Influence of Nucleotides on the Cold Stability of Chloroplast Coupling Factor 1†

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ABSTRACT: Chloroplast coupling factor 1 (CF_1), a cold-labile enzyme, contains six nucleotide binding sites. These sites are located at the α/β interfaces of the $\alpha_3\beta_3$ heterohexamer. The cold lability of CF_1 is decreased by the presence of nucleotides in the medium. We have studied the influence of both different nucleotides and different binding sites on the cold dissociation of CF_1. To monitor the dissociation of CF_1 during cold treatment, 8-anilino-1-naphthalenesulfonic acid (ANS) was employed. The increase in ANS fluorescence during cold treatment is the result of increased accessibility of intersubunit hydrophobic regions as the complex dissociates. Mg^{2+}-adenosine triphosphates, tightly bound to CF_1, markedly stabilize the enzyme in the cold. ADP only protects CF_1 from dissociating in the cold when it is bound to the loose sites or when it is bound in conjunction with Mg^{2+}. CF_1 that contained 2 mol of ADP/mol and little bound Mg^{2+} was nearly as cold labile as CF_1 that contained just 0.2 mol of ADP/mol. When about one of the two bound ADPs was replaced with adenylyl β,γ-imidodiphosphate (AMP-PNP), some protection from cold dissociation was observed. These results show that the site(s) occupied, as well as the nucleotides they contain, strongly influence(s) the structural stability of CF_1.

In mitochondria, bacteria, and chloroplasts, electron transport is coupled to synthesis of ATP by the F_1-F_o-ATP synthase. F_o, which is integrated in the membrane, acts as a proton channel. F_1 contains the nucleotide binding sites. F_1 is composed of five different subunits, α through ϵ in order of decreasing molecular weight, with a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. F_1 in solution acts as an ATPase [for reviews, see McCarty and Moroney (1985), Futai et al. (1989), and Pedersen and Amzel (1993)].

The F_1-ATPases are cold-labile complexes. During incubation in the cold, the ATPase activity of F_1 decreases presumably due to dissociation of the complex. The influence of intersubunit contacts on the cold stability of chloroplast coupling factor 1 (CF_1)[1] was investigated earlier (Hightower & McCarty, 1996). It was shown that, while modification of the γ subunit does not affect cold stability, removal of the δ subunit and truncation of the C-termini of the β subunits increase the cold lability of CF_1. The presence of nucleotides in the medium stabilizes CF_1 in the cold.

There are six nucleotide binding sites on CF_1, located at the α/β interfaces. The characteristics of these sites have been and are still being investigated (Bruist & Hammes, 1981; Shapiro et al., 1991; J. Digel, unpublished results). In general, the sites can be grouped into two classifications, tight binding and loose binding. Four of the sites bind nucleotides tightly. Even though the effect of nucleotides in the medium on the cold lability of CF_1 has been studied (Posorske & Jagendorf, 1976), the influence of specific bound nucleotides has not been established. For a variety of reasons, it is difficult to examine the effect of bound nucleotides on cold lability by monitoring ATPase activity. Therefore, new methods must be developed to investigate the influence of nucleotides on the stability of CF_1.

Many multimeric proteins are cold labile. The dissociation of these complexes in the cold is thought to be a consequence of the hydrophobic interactions between the subunits. As the temperature decreases, the strength of these interactions decreases and the complex dissociates [for a review, see Privalov (1990)]. The structure of CF_1 is believed to be dominated by hydrophobic intersubunit contacts.

For many years, 8-anilino-1-naphthalenesulfonic acid (ANS) has been used to study the structure of proteins. By itself, ANS is not very fluorescent in aqueous solutions. Its fluorescence increases after it binds to a membrane or a hydrophobic region on a protein [for a review, see Slavik (1982)]. ANS has been employed to study protein denaturation and refolding as well as complex assembly. Among other things, it has been used to inhibit viral capsid assembly (Teschke et al., 1993) and to monitor the exposure of hydrophobic surfaces on the chaperonin GroEL (Gibbons & Horowitz, 1995; Ybarra & Horowitz, 1995). ANS has even been used to study the interaction of nucleotides with rubisco activase (Wang & Portis, 1991). Because of its inability to bind to single hydrophobic residues, ANS has been useful for studying the denaturation and renaturation of proteins (Semisotnov et al., 1991). ANS binding could be used as a convenient assay of the cold dissociation of CF_1 loaded with a variety of nucleotides. The dissociation of the subunits would lead to the exposure of hydrophobic surfaces to the medium. ANS would then bind to these surfaces, resulting in increased fluorescence. By using ANS, the influence of nucleotides on the cold lability of CF_1 could be determined without activity assays.

As determined here, ANS binding is a valid method for studying the cold dissociation of CF_1. By employing this method, we have determined the influence of bound nucleotides on the cold lability of CF_1. The presence or absence of ADP at the tight ADP and tight exchangeable sites does

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; ANS, 8-anilino-1-naphthalenesulfonic acid; AMP-PNP, adenylyl β,γ-imidodiphosphate; $CF_1(-\epsilon)$, CF_1 lacking the ϵ subunit.

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not greatly affect the cold dissociation of CF_1. The binding of Mg^{2+} to nucleotides already present at these sites does decrease the cold lability of CF_1. Bound adenine nucleoside triphosphates provide significant stabilization to CF_1. By titrating ADP loaded CF_1 with ADP, the ability of nucleotides at the loose sites to protect CF_1 from cold dissociation was determined.

MATERIALS AND METHODS

CF_1 was isolated from market spinach by a modification of the procedure of Shapiro and McCarty (1990) (Soteropoulos et al., 1994). The Zeta prep disk was replaced by a 5.4 cm × 14 cm column of Toyopearl QAE-550C resin. The ribulose bisphosphate carboxylase/oxygenase contaminant was removed by immunoaffinity chromatography (Soteropoulos et al., 1992). $CF_1(-\epsilon)$ was prepared as described previously (Richter et al., 1984, 1985; Soteropoulos et al., 1994). $CF_1(-\epsilon)$ was depleted of tightly bound nucleotides [$CF_1(-\epsilon)$ NTD)] by treatment with alkaline phosphatase (Digel et al., 1996).

CF_1 was stored at pH 8.0 as 50% ammonium sulfate precipitates in the presence of 1 mM ATP and 2 mM EDTA. Before use, the CF_1 was desalted by passage through two consecutive 3 mL Sephadex G-50 centrifuge columns (Penefsky, 1977). Protein concentrations were determined by the procedure of Lowry et al. (1951).

Cold dissociation was studied by ANS fluorescence using an SLM/Aminco SPF-500 spectrofluorometer modified by OLIS. The temperature in the cuvette was approximately 0 °C. CF_1 was diluted to 50 μg/mL (1 mL total) in cold buffer containing 50 mM Tris-HCl (adjusted to pH 8.0 at 20 °C), 200 mM NaCl, and 30 μM ANS with or without (Mg^{2+} loaded samples) 5 mM EDTA to begin the assay. Free acid ANS that had been passed through a 1.5 cm x 3 cm column of Chelex 100 was used for all experiments. The ANS was excited at 360 nm, and the fluorescence emission was monitored at 430 nm. This emission wavelength was selected because it gave the maximum difference in the fluorescence of free and bound ANS. The fluorescence of bound ANS was substantially blue-shifted.

To study the effect of bound nucleotides (see Table 1 for the nomenclature of the nucleotide binding sites) on the cold dissociation of CF_1, protein (3−17.5 μM) was preloaded with a variety of nucleotides as described below. All nucleotide loading was performed in 50 mM Tris-HCl (pH 8.0). The ADP used for all of the experiments was purified by chromatography on a Dowex 1-Cl column (Cohn, 1957). As determined by high-performance liquid chromatography, no ATP was present in the ADP.

Tight ADP and Tight Exchangeable Sites. The tight exchangeable site was loaded by incubation of CF_1 with ADP (1 mM) or AMP-PNP (5 mM) in the presence of 5 mM EDTA for 2 h or overnight, respectively. The excess and loosely bound nucleotides were removed by passing the CF_1 through two consecutive 3 mL Sephadex G-50 centrifuge columns.

To load both the tight exchangeable site and the tight ADP site with Mg^{2+}-nucleotides, the CF_1 was first loaded with ADP or AMP-PNP in the presence of EDTA as above. After removal of the EDTA and excess nucleotides, the enzyme was incubated with 1 mM $MgCl_2$ for 1 h. The excess $MgCl_2$ was removed by two consecutive 3 mL Sephadex G-50 centrifuge columns.

To differentiate between stabilization due to Mg^{2+} binding to ADP in the tight ADP site and the tight exchangeable site, CF_1 was prepared with the tight ADP site or both sites filled. CF_1 with ADP only at the tight ADP site was prepared by treating the protein with 100 mM Na_2SO_3, 5 mM EDTA for 10 min (Digel & McCarty, 1995). The protein was passed through two consecutive 3 mL Sephadex G-50 centrifuge columns equilibrated with 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and 100 mM Na_2SO_3 to remove any released nucleotides. The Na_2SO_3/EDTA treatment was repeated, and the Na_2SO_3 and EDTA were removed by three consecutive 3 mL Sephadex G-50 centrifuge columns. Half of the CF_1 was then incubated with 1 mM $MgCl_2$ for 1 h. Excess and loosely bound Mg^{2+} was removed by three consecutive 3 mL Sephadex G-50 centrifuge columns. CF_1 with ADP bound at both sites was prepared by incubating the protein in 0.5 mM ADP, 5 mM EDTA for 1 h. After removing the EDTA and the excess and loosely bound ADP, half of the sample was treated with $MgCl_2$ as described above.

Mg^{2+}-ATP Sites. The Mg^{2+}-ATP sites were loaded with Mg^{2+}-ADP (1 mM ADP, 1 mM $MgCl_2$; 2 or 5 h), Mg^{2+}-ATP (5 mM ATP, 0.5 mM $MgCl_2$; 5 h), or Mg^{2+}-AMP-PNP (5 mM AMP-PNP, 5 mM $MgCl_2$; overnight). The 2 h incubation with ADP and $MgCl_2$ was performed in the presence of 20 μM P^1,P^5-di(adenosine-5′) pentaphosphate to inhibit adenylate kinase. The excess nucleotides and $MgCl_2$ were removed by passing the protein through two consecutive 3 mL Sephadex G-50 centrifuge columns.

Mg^{2+} contents were determined by atomic absorption on a Perkin-Elmer Model 4000 using flame detection. An equal volume of 2 N HNO_3 (trace metal grade) was added to the protein samples (800 μL, 9.8−12.3 mg/mL). The samples were centrifuged for 30 min in a microcentrifuge, and the supernatants were collected. The pellet was washed twice by resuspending it in 200 μL of 1 N HNO_3, centrifuging for 30 min, and removing the supernatant. The combined supernatants were centrifuged for 30 min and placed into another tube to eliminate any particulate matter.

Nucleotide contents were determined by high-performance liquid chromatography as described previously (Moal et al., 1989; Soteropoulos et al., 1994) using a Chromega chromegabond mc 18/5 4.6 mm × 10 cm reversed-phase column on a Beckman 342 liquid chromatograph. The molecular weight of CF_1 is 400 000 (Moroney et al., 1983).

Toyopearl QAE-550C resin was purchased from Supelco (Bellefont, PA).

RESULTS

ANS binds to hydrophobic patches on proteins. Upon binding to a hydrophobic surface, the fluorescence emission of the ANS increases. This increase in the fluorescence of bound ANS is evident in Figure 1. Upon addition of CF_1 to buffer containing ANS, the fluorescence increases. The initial jump in ANS fluorescence is due to binding of ANS to exposed hydrophobic surfaces on intact CF_1. Following the initial jump, there is a further increase in the ANS fluorescence over time during cold treatment. Most of the intersubunit contacts in CF_1 are hydrophobic. As the complex dissociates in the cold, more hydrophobic residues

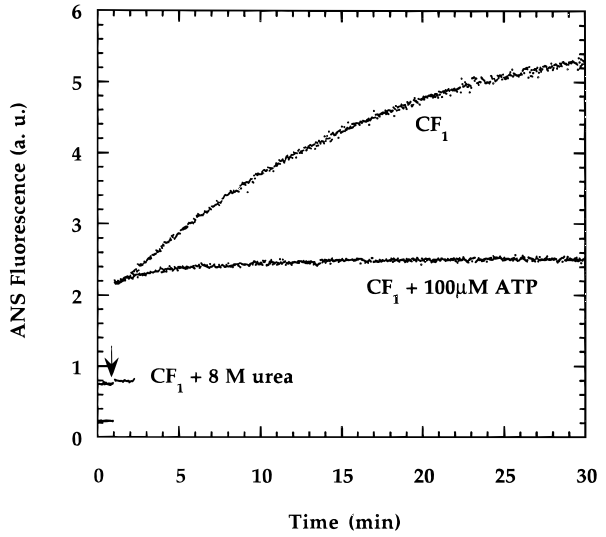

FIGURE 1: Feasibility of monitoring the cold dissociation of CF_1 by ANS fluorescence. The cold dissociation of CF_1 with and without the addition of 100 μM ATP or 8 M urea was followed by ANS fluorescence. The CF_1 was incubated in a buffer at 0 °C containing 50 mM Tris-HCl (adjusted to pH 8.0 at 20 °C), 200 mM NaCl, 5 mM EDTA, and 30 μM ANS. For the ATP addition, the buffer also contained 100 μM ATP. The urea run was performed in 50 mM Tris-HCl (adjusted to pH 8.0 at 20 °C), 200 mM NaCl, 5 mM EDTA, 30 μM ANS, and 8 M urea. The arrow indicates the addition of CF_1 to a final concentration of 50 μg/mL.

become accessible for ANS binding, and the fluorescence increases. It has previously been shown that the presence of ATP in the medium stabilizes CF_1 in the cold (McCarty & Racker, 1966; Posorske & Jagendorf, 1976; Hightower & McCarty, 1996). With 100 μM ATP in the dissociation buffer, there is very little increase in the ANS fluorescence after the initial increase. The process that is monitored by the change in the ANS fluorescence is dissociation and not denaturation. When CF_1 is added to buffer containing 8 M urea, there is no increase in the ANS fluorescence because ANS does not bind to single hydrophobic residues.

ANS does not appear to influence the stability of CF_1. There is no detectable effect of ANS on the cold inactivation of $CF_1(-\epsilon)$ (data not shown). Adding ATP, 100 μM final concentration, to CF_1 that has been cold treated for 30 min does not greatly affect the fluorescence of the sample. This observation indicates that ANS binding is not affected by the presence of nucleotides in the medium and that the ANS is probably not bound to the nucleotide binding sites. There was no increase in ANS fluorescence after the initial jump when CF_1 was incubated with ANS at room temperature under the same conditions used for dissociation. Using ANS is a legitimate method for studying the cold dissociation of CF_1.

Effect of Nucleotides on the Cold Dissociation of CF_1. Previously, the loss of ATPase activity has been used to investigate the cold stability of CF_1 (McCarty & Racker, 1966; Posorske & Jagendorf, 1976; Hightower & McCarty, 1996). Studying the influence of bound nucleotides on cold lability by measuring ATPase activity presents several problems. It is necessary to use activated forms of CF_1, such as $CF_1(-\epsilon)$, to assay the ATPase activity during cold inactivation. In addition, activation of CF_1 may change the characteristics of the nucleotide binding sites. The presence of nucleotides at some of the binding sites also inhibits the ATPase activity (Du & Boyer, 1990).

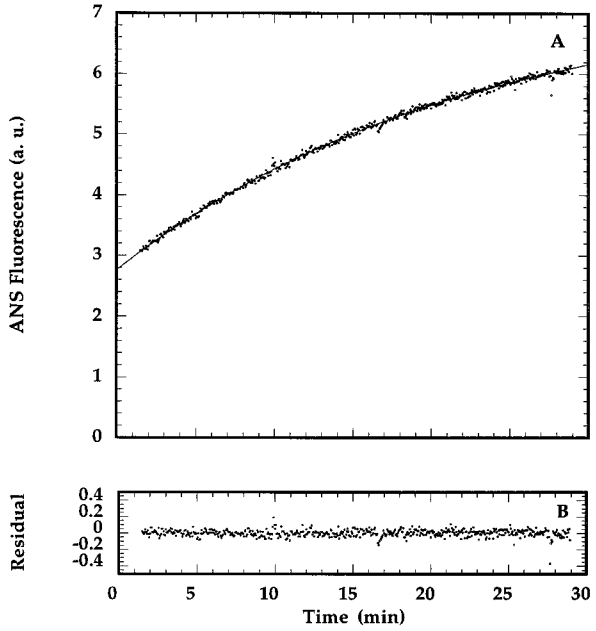

FIGURE 2: Example curve fit for the cold dissociation of CF_1. The ANS fluorescence increase for the cold dissociation of CF_1 was fit to eq 1 (A). The solid line is the curve fit. The residual of the curve fit is in panel B.

ANS was used to monitor the cold dissociation of CF_1 containing bound nucleotides and CF_1 with nucleotides present in the medium. The increase in ANS fluorescence during dissociation was fit to the following equation using KaleidaGraph version 3.0:

$$F(t) = F_{max}[1 - \exp(-kt)] + F_{min} \quad (1)$$

where $F(t)$ is the ANS fluorescence at time t, F_{max} is the extent of the fluorescence change, and F_{min} is the fluorescence immediately after the addition of CF_1. CF_1 was added after establishing a base line for 1 min. To eliminate variations caused by mixing, the first 1.5 min of the run after the addition of the protein was masked in the curve fits. The F_{min} value is the extrapolation of the curve fit to the addition of the protein and represents the initial ANS fluorescence due to binding to intact CF_1. There was little change in the value of F_{min} with loading of the nucleotide binding sites. The fits to eq 1 have R values that, with the exception of Mg^{2+}-ATP- and Mg^{2+}-AMP-PNP-loaded CF_1, range from 0.997 to 0.999. A sample curve fit for CF_1 and the residual of the fit are shown in Figure 2.

The characteristics of the nucleotide binding sites on CF_1 have been extensively studied (Bruist & Hammes, 1981; Shapiro et al., 1991). The binding sites were designated N1 through N6 and grouped into three classes of two sites each. After further investigation of the properties of the binding sites, these designations are no longer appropriate. Table 1 lists the characteristics of the sites as well as their new names and the old "N" designations. The new names for the sites will be used exclusively in this paper.

Tight ADP Site. As isolated, CF_1 contains approximately 1.5 mol of ADP tightly bound per mole of CF_1. The ADP is bound at two sites: the tight ADP site and the tight exchangeable site. Even though the nucleotide can be emptied from the tight exchangeable site, the ADP in the tight ADP site is very hard to remove. However, this ADP is not required for the structural integrity of CF_1. $CF_1(-\epsilon)$

Table 1: Nomenclature of the Nucleotide Binding Sites

characteristics	name	former designation
slowly dissociating (tight)		
(1) exchangeable with nucleotides in the medium	tight exchangeable	N1
(2) non (or slowly) exchangeable		
(A) Mg^{2+}-ATP preferred ligand[a]	Mg^{2+}-ATP	N2, N5
(B) ADP preferred ligand	tight ADP	N4
rapidly dissociating (loose)		
(1) high affinity	loose high-affinity	N3
(2) low affinity	loose low-affinity	N6

[a] This site will also bind ADP in the presence of Mg^{2+}, but with much less affinity than Mg^{2+}-ATP.

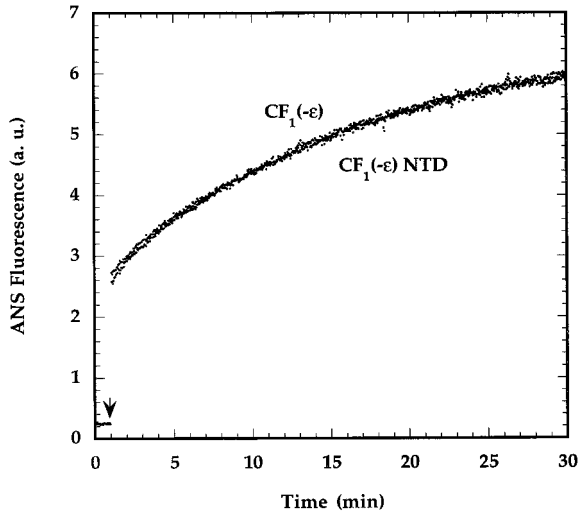

FIGURE 3: Influence of nucleotide at the tight ADP site on cold dissociation. $CF_1(-\epsilon)$ and nucleotide-depleted $CF_1(-\epsilon)$ [$CF_1(-\epsilon)$ NTD] were used. The arrow indicates the addition of CF_1 to 50 μg/mL. The dissociation buffer contained 50 mM Tris-HCl (adjusted to pH 8.0 at 20 °C), 200 mM NaCl, 5 mM EDTA, and 30 μM ANS.

that has been depleted of nucleotide ($CF_1(-\epsilon)$ NTD) is very stable (Figure 3). There is no gross difference in cold lability of the CF_1 after depletion of the nucleotides from the tight ADP site. The same result has been obtained when ATPase activity is used to monitor cold stability (Digel et al., 1996). A slight difference is apparent from the ANS assay. The rate constant (k) for $CF_1(-\epsilon)$ NTD (0.0675 ± 0.0025 min^{-1}) is higher than it is for $CF_1(-\epsilon)$ (0.0552 ± 0.0015 min^{-1}) (Table 2). Even though this increase in the rate constant may be significant, removal of the ADP from the tight ADP site does not greatly destabilize the CF_1.

Tight Exchangeable Site. The tight exchangeable site can be specifically loaded with nucleotides if CF_1 is incubated with the nucleotide in the presence of EDTA. The presence of ADP at both the tight ADP and tight exchangeable sites does not affect the cold lability of CF_1. Neither the extent nor the rate constant is affected by filling both of the nucleotide binding sites with ADP (Table 2). There is significant stabilization when the ADP at the tight exchangeable site is replaced by adenylyl β,γ-imidodiphosphate, AMP-PNP, a nonhydrolyzable analogue of ATP. The initial velocity (V_0) for dissociation is 0.187 ± 0.015 F·min^{-1} for AMP-PNP loaded CF_1 and 0.292 ± 0.011 F·min^{-1} for unloaded CF_1. This increased stability is manifested by a decrease in the extent of the fluorescence increase (Table 2). The extent drops from 4.48 ± 0.10 F for CF_1 to 2.84 ± 0.13 F when 0.8 mol of AMP-PNP is bound per mole of CF_1. There is no significant change in the rate constant.

If CF_1 with nucleotides at the tight ADP and/or tight exchangeable sites is incubated with $MgCl_2$, the Mg^{2+} can bind to the nucleotides (Digel & McCarty, 1995). Stabilization in the cold is observed for both ADP- and AMP-PNP-loaded CF_1 after the Mg^{2+} is bound. For CF_1 with 2 mol of ADP/mol of protein, the V_0 decreases from 0.212 ± 0.007 F·min^{-1} to 0.099 ± 0.006 F·min^{-1} with bound Mg^{2+} (Table 3). The change in the initial velocity is not due to a change in the extent of the fluorescence increase. Instead, the rate constant is affected (0.0442 ± 0.0013 min^{-1} versus 0.0207 ± 0.0020 min^{-1}). Since AMP is not bound by CF_1, the AMP detected during HPLC analysis must have been formed by adenylate kinase during preparation of the nucleotide sample after the Mg^{2+} and ADP were released from the CF_1. For AMP-PNP-loaded CF_1, without Mg^{2+} present, the fluorescence extent is decreased, but the rate constant is not affected. After Mg^{2+} binding, the rate constant decreases from 0.0385 ± 0.0046 min^{-1} to 0.0241 ± 0.0033 min^{-1} (Table 3). For this form of the enzyme, ADP is present at the tight ADP site, and AMP-PNP is bound at the tight exchangeable site.

To determine whether one or both sites need to be filled for Mg^{2+}-ADP to stabilize the complex, CF_1 was prepared that contained ADP at only the tight ADP site or at both the tight ADP and tight exchangeable sites. Without treatment with $MgCl_2$, the CF_1 contained very low levels of Mg^{2+}, 0.03 and 0.14 mol of Mg^{2+}/mol of CF_1, respectively (Table 4). After Mg^{2+} treatment, the Mg^{2+} content of the CF_1 was similar to the nucleotide content. The presence of Mg^{2+}-ADP in the tight ADP site did provide some stability to the CF_1. However, having both sites filled with Mg^{2+}-ADP caused a dramatic decrease in the V_0 (60.7%) relative to CF_1 with only ADP at the sites. It is important to note that the Mg^{2+} is bound to the CF_1 and is not present in the medium.

Mg^{2+}-ATP Sites. There are two nucleotide binding sites on CF_1, the Mg^{2+}-ATP sites, that have a high affinity for Mg^{2+}-nucleotides. It is possible to load these sites with Mg^{2+}-ADP, Mg^{2+}-ATP, or Mg^{2+}-AMP-PNP. The affinity of these sites for Mg^{2+}-ADP is lower than it is for Mg^{2+}-adenosine triphosphates. It was previously shown that preloading CF_1 with Mg^{2+}-nucleotides provides significant stabilization during thermal denaturation (Wang et al., 1993; Hightower & McCarty, 1996).

It is not clear whether binding of Mg^{2+}-ADP at the Mg^{2+}-ATP sites provides structural stability to CF_1. In the thermal stability studies (Wang et al., 1993), there was no differentiation between the effect of Mg^{2+}-ADP bound at the tight ADP and tight exchangeable sites and at the Mg^{2+}-ATP sites. As shown in Table 3, Mg^{2+} can bind to ADP present in the tight ADP and tight exchangeable sites and significantly decrease the cold lability of CF_1. The same is observed when CF_1 is loaded with ADP in the presence of Mg^{2+} to fill the Mg^{2+}-ATP sites as well as the other tight sites. There does not appear to be significant stabilization from Mg^{2+}-ADP at the Mg^{2+}-ATP sites. When Mg^{2+} is bound to ADP in the tight ADP and tight exchangeable sites, the rate constant is decreased, but the extent is not affected. Similarly, the rate constant for CF_1 loaded with Mg^{2+}-ADP at the Mg^{2+}-ATP sites (0.0262 ± 0.0011 min^{-1}) is lower than the rate constant for unloaded CF_1 (0.0577 ± 0.011 min^{-1}) (Table 5). There may be a slight decrease in the extent, but as explained later,

Table 2: Effect of Nucleotides at the Tight ADP and Tight Exchangeable Sites on Cold Dissociation

CF_1 sample	nucleotide content (mol/mol of CF_1) ADP	AMP-PNP	n[a]	extent (F)	k (min^{-1})	V_0 ($F \cdot min^{-1}$)
$CF_1(-\epsilon)$	1.6		4	3.98 ± 0.06	0.0552 ± 0.0015	0.220 ± 0.007
$CF_1(-\epsilon)$ NTD[b]	0.2		4	3.79 ± 0.04	0.0675 ± 0.0025	0.256 ± 0.009
CF_1	1.6		5	4.13 ± 0.12	0.0603 ± 0.0017	0.249 ± 0.009
ADP-loaded CF_1	1.9		5	4.19 ± 0.29	0.0568 ± 0.0017	0.237 ± 0.014
CF_1	1.6		5	4.48 ± 0.10	0.0652 ± 0.0017	0.292 ± 0.011
AMP-PNP-loaded CF_1	0.9	0.8	5	2.84 ± 0.13	0.0656 ± 0.0032	0.187 ± 0.015

[a] n is the number of assays with the same preparation. [b] $CF_1(-\epsilon)$ NTD also contained 0.1 mol of AMP/mol of $CF_1(-\epsilon)$.

Table 3: Stabilization of CF_1 in the Cold by Binding Mg^{2+} to Nucleotides at the Tight ADP and Tight Exchangeable Sites

CF_1 sample	nucleotide content[a] (mol/mol of CF_1) ADP	AMP-PNP	n[b]	extent (F)	k (min^{-1})	V_0 ($F \cdot min^{-1}$)
ADP-loaded CF_1	2.0		3	4.81 ± 0.10	0.0442 ± 0.0013	0.212 ± 0.007
CF_1 loaded with ADP, then Mg^{2+}	1.9		3	4.88 ± 0.29	0.0207 ± 0.0020	0.099 ± 0.006
AMP-PNP-loaded CF_1	1.0	1.0	4	3.34 ± 0.20	0.0385 ± 0.0046	0.128 ± 0.012
CF_1 loaded with AMP-PNP, then Mg^{2+}	1.0	1.0	4	1.99 ± 0.06	0.0241 ± 0.0033	0.048 ± 0.008

[a] Small amounts of AMP (0.05 and 0.07 mol/mol of CF_1, respectively) were detected for ADP-loaded CF_1 and CF_1 loaded with ADP and then Mg^{2+}. [b] n is the number of assays with the same preparation.

Table 4: Stabilization of CF_1 by Bound Mg^{2+}-ADP

nucleotide content (mol of ADP/mol of CF_1)	Mg^{2+} content (mol of Mg^{2+}/mol of CF_1)	V_0 ($F \cdot min^{-1}$)	% inhibition V_0
1.1	0.03	0.452 ± 0.018	
1.2	1.24	0.367 ± 0.004	18.8
2.4	0.14	0.341 ± 0.016	
2.2	1.79	0.134 ± 0.003	60.7

this decrease is probably due to the 0.1 mol of Mg^{2+}-ATP/mol of CF_1 bound to the protein and not the Mg^{2+}-ADP. Even though the preloading was performed in the presence of 20 μM P^1,P^5-di(adenosine-5′) pentaphosphate to inhibit the activity of a trace amount of adenylate kinase (Lienhard & Secemski, 1973), a small amount of ATP was still produced.

In the presence of Mg^{2+}, adenylate kinase converts ADP to ATP which can then bind to the CF_1 as Mg^{2+}-ATP. This Mg^{2+}-ATP is most likely bound at a Mg^{2+}-ATP site because ATP bound at the tight exchangeable site is hydrolyzed to ADP. When 0.1 mol of Mg^{2+}-ATP is bound per mole of CF_1, there is little change in the extent of the fluorescence increase (Table 5). However, when 1.0 mol of Mg^{2+}-ATP is bound per mole of CF_1, there is a significant decrease in the extent (4.95 ± 0.10 F for CF_1 versus 2.46 ± 0.11 F for Mg^{2+}-ADP-loaded CF_1). These results suggest that the Mg^{2+}-ATP sites do not need to be completely filled with Mg^{2+}-ATP for the CF_1 to be significantly stabilized in the cold.

When the Mg^{2+}-ATP sites are filled with Mg^{2+}-ATPs, CF_1 is almost fully protected from cold dissociation. There is little or no increase in the ANS fluorescence over time after addition of the protein to the dissociation buffer. Both the extent and the rate constant are strongly affected (Table 6). For the Mg^{2+}-AMP-PNP-loaded enzyme, the runs could not be fit to eq 1.

Loose Sites. CF_1 has two sites which bind nucleotides loosely. The affinity of these sites for nucleotides is dramatically different. As observed by Bruist and Hammes (1981), one site, the loose high-affinity site, has a micromolar K_d for ADP and ATP. It has been speculated that the other site, the loose low-affinity site, has a $K_d > 200$ μM. Having 100 μM ATP in the medium fully protects CF_1 from cold dissociation (Figure 1). To investigate this protection, CF_1 was preloaded with ADP in the presence of EDTA to fill the tight ADP and tight exchangeable sites. By nucleotide analysis, there was 2.3 mol of ADP/mol of CF_1. The protein was then dissociated in the presence of increasing concentrations of ATP-free ADP to study the influence of nucleotides at the loose sites on the cold lability. The results of the titration are shown in Figure 4. The stability of CF_1 increases with higher concentrations of ADP in the medium. Half-maximal protection is provided by an ADP concentration of approximately 17 μM. This ADP is most likely binding to the loose sites since the tight ADP and tight exchangeable sites were preloaded and the assay was performed in the presence of 5 mM EDTA. The curve fits for the titrations illustrate that binding of ADP to one or both of the loose sites predominantly affects the extent of the fluorescence increase, not the rate constant.

Table 7 is a summary of how various nucleotides at different binding sites affect the cold dissociation of CF_1. The extent of the ANS fluorescence increase is affected by the binding of adenosine triphosphates at the tight exchangeable site or the Mg^{2+}-ATP sites or ADP binding, at high concentrations, to the loose sites. Mg^{2+}-adenine nucleotides are required, at either the tight exchangeable or the Mg^{2+}-ATP sites, to observe a dramatic effect on the rate constant. The decrease in the extent may be indicative of a tightening of the CF_1 structure so that less hydrophobic surfaces are accessible to the ANS. The decreased rate constant may be caused by a slower dissociation of a subunit or subunits from the rest of the complex.

DISCUSSION

Nucleotides are very important for stabilizing the structure of CF_1. The nucleotide binding sites are proposed to be at the interfaces of the α and β subunits with both subunits interacting with the nucleotides (Abrahams et al., 1994). Therefore, it is not surprising that the presence of bound nucleotides or nucleotides in the medium would protect CF_1 from dissociating in the cold. The influence of nucleotides

Table 5: Effect of Mg^{2+}-ADP Bound at the Mg^{2+}-ATP Sites on Cold Dissociation

CF_1 sample	nucleotide content (mol/mol of CF_1) ADP	ATP	n[a]	extent (F)	k (min^{-1})	V_0 ($F \cdot min^{-1}$)
CF_1	1.8		4	4.70 ± 0.12	0.0577 ± 0.0011	0.271 ± 0.007
Mg^{2+}-ADP-loaded CF_1	3.4	0.1	4	4.44 ± 0.06	0.0262 ± 0.0011	0.116 ± 0.005
CF_1	1.7		5	4.95 ± 0.10	0.0657 ± 0.0031	0.325 ± 0.016
Mg^{2+}-ADP-loaded CF_1[b]	2.4	1.0	5	2.46 ± 0.11	0.0250 ± 0.0011	0.062 ± 0.003

[a] n is the number of assays with the same preparation. [b] 0.03 mol of AMP/mol of CF_1 was detected in this sample.

Table 6: Effect of Mg^{2+}-Adenosine Triphosphates Bound at the Mg^{2+}-ATP Sites on Cold Dissociation

CF_1 sample	nucleotide content (mol/mol of CF_1) ADP	ATP	AMP-PNP	n[a]	extent (F)	k (min^{-1})	V_0 ($F \cdot min^{-1}$)
CF_1	1.3			6	2.49 ± 0.10	0.0858 ± 0.0025	0.213 ± 0.011
Mg^{2+}-ATP-loaded CF_1[b]	1.6	1.6		4	0.36 ± 0.08	0.0266 ± 0.0073	0.009 ± 0.002
CF_1	1.7			4	4.63 ± 0.12	0.0457 ± 0.0005	0.212 ± 0.004
Mg^{2+}-AMP-PNP-loaded CF_1	1.3		2.8	3	~0	~0	~0

[a] n is the number of assays with the same preparation. [b] 0.05 mol of AMP per mole of CF_1 was detected.

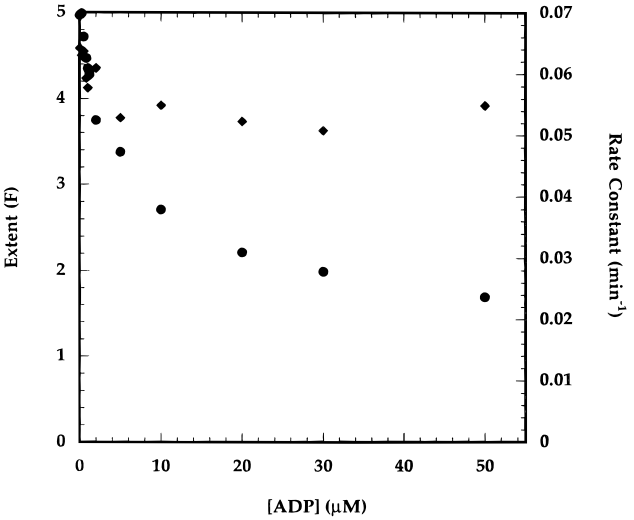

FIGURE 4: Titration of ADP-loaded CF_1 with ADP. CF_1 was preloaded with ADP in EDTA so that it contained 2.3 mol of ADP bound at the tight ADP and tight exchangeable sites per mole of CF_1. The dissociation buffer contained 50 mM Tris-HCl (adjusted to pH 8.0 at 20 °C), 200 mM NaCl, 5 mM EDTA, 30 μM ANS, and increasing concentrations of ADP. CF_1 was added to the cold buffer to initiate the dissociation. The extent (●) and the rate constant (◆) values were derived from curve fits to eq 1.

on the cold lability of CF_1 was previously investigated by Posorske and Jagendorf (1976). Their analysis did not give a clear picture of stabilization by nucleotides. Since the nucleotide content of the samples was not routinely determined, there is no way to differentiate between binding at the various sites. In addition, our laboratory has established that there is enough Mg^{2+} in the buffers to cause a significant decrease in the nucleotide exchange rate (J. Digel, unpublished result). While some of the conclusions made in the Posorske and Jagendorf paper, such as the Mg^{2+} requirement for stabilization by ADP, are undoubtedly accurate, a more careful study of the influence of nucleotides on the cold dissociation of CF_1 was necessary.

The structural integrity of CF_1 is dominated by intersubunit contacts. The ADP that is tightly bound to the complex when it is isolated from the thylakoid membranes is very hard to remove. It would be logical to assume that this nucleotide must be important for the structural stability of the complex. However, CF_1 can be depleted of almost all of its nucleotides

Table 7: Summary of the Influence of Bound Nucleotides on the Cold Dissociation of CF_1

tight[a]	Mg^{2+}-ATP	loose	extent[b]	k[b]
ADP			1.4	5.8
ADP & AMP-PNP			36.6	0
ADP & Mg^{2+}			0	53.2
ADP, AMP-PNP, & Mg^{2+}			40.4	37.4
ADP & Mg^{2+}	Mg^{2+}-ADP & -ATP		5.5	54.6
ADP & Mg^{2+}	Mg^{2+}-ADP & -ATP		50.3	61.9
ADP & Mg^{2+}	Mg^{2+}-ATP		85.5	69.0
ADP, AMP-PNP, & Mg^{2+}	Mg^{2+}-AMP-PNP		100	100
ADP		ADP	66.0	14.5

[a] "Tight" implies both the tight ADP and tight exchangeable sites. [b] These values represent the percent inhibition of the extent or the rate constant.

without seriously affecting the cold lability of the protein as assayed by either ATPase activity (Digel et al., 1996) or ANS fluorescence. Moreover, ADP can be loaded into the tight ADP and tight exchangeable sites without increasing the cold stability of CF_1. Since the presence of nucleotides is not critical for maintaining the stability of CF_1, the interactions of the subunits must be responsible for the structural integrity. It was previously shown that elimination of certain intersubunit contacts markedly increased the cold lability of CF_1 (Hightower & McCarty, 1996).

In contrast to the ADP, AMP-PNP bound at any of the sites stabilizes CF_1. It is possible that the larger triphosphate group is a better bridge between the subunits than the diphosphate. The structure of the CF_1 may be tighter when triphosphates are bound to the nucleotide sites. Of all the bound nucleotides tested here, the preferred nucleotides for stabilizing the structure of CF_1 during cold treatment are the Mg^{2+}-adenosine triphosphates.

The cold lability of CF_1 can be diminished by having nucleotides present in the medium. In this study, the influence of nucleotides at the loose sites was monitored after preloading the tight ADP and tight exchangeable sites with ADP. Half-maximal protection was reached at approximately 17 μM ADP. The dissociation constants for the loose sites have been reported to be micromolar and greater than 200 μM (Bruist & Hammes, 1981). The process monitored by the change in ANS fluorescence involves stabilization of the complex as well as binding of the nucleotide. In addition, the cold dissociation was performed under much harsher

conditions, low temperature and high salt concentrations, than the binding studies.

Possibly the most intriguing finding from this work is the ability of Mg^{2+} to bind to nucleotides in the tight ADP and tight exchangeable sites and stabilize the CF_1. Posorske and Jagendorf (1976) showed that ADP in the medium was not as effective as ADP and Mg^{2+} for preventing the cold inactivation of CF_1. We have shown that ADP bound at the tight ADP and the tight exchangeable sites does not have an effect on the cold dissociation of CF_1. However, ADP in conjunction with Mg^{2+} can provide stability. The ability of AMP-PNP bound at the tight exchangeable site, with or without Mg^{2+}, to protect CF_1 in the cold may provide a clue to the mechanism of stabilization by Mg^{2+}-ADP. There are undoubtedly fewer contacts between a diphosphate and the binding site than between a triphosphate and the binding site. The presence of Mg^{2+} may provide additional contact between the α and β subunits and the ADP in the binding sites that causes an increase in the stability of the complex.

Even though this work provides insight into why CF_1 does or does not dissociate in the cold, it does not explain how it dissociates. For a more comprehensive interpretation of the subunit and nucleotide effects on the cold lability of CF_1, it will be necessary to gain a better understanding of the mechanism of cold dissociation. Work is in progress to determine what part or parts of the complex dissociate during cold treatment. Unfortunately, it is not possible at this time to assign the characteristics of each binding site of CF_1 to the nucleotide binding sites resolved in the crystal structure of bovine mitochondrial F_1 (Abrahams et al., 1994). If the interactions between individual residues in the nucleotide binding sites and the nucleotides and Mg^{2+} can be established, it may be possible to establish how bound nucleotides protect CF_1 from dissociating in the cold.

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REFERENCES

Abrahams, J. P., Leslie, A. G. W., Lutter, R., & Walker, J. E. (1994) *Nature 370*, 621−628.

Bruist, M. F., & Hammes, G. G. (1981) *Biochemistry 20*, 6298−6305.

Cohn, W. E. (1957) in *Ion Exchangers in Organic and Biochemistry* (Calman, C., & Kressman, T. R. E., Eds.) pp 345−359, Interscience, New York.

Digel, J. G., & McCarty, R. E. (1995) *Biochemistry 34*, 14482−14489.

Digel, J. G., Kishinevsky, A., Ong, A. M., & McCarty, R. E. (1996) *J. Biol. Chem.* (in press).

Du, Z., & Boyer, P. D. (1990) *Biochemistry 29*, 402−407.

Futai, M., Noumi, T., & Maeda, M. (1989) *Annu. Rev. Biochem. 58*, 111−136.

Gibbons, D. L., & Horowitz, P. M. (1995) *J. Biol. Chem. 270*, 7335−7340.

Hightower, K. E., & McCarty, R. E. (1996) *Biochemistry 35*, 4852−4857.

Lienhard, G. E., & Secemski, I. I. (1973) *J. Biol. Chem. 248*, 1121−1123.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951*) J. Biol Chem. 193*, 265−275.

McCarty, R. E., & Racker, E. (1966) *Brookhaven Symp. Biol. 19*, 202−214.

McCarty, R. E., & Moroney, J. V. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A., Ed.) 2nd ed., pp-383−413, Plenum Publishing Corp., New York.

Moal, J., Le Coz, J. R., Samain, J. F., & Daniel, J. Y. (1989) *Comp. Biochem. Physiol. 93B*, 307−316.

Moroney, J. V., Lopresti, L., McEwen, B. F., McCarty, R. E., & Hammes, G. G. (1983*) FEBS Lett. 158*, 58−62.

Pedersen, P. L., & Amzel, L. M. (1993) *J. Biol. Chem. 268*, 9937−9940.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891−2899.

Posorske, L., & Jagendorf, A. T. (1976) *Arch. Biochem. Biophys. 177*, 276−283.

Privalov, P. L. (1990) *Crit. Rev. Biochem. Mol. Biol. 25*, 281−305.

Richter, M. L., Patrie, W. J., & McCarty, R. E. (1984) *J. Biol. Chem. 259*, 7371−7373.

Richter, M. L., Snyder, B., McCarty, R. E., & Hammes, G. G. (1985) *Biochemistry 24*, 5755−5763.

Semisotnov, G. V., Rodionova, N. A., Razgulyaev, O. I., Uversky, Gripas, A. F., &Gilmanshin, R. I. (1991) *Bioploymers 31*, 119−128.

Shapiro, A. B., & McCarty, R. E. (1990) *J. Biol. Chem. 265*, 4340−4347.

Shapiro, A. B., Huber, A. H., & McCarty, R. E. (1991) *J. Biol. Chem. 266*, 4194−4200.

Slivak, J. (1982) *Biochim. Biophys. Acta 694*, 1−25.

Soteropoulos, P., Süss, K.-H., & McCarty, R. E. (1992) *J. Biol. Chem. 267*, 10348−10354.

Soteropoulos, P., Ong, A. M., & McCarty, R. E. (1994) *J. Biol. Chem. 269*, 19810−19816.

Teschke, C. M., King, J., & Prevelige, P. E., Jr. (1993) *Biochemistry 32*, 10658−10665.

Wang, Z.-Y., & Portis, A. R., Jr. (1991) *Biochim. Biophys. Acta 1079*, 263−267.

Wang, Z.-Y., Freire, E., & McCarty, R. E. (1993) *J. Biol. Chem. 268*, 20785−20790.

Ybarra, J., & Horowitz, P. M. (1995) *J. Biol. Chem. 270*, 22962−22967.

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