to compare the initial reaction rates. The results are shown in Fig. 2 A–C.

When no inhibitor was added (open symbols), the rate of binding was faster than the rate of hydrolysis in all experiments; this is in accordance with published results [9,12,13]. Both binding and hydrolysis were inhibited by ammonium chloride (Fig. 2A) or Reactive Blue 2 (Fig. 2B) but binding was always faster than hydrolysis. With thiosulfate, however, binding was nearly completely inhibited while ATP hydrolysis was activated (Fig. 2C). The result of this experiment clearly demonstrates that tight binding and ATP hydrolysis are independent processes, at least under the conditions of this experiment.

In an additional experiment, CF_1 was preincubated with DTT, $MgCl_2$, and unlabeled ATP overnight. According to Ref. 4, MgATP becomes tightly bound to site 2 under these conditions. The following day, free nucleotides and magnesium ions were removed by gel filtration,a nd the CF_1 was incubated with [^{14}C]ATP in the presence of $CaCl_2 \pm$ thiosulfate. Under these conditions, only the bound ADP is exchangeable (Refs. 3 and 4; see also unpublished data). After 30 and 60 s aliquots were either deproteinized or passed over Sephadex columns. The result of this experiment is give in Table IV.

While ATP hydrolysis is not affected by preincubation, binding of [^{14}C]nucleotides to the enzyme is dramatically reduced (0.04 AdN bound per CF_1 vs. 1.3 ATP hydrolysed per CF_1 in 30 s). Thiosulfate activated ATP hydrolysis by MgATP-containing CF_1 as before, but the effect on the exchange reaction cannot be determined because only 0.04 nucleotides were bound during the incubation without thiosulfate.

Discussion

The experiments presented in this paper clearly demonstrate that under certain conditions, the rate of ATP hydrolysis may be higher than incorporation of labeled medium substrate into tight binding sites. It is therefore unlikely that ATP hydrolysis requires an intermediately bound ATP which is hydrolysed and released only after bind-

ing of another ATP molecule to an open (reversible) binding site. It might well be that ATP hydrolysis can occur via different ways, and that the above-described experiments represent the so-called 'single-site catalysis' as described for mitochondrial F_1-ATPases [25,26]. However, previous experiments at higher ATP concentrations in the presence of thiosulfate indicated that even under these conditions, nucleotide exchange is slower than ATP hydrolysis.

We conclude from our data that ATP hydrolysis is catalyzed by one of the reversible binding sites; either site 2 or site 3 (B or C), or another site is possible. The experiment in which CF_1 was preincubated with MgATP (i.e., site 2 is occupied and no exchange occurs during hydrolysis) demonstrates that only site 3 or another site may catalyze ATP hydrolysis. Site 3 is probably involved in the exchange reaction of site-1 bound ADP: medium ADP is required to release formerly bound ADP from its site. The affinity of the reversible site for adenine nucleotides (3–5 μM, Ref. 4) is in the range of the half-maximal effect of nucleotide binding [13]. It was described in a preceding paper that after binding of a nucleotide to the reversible site, a second nucleotide molecule is involved in the process of tight binding [13]. The simplest explanation is that reversible binding of medium nucleotide to site 3 induces the dissociation of ADP from site 1 followed by re-occupation of site 1 by a second (labeled) medium nucleotide and conversion of the transitorily open site 1 into a tight site.

Since it was found that the reversible binding of nucleotides to site B and C (sites 2 and 3) is inhibited by pyrophosphate [5], and that the early phase of tight nucleotide binding is inhibited by thiosulfate and pyrophosphate (Tables II and III), we assume that the observed effect of pyrophosphate or thiosulfate on the exchange of tightly bound ADP occurs on one of the reversible sites, i.e., site 3 when MgATP is bound to site 2. The fact that ATP hydrolysis is nearly not inhibited by pyrophosphate [5] or even activated by thiosulfate (this paper) may indicate that the reversible site 3 of the coupling factor is blocked by these anions; binding of medium ATP is therefore inhibited. If this is the case, a site different from sites 1, 2 and 3 catalyzes ATP hydrolysis.

Sites which were assumed to be catalytic (and which have lower affinity for adenine nucleotides) were found by Hisabori and Sakurai [5], and by Xue et al. [27] on isolated CF_1. From our results presented in this paper, we propose that the three high-affinity sites 1–3 have low (if any) catalytic activity, and that one or more additional sites are involved in rapid ATP hydrolysis. To obtain three-fold symmetry as was found for the high-affinity sites [28], three additional catalytic sites might be present on CF_1. A total of six sites – three high-affinity sites and three catalytic sites – were also found on the mitochondrial and bacterial F_1-ATPases [29,30] which might represent a common feature of all coupling factor ATPases.

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