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Evidence for two tightly bound nucleotides on thylakoid-bound chloroplast coupling factor 1 (CF_1): one ADP – exchangeable upon illumination – and one non-exchangeable MgATP

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Isolated chloroplast coupling factor 1 contains a tightly bound ADP (which is exchangeable for labeled medium ADP or ATP) but contains two tightly bound nucleotides (one ADP and one ATP) after incubation with radioactive ATP in the presence of magnesium ions (Bruist, M.F. and Hammes, G.G. (1981) Biochemistry 20, 6298–6305). On the membrane-bound enzyme, only one nucleotide (mainly ADP) was found to be exchangeable during incubation with labeled ATP in the light (Bickel-Sandkötter, S. and Strotmann, H. (1981) FEBS Lett. 125, 188–192). This paper demonstrates that membrane-bound CF_1 contains another tight binding site, occupied with non-exchangeable ATP in addition to the tight ADP site (exchangeable in the light). During isolation, the incubation of CF_1 with EDTA causes the loss of the tightly bound ATP so that the normal isolation procedure yields an enzyme species with only one tightly bound nucleotide molecule (ADP). If EDTA is omitted during isolation, the additional ATP can be detected easily by the luciferin-luciferase method. Reconstitution of isolated CF_1 containing two tightly bound nucleotides with CF_1-free membranes results in a membrane-bound coupling factor with two radioactive nucleotides. Because the exchange of this ATP is very slow, it is assumed that the tightly bound ATP has structural rather than catalytic or regulatory functions.

Introduction

The chloroplast ATP synthase or coupling factor CF_1 contains nucleotide binding sites for catalytic binding of ADP (or ATP) as well as for regulatory binding. Number and properties of binding sites are still a matter of controversy [1–6]. For the membrane-bound CF_1, two different types of binding site were found [7]: site A binds ADP tightly (and ATP less tightly) and becomes a reversible, high-affinity (about 5 μM) binding site during illumination of thylakoids; site B binds nucleotides reversibly (K_d about 30 μM) during energization of the membranes. Tight binding of ADP to site A inhibits the enzyme, while tight ATP binding has no effect; therefore site A has mainly regulatory but also some catalytic functions. Site B is presumably the main catalytic site; its affinity for ADP is in the same range as the K_m(ADP) in photophosphorylation.

On the thylakoid-bound CF_1, only one tight nucleotide site was found [2,8]; the bound ADP

Abbreviations: CF_1, chloroplast coupling factor 1; CF_0F_1, chloroplast ATP synthase complex; Chl, chlorophyll; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetate; Mes, 4-morpholineethanesulfonate; TNP-ATP, 2′(3′)-trinitrophenyl-ATP; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine.

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was assumed to be identical to the ADP molecule found on site 1 of the isolated coupling factor [9–14]. Several groups reported that more than one nucleotide is bound to the coupling factor after isolation [1,2], but nothing is known about the properties of these additional binding sites.

With the isolated enzyme, tight and reversible binding of nucleotides was investigated in different groups [3–5,9,15]. It is obvious that different sites are responsible for tight and reversible binding of nucleotides. Of the two tight sites, only the tight ADP site (site 1; Ref. 3) has some catalytic activity [16,17]; the MgATP site (site 2) has neither ATPase activity [3] nor any influence on the activity of the other sites [17]. The site for reversible binding of nucleotides (site 3) seems to be the main site for ATP hydrolysis [3,16].

The data from the literature might be summarized in the following way:

	Isolated CF_1	Membrane-bound CF_1
Tight/exchangeable ADP site	site 1	site A
Tight/nonexchangeable MgATP site	site 2	? (this paper)
Reversible/catalytic site	site 3	site B

The site for ATP synthesis on the isolated CF_1 is unknown. ATP is formed either from bound ADP after addition of phosphate [18], or from medium ADP after addition of ADP and phosphate [19]. The F_1 from the thermophilic bacterium PS3 is able to form enzyme-bound ATP from medium ADP and phosphate even though no bound nucleotides were detected after isolation of the enzyme [20].

This paper demonstrates that membrane-bound and freshly isolated CF_1 contains more than one adenine nucleotide molecule; it is assumed that in addition to the known ADP on site A, one ATP is present on the enzyme before isolation which does not exchange during illumination of thylakoids. Storage of the isolated enzyme in the presence of EDTA leads to dissociation of the bound ATP molecule.

Materials and Methods

Chloroplast coupling factor CF_1 was isolated in large-scale preparations from spinach leaves as described [21]; it 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 (1.5 × 15 cm) equilibrated with the Tris-EDTA buffer. This CF_1 has one ADP bound per enzyme and is called 'standard CF_1'.

Small-scale preparations of CF_1 for immediate use ('freshly isolated CF_1') were carried out with thylakoids from about 100 g of spinach leaves. CF_1 was eluted from the DEAE-cellulose column with 50 mM Mes pH 6.5, 1 mM EDTA, 400 mM NaCl (no ATP added) and was used directly without ammonium sulfate precipitation. The peak fraction was detected by absorption at 277 nm, and aliquots were desalted by passage through the Sephadex G-50 column. The purity of those preparations as judged from SDS gels was greater than 95%. When the effect of ammonium sulfate precipitation on bound nucleotides was studied, solid ammonium sulfate (3 g/10 ml) was added to the Mes-EDTA-NaCl fractions without addition of ATP; the fractions were stored at 4°C.

Thylakoids were isolated essentially as described in Ref. 10, and washed with 10 mM Tricine (pH 8), 50 mM NaCl, 5 mM $MgCl_2$ 3–5 times. Aliquots were taken for determination of chlorophyll [22] and nucleotides (see below).

Chloroform-extraction of CF_1 was carried out according to Ref. 23. Thylakoids isolated as described above were resuspended in Tricine-NaCl-$MgCl_2$ buffer (chlorophyll content about 2 mg/ml) and stirred vigorously for 15 s with half the volume of chloroform. After centrifugation at 2000 × *g* at 4°C for 10 min, the yellow-green supernatant was degassed with an aspirator, applied to a DEAE-cellulose column, eluted with a step gradient of NaCl and desalted on a Sephadex G-50 column as described for EDTA-extracted CF_1. The purity of the chloroform-extracted CF_1 was about 90%.

CF_0F_1 was isolated by the procedure of Pick and Racker [24,25], modified by Fromme and

Gräber (unpublished results). ATP was omitted from all media; after the 33–45% ammonium sulfate precipitation of crude CF_0F_1, the sediment was dissolved in 0.2 M sucrose, 20 mM Tricine (pH 8), 3 mM $MgCl_2$ (freezing medium of Ref. 25) and precipitated again with ammonium sulfate (50% saturation). After storage at 4°C for several days, an aliquot was centrifuged, dissolved in 50 mM Tris (pH 8), 0.1% Triton X-100 and desalted on a Sephadex G-50 column equilibrated with the same buffer. The slightly yellow-green fraction was analyzed for protein and nucleotides. The purity of this kind of preparation was about 80–90% as judged from SDS gels.

Determination of enzyme-bound (unlabeled) nucleotides was carried out after addition of perchloric acid and neutralization with KOH with the luciferin-luciferase method as described [27].

ATPase activity of CF_1 was measured in a medium containing 50 mM Tris (pH 8), 1 mM $CaCl_2$, 1 mM ATP, 20% ethanol (v/v), and 4 μg CF_1 at 37°C. Phosphate was determined as described [21,28].

Tight binding of nucleotides to isolated CF_1 was studied by incubation of desalted coupling factor (0.4–1 mg/ml) in Tris-EDTA buffer with [8-^{14}C]adenine nucleotides (2.1 GBq/mmol; Amersham Buchler); excess free nucleotides were removed by centrifugation of the sample through one or two 1-ml-Sephadex G-50 columns as described [15].

Radioactivity was measured in liquid scintillation cocktail (Beckman Optifluor); protein was determined according to Lowry et al. [29] with bovine serum albumin as standard. An extinction coefficient of 0.483 cm^2/mg at 277 nm was used [3]; a molecular mass of 400 kDa was used for CF_1 [30] and of 557 kDa for the CF_0F_1 (Fromme, P., Boekema, E.J. and Gräber, P., unpublished results). Sodium bromide particles were prepared from spinach thylakoids by NaBr treatment according to Ref. 32. The chlorophyll concentration was determined spectrophotometrically [22], and thin-layer chromatography was carried out on PEI cellulose plates as described in Ref. 15. Magnesium determination was carried out with the dye Eriochrome Black T [33].

Results and Discussion

Nucleotide content of freshly isolated CF_1 (EDTA-extracted)

Roy and Moudrianakis [1] and Harris and Slater [2] reported that isolated CF_1 contains about one ADP and one ATP per enzyme molecule. Only one ADP/enzyme was found by other groups [9–11,13]. Bruist and Hammes [3] reported that incubation of CF_1 with radioactive ATP (in the presence of magnesium) leads to an enzyme species with two tightly bound nucleotides – one ADP and one ATP. The amount of bound ATP is decreased after precipitation with ammonium sulfate and storage in Tris-EDTA buffer [3]. Therefore we assumed that membrane-bound CF_1 contains one ATP and one ADP, and the storage of freshly isolated CF_1 causes the loss of the ATP molecule leading to an enzyme with one ADP bound.

Direct determination of bound nucleotides. To determine the amount of bound nucleotides, CF_1 was extracted from washed thylakoids in 0.75 mM EDTA; the protein was bound to DEAE-cellulose and eluted with 0.4 M sodium chloride in Mes-EDTA buffer. No ATP was added during isolation. This freshly isolated CF_1 was passed through a Sephadex G-50 column equilibrated with Tris-EDTA buffer and was precipitated with perchloric acid. After storage in the freezer overnight, the amount of nucleotides in the supernatant was determined using the luciferin-luciferase method.

Freshly isolated CF_1 contained no AMP, 0.9–1.12 ADP, and between 0.3 and 0.6 ATP per enzyme. After precipitation of the freshly isolated enzyme with ammonium sulfate and storage for several days at 4°C, 0.8–1.04 ADP were found, while only 0–0.05 ATP and no AMP were bound to the enzyme.

Direct determination of bound magnesium. The Mg content of CF_1-preparations was determined with the dye Eriochrome Black T. Freshly prepared CF_1, and CF_1 stored under ammonium sulfate for 1 week were desalted by passage through a Sephadex G-50 column equilibrated with water. To increase the magnesium concentration, the protein fractions were freeze-dried and subjected to the magnesium determination.

While freshly isolated CF_1 contained about 0.5

TABLE I

BINDING OF [^{14}C]ATP TO FRESHLY ISOLATED AND STANDARD CF_1

Freshly isolated and standard CF_1 were desalted over a Sephadex G-50 column and eluted with Tris-EDTA-buffer. The incubation assay contained 23 μM [^{14}C]ATP, 5 mM $MgCl_2$ when indicated, and 0.4 mg CF_1/ml. After incubation overnight at room temperature, two 100-μl samples were passed through two subsequent Sephadex centrifugation columns and analysed for radioactivity and protein.

	Labeled nucleotides incorporated after incubation with	
	[^{14}C]ATP	[^{14}C]ATP + $MgCl_2$
Freshly isolated CF_1		
experiment 1	1.10	1.21
experiment 2	1.08	1.32
Standard CF_1		
experiment 1	0.98	2.02
experiment 2	1.10	2.21

Mg per enzyme, the enzyme stored under ammonium sulfate contained nearly no magnesium. The Mg content of the enzyme correlates well with the amount of ATP determined by the luciferin-luciferase method giving an MgATP stoichiometry of about 1.

Binding of ^{14}C-nucleotides. When standard CF_1 (containing one unlabeled ADP; see above) is incubated with labeled ADP, 0.6–1.1 [^{14}C]ADP per CF_1 are routinely observed by radioactivity, and 0.9–1.2 ADP per CF_1 by the luciferin-luciferase assay. Nearly no AMP or ATP were detected under these conditions. If $MgCl_2$ and [^{14}C]ATP are used in the exchange experiment, up to two radioactive nucleotides are found while the total amount of nucleotides (determined by luciferin-luciferase) is about 1.9–2.3 per CF_1. These results indicate that the unlabeled ADP found on isolated CF_1 is replaced by labeled ADP or ATP, in the presence or absence of Mg ions. With $MgCl_2$ + [^{14}C]ATP, an additional site is occupied in accordance with the data of Bruist and Hammes [3].

Preincubation of the enzyme with unlabeled ATP in the presence of Mg ions and subsequent incubation with labeled ATP (+$MgCl_2$) gives about one bound labeled nucleotide but two nucleotides with the luciferin-luciferase method.

TABLE II

EFFECT OF TNP-ATP PREINCUBATION ON ATPASE ACTIVITY OF CF_1

Stored CF_1 was desalted and incubated with $MgCl_2$ (5 mM), ATP (25 μM), or TNP-ATP (5 μM). Protein concentration during incubation was 0.6 mg/ml. After 30 min, either water, ATP, or TNP-ATP was added to the samples. 30 min later, the ATPase activity of each sample was tested in a medium according to Material and Methods. Freshly isolated CF_1 was desalted prior to use by passage over a Sephadex column and was eluted with Tris-EDTA buffer.

	CaATPase activity (μmol P_i/mg protein per min) after a second incubation with:		
	–	ATP	TNP-ATP
Standard CF_1			
First incubation with			
$MgCl_2$	5.4	6.4 (100%)	1.2 (19%)
$MgCl_2$ + ATP	6.0	5.7 (100%)	5.7 (100%)
$MgCl_2$ + TNP-ATP	1.4	1.5	1.3
Freshly isolated CF_1			
First incubation with			
$MgCl_2$	9.8	9.2 (100%)	5.8 (63%)

Thus it is concluded that occupation of site 2 (the tight MgATP site) with MgATP is nearly irreversible under these conditions but does not interfere with the exchange of nucleotides on site 1 [3,17].

Since tightly bound MgATP is not replaced by radioactive ATP, binding of [^{14}C]ATP (+$MgCl_2$) to freshly isolated CF_1 might be a measure for the amount of bound (unlabeled) MgATP.

Immediately after a small-scale isolation of CF_1, the enzyme was desalted using Tris-EDTA buffer and was incubated with 10–50 μM [^{14}C]ATP ± $MgCl_2$. Free nucleotides were removed by passage through one or two Sephadex columns, and the amount of bound radioactive nucleotides was determined (Table I).

Freshly isolated CF_1 bound about one radioactive AdN per enzyme in the absence of Mg, and about 1.2–1.3 AdN/enzyme in the presence of $MgCl_2$. Standard CF_1, however, bound about one AdN in the absence and two in the presence of $MgCl_2$ indicating that freshly isolated coupling factor might contain a tightly bound MgATP.

Indirect determination of bound MgATP. Binding of the ATP analog trinitrophenyl-ATP (TNP-

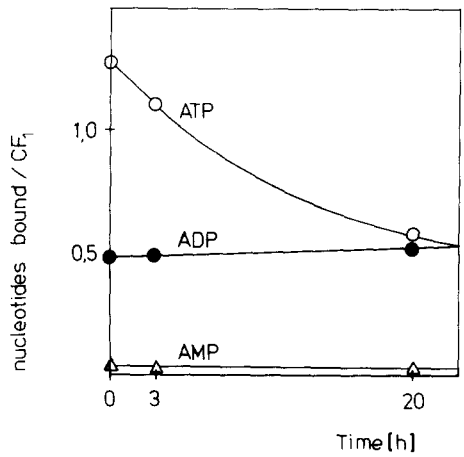

Fig. 1. Release of tightly bound nucleotides. CF_1 was preincubated with [^{14}C]ATP and $MgCl_2$ for 2 h. Free nucleotides were removed by gel filtration. At the times indicated, samples were centrifuged through Sephadex columns. Aliquots of the eluate were analyzed for nucleotides, protein, and (after $HClO_4$ precipitation of protein) chromatographed to determine the relative amounts of AMP, ADP, and ATP.

ATP) in the presence of Mg^{2+} to the isolated standard CF_1 inhibits the calcium-dependent ATPase activity of the enzyme [34]. CF_1 was therefore incubated with TNP-ATP, and the ethanol-activated Ca-ATPase activity was determined. Table II shows that preincubation of standard CF_1 with $MgCl_2$ (5 mM) and a second incubation with TNP-ATP (5 μM, in the presence of $MgCl_2$) leads to inhibition of the ATPase (1.2 vs 6.4 μmol P_i mg protein per min). If the standard CF_1 was preincubated with $MgCl_2$ (5 mM) and ATP (25 μM), no inhibition by a second incubation with TNP-ATP was detected (Table II). Incubation with TNP-ATP in the absence of $MgCl_2$ did not inhibit (data not shown).

If freshly isolated CF_1 was preincubated with $MgCl_2$, and TNP-ATP was added as before, an intermediate effect on the ATPase activity was observed (37% inhibition vs. 81% inhibition with the standard CF_1). This might be explained that about half of the freshly isolated enzyme molecules contain tightly bound MgATP thus preventing inhibition by MgTNP-ATP binding.

Stability of the ATP-enzyme complex

Bruist and Hammes [3] described that nucleotides bound to the tight MgATP site are not exchanged or released during prolonged incubation in a medium containing EDTA ± nucleotides. However, precipitation of the enzyme with ammonium sulfate and storage in EDTA-ATP buffer leads to the release of bound MgATP [3]. In the experiment shown in Fig. 1, stored CF_1 was incubated with [^{14}C]ATP and $MgCl_2$ for 2 h. Free nucleotides were removed by passage through a Sephadex column equilibrated with Tris-EDTA buffer. From this incubation assay, samples were taken after 3 and 20 h, passed through centrifugation columns and analyzed for protein, radioactivity, and relative amounts of AMP, ADP and ATP by thin-layer chromatography.

In this experiment, nucleotides are lost during incubation of the enzyme in Tris-EDTA buffer. It is obvious that only the amount of bound ATP is decreased while the amount of ADP remains constant. In contrast to the above cited results [3], this experiment shows a final nucleotide distribution of about 0.5 ADP and 0.5 ATP bound to the enzyme.

Therefore, it seems possible that the membrane-bound coupling factor contains one nucleotide on the ADP site (site A) and one on the MgATP site. The ATP molecule is lost during isolation and storage, and the amount of nucleotides is decreased to one nucleotide per enzyme.

Preparations of coupling factors without EDTA incubation

EDTA which is present during the isolation procedure seems to be responsible for the slow release of ATP from the coupling factor. Therefore the amount of ATP and ADP was determined on washed thylakoids, on the isolated CF_0F_1 complex, and on CF_1 isolated by extraction with chloroform. In all cases, no EDTA is necessary during the preparation, and no nucleotides were added to the isolation media. The results of these determinations are given in Table III.

On washed thylakoids, the amount of ATP was higher than the amount of ADP resulting in an ATP/ADP ratio of 1.26. Using a stoichiometry of 0.42 mg CF_1 per mg chlorophyll [35], i.e., 1.05 nmol CF_1/mg Chl, the experiment gave more than one ATP (and ADP) per CF_1. However, the CF_1/chlorophyll-ratio changes depending on growth conditions [36] so that an absolute number

TABLE III

NUCLEOTIDE CONTENT OF THYLAKOIDS, OF CF_0F_1 AND OF CHLOROFORM-EXTRACTED CF_1

Thylakoids, CF_0F_1, and chloroform-extracted CF_1 were isolated as described in Materials and Methods and were analyzed for the amount of nucleotides.

	ATP	ADP	ATP/ADP
	(nmol/nmol CF_1)		
Thylakoids	1.2	0.95	1.26
CF_0F_1	0.83	0.97	0.86
Chloroform-CF_1	1.08	1.25	0.87

of bound nucleotides cannot be given.

The CF_0F_1 preparation showed somewhat less than one ATP and one ADP per enzyme which might be explained by some protein contaminations. However, the molecular mass of the complex as taken from the literature ranges from 435 to 557 kDa [24,31,37] so that the absolute values of bound nucleotides in Table III have to be taken with care. The ATP/ADP ratio is about 0.86 indicating an equal amount of both nucleotide species.

CF_1 extracted with chloroform contained about 1 ATP and 1 ADP giving again an ATP/ADP ratio of 0.87.

All results are in agreement with the interpretation that membrane-bound CF_1 contains one ATP (as MgATP) and one ADP per enzyme molecule.

Reconstitution of thylakoids with ^{14}C-labeled CF_1

No methods are available for direct incorporation of radioactive nucleotides into the MgATP site of membrane-bound CF_1. Therefore, standard CF_1 was incubated with [^{14}C]ATP in the presence of magnesium ions, and the resulting enzyme with two nucleotides bound was used for reconstitution with CF_1-free membranes ('sodium bromide particles'; Ref. 32). Table IV shows that the reconstituted membranes contained 1.7–2.0 nmol ^{14}C-nucleotides per mg chlorophyll or 1.6–1.9 nucleotides per coupling factor using the stoichiometry 1.05 nmol CF_1 per mg chlorophyll [35]. Therefore it is reasonable to assume that the membrane-bound enzyme contains at least two 'tight' binding sites for nucleotides – one of them (site A) seems to be identical with site 1 of the isolated

TABLE IV

NUCLEOTIDE CONTENT OF ISOLATED STANDARD CF_1 AFTER INCUBATION WITH Mg[^{14}C]ATP AND OF CF_1-DEPLETED MEMBRANES RECONSTITUTED WITH [^{14}C]CF_1

Standard CF_1 was incubated with [^{14}C]ATP (10 μM) and $MgCl_2$ (5 mM) for 2 h, passed through two subsequent Sephadex centrifugation columns, and reconstituted with CF_1-depleted thylakoids (NaBr-particles). The [^{14}C]AdN content was determined before and after reconstitution as described in Materials and Methods.

	Bound nucleotides	
	isolated enzyme (nmol/nmol CF_1)	after reconstitution (nmol/mg Chl)
Experiment 1	2.5	1.9
Experiment 2	2.2	1.7
Experiment 3	1.9	2.0

enzyme (9–14). The identity of site 1 (tight ADP site) with the tight site of membrane-bound CF_1 (site A) was assumed by several groups; it was demonstrated by Strotmann et al. [10] and Magnusson and McCarty [12] that illumination of thylakoids in the presence of [^{14}C]ATP or ADP and isolation of the coupling factor gives an enzyme species with tightly bound [^{14}C]ADP. This tightly bound, labeled nucleotide can be exchanged during incubation with unlabeled ADP or ATP (Soffner, C. and Schumann, J., unpublished data). Selman and Selman-Reimer [14] showed that incorporation of nucleotides into the isolated CF_1 followed by reconstitution with thylakoids and illumation induces the release of labeled nucleotides.

The non-exchangeable tight site of the membrane-bound CF_1 (MgATP site) cannot be seen in normal exchange experiments with thylakoids in the dark or in the light because thylakoids bind only one nucleotide per coupling factor after incubation with ^{14}C-nucleotides in the light [10,12,38,39]. On the other hand, more than one tightly bound AdN per CF_1 was found frequently (up to 3 nmol AdN per mg Chl; Refs. 2, 7, 8, 10 and 31). If an exchange of labeled ATP with the tightly bound MgATP occurs, it is very slow compared to binding of ADP (or ATP) to the normal tight site (site A), but more experimental data are necessary for the separation of the two binding

processes. An energy-independent exchange into one of the tight sites was observed during dark incubation of membranes with radioactive nucleotides [39], but no reillumination was carried out to measure the exchangeability of these tightly bound nucleotides.

Dark binding of ATP leads to a tightly bound nucleotide which is barely exchangeable in the light (Lohse, D., personal communication), indicating that indeed an exchange of radioactive nucleotides into the tight MgATP site might be possible on the membranes. Preliminary results with the reconstituted system indicated that tightly bound MgATP is not released into the medium during short-time illumination and is not exchanged for unlabeled ADP or ATP (data not shown). Therefore, we assume that the exchange of tightly bound ATP on the membrane-bound chloroplast coupling factor is much slower than catalytic or regulatory processes on the enzyme; the tightly bound MgATP might have structural rather than regulatory functions.

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