Structural Stability of Chloroplast Coupling Factor 1 as Determined by Differential Scanning Calorimetry and Cold Inactivation†

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ABSTRACT: At least part of the γ subunit of the catalytic portion of the chloroplast ATP synthase (CF_1) is present in the middle of the $\alpha_3\beta_3$ heterohexamer. Interactions of the α/β subunits with the γ subunit stabilize the hexameric structure. Surprisingly, neither reduction of the γ disulfide nor selective proteolysis of α, β, and γ affects the thermal stability of EDTA-treated CF_1 preparations, as determined by differential scanning calorimetry. Dissociation of the enzyme in the cold may be monitored by loss of the ATPase activity of CF_1 depleted of its ϵ subunit [$CF_1(-\epsilon)$]. The rate of cold inactivation of ATPase activity of reduced and alkylated $CF_1(-\epsilon)$ treated with trypsin in solution was much faster than that of $CF_1(-\epsilon)$ (8.1 versus 38.7 min, respectively, for 50% loss of activity). The increased cold lability of the trypsin-treated enzyme was not a consequence of the cleavage of the γ. CF_1 incubated with trypsin under conditions in which γ is not cleaved was as cold labile as CF_1 with cleaved γ. Instead, loss of the δ subunit and a few residues from the C-termini of the β subunits were responsible for the increased cold lability of the enzyme.

Proton-linked ATP production is catalyzed by the ATP synthase, F_1F_o. The efflux of protons through the membrane integrated F_o component provides the energy for the synthesis of ATP from ADP and P_i. F_1 contains the catalytic sites and regulatory regions. In bacteria and chloroplasts, it is comprised of five different subunits, α through ϵ in order of decreasing molecular weight, with a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. In solution, the F_1 acts as an ATPase hydrolyzing ATP to ADP and P_i.

Much effort has been made to understand the functions of the individual subunits of F_1-ATPases. The nucleotide binding sites are located at the interfaces of the α and β subunits (Abrahams et al., 1994). The functions of the small subunits, γ, δ, and ϵ, are similar for both bacterial and chloroplast F_1. The γ subunit is considered to be involved in regulation of both ATP synthesis and hydrolysis as well as binding of the ϵ subunit. The δ subunit has been implicated in binding F_1 to F_o. The ϵ subunit of chloroplast F_1 and *Escherichia coli* F_1 is required for ATP synthesis and acts as an inhibitor of ATPase activity [for reviews, see Futai et al., (1989) and McCarty (1995)].

The γ subunit seems to be important for the both the structure and the function of the F_1-ATPases. Differential scanning calorimetry showed that removal of the δ and ϵ subunits did not significantly affect the temperature at the maximum excess heat capacity (T_m),[1] suggesting that the structural core of the enzyme is $\alpha_3\beta_3\gamma$ (Wang et al., 1993). Reconstitution work with F_1 subunits from *E. coli* (Futai, 1977; Dunn & Futai, 1980), plants (Chen & Jagendorf, 1994; Gao et al., 1995), and the thermophilic bacterium PS3 (Yoshida et al., 1977) have shown that stable, active $\alpha_3\beta_3\gamma$ complexes can be reformed. Even though $\alpha_3\beta_3\delta$ complexes have been reported for PS3, they are not as stable as the native enzyme or the $\alpha_3\beta_3\gamma$ (Yoshida et al., 1977; Yokoyama et al., 1989).

Nucleotides stabilize the enzyme during both cold inactivation and thermal denaturation. The F_1-ATPases are cold labile complexes. Incubation of isolated F_1 in the cold causes it to dissociate into its consistuent subunits and lose its ability to hydrolyze ATP (McCarty & Racker, 1966; Penefsky & Warner, 1965; R. E. McCarty, unpublished results). The addition of nucleotides has been shown to protect CF_1 in the cold (McCarty & Racker, 1966; Posorske & Jagendorf, 1976). Occupancy of the nucleotide binding sites (Wang et al., 1993) or the presence of nucleotides in the medium (Farron & Racker, 1970; Smith et al., 1986) protects the protein from thermal denaturation.

We have investigated the influence of the γ subunit on the structural stability of CF_1. Reduction of the γ disulfide and cleavage of γ subunit by trypsin into two or three fragments do not significantly affect either the thermal or the cold stability of CF_1. The δ subunit and the C-terminal region of β, as well as nucleotides, are important for the structural stability of CF_1.

MATERIALS AND METHODS

CF_1 was prepared from market spinach as previously described (Shapiro & McCarty, 1990; Soteropoulos et al., 1994; Digel & McCarty, 1995). Contaminating ribulose bisphosphate carboxylase/oxygenase was removed by immunoaffinity chromatography (Soteropoulos et al., 1992). All forms of CF_1 were stored at 4 °C in pH 8.0 buffers as 50% ammonium sulfate precipitates with 1 mM ATP and 2 mM EDTA.

δ and ϵ were removed from CF_1 and CF_1 with truncated β as described in Richter et al. (1986). The ϵ subunit was

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; $CF_1(-\epsilon)$, CF_1 lacking the ϵ subunit; T_m, the temperature at the maximum excess heat capacity; β^t, β subunit with C-terminal truncation; $\gamma_{(27,10)}$, γ subunit cleaved into the 27 and 10 kDa fragments; α^t, α subunit with a 16 residue N-terminal truncation; $\gamma_{(8,15,10)}$, γ subunit cleaved into the 8, 15, and 10 kDa fragments; TPCK-trypsin, trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone; ACMA, 9-amino-6-chloro-2-methoxyacridine; $t_{50\%}$, time for the loss of 50% of the ATPase activity; HPLC, high-performance liquid chromatography.

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removed from all other types of protein by published procedures (Richter et al., 1984, 1985; Soteropoulos et al., 1994).

Membrane-bound CF_1 was treated with trypsin in the dark ($\alpha_3\beta^t_3\gamma\delta$) or during illumination ($\alpha_3\beta^t_3\gamma_{(27,10)}\delta$) for 3−5 min as described elsewhere (Schumann et al., 1985; Soteropoulos et al., 1992). Soluble $CF_1(-\epsilon)$ that had been reduced, alkylated with iodoacetic acid, and then trypsin treated ($\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$) was prepared as described in the preceding paper (Hightower & McCarty, 1996a).

CF_1 with α and β truncated but intact γ ($\alpha^t_3\beta^t_3\gamma$) was prepared by trypsin treatment of full-subunit CF_1 as described by Moroney and McCarty (1982a) with the following modifications: After a 5 min incubation with TPCK-trypsin (1 μg of trypsin:100 μg of CF_1), the trypsin was removed from the protein by passing the solution through a 1 mL column of soybean trypsin inhibitor bound to 4% beaded agarose. The ϵ subunit was then removed from the protein as above. The presence of the ϵ subunit protects γ from trypsin cleavage but does not affect the truncation of either α or β (Richter et al., 1985). The δ subunit is digested during trypsin treatment.

Reduction of the γ disulfide and alkylation of the cysteine residues were performed as in Soteropoulos et al. (1994). After incubation with iodoacetic acid, the protein was passed through a 3 mL Sephadex G-50 centrifuge column (Penefsky, 1977) to remove the iodoacetic acid. Dithiothreitol was added to 2 mM, and the protein was passed through another centrifuge column.

The δ subunit was prepared as described in Xiao and McCarty (1989). After isolation, the δ was dialyzed against 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, concentrated with Aquacide, and then stored at −20 °C. δ was reconstituted with $\alpha_3\beta_3\gamma$, $\alpha_3\beta^t_3\gamma$, and $\alpha^t_3\beta^t_3\gamma$ by incubating the protein at 2 mol of δ:1 mol of CF_1 for 10 min at 25 °C. The protein was passed through a 3 mL Sephadex G-50 centrifuge column and then precipitated with ammonium sulfate, with 1 mM ATP and 2 mM EDTA, to remove any unbound δ. Light dependent proton uptake of reconstituted CF_1 on thylakoid membranes was used to determine if the isolated δ was still functional. ΔpH was monitored by a modification of the procedure described by Richter et al. (1984). Quinacrine was replaced with 1 μM 9-amino-6-chloro-2-methoxyacridine (ACMA) and 1 mM diaminodurene·2HCl was used instead of *N*-methylphenazonium methosulfate.

Differential scanning calorimetry was performed in the Biocalorimetry Center in the Department of Biology, Johns Hopkins University. Protein was desalted by passage through a 3 mL Sephadex G-50 centrifuge column equilibrated with 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and was then incubated in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl with 0.5 mM $MgCl_2$, 5 mM ATP for 5 h at room temperature. Excess Mg^{2+}-ATP was removed by passing the protein through two 3 mL Sephadex G-50 centrifuge columns equilibrated with 50 mM Tris-HCl (pH 8.0), 50 mM NaCl. The EDTA treated protein was kept as an ammonium sulfate precipitate in the presence of 1 mM ATP and 2 mM EDTA. The protein was desalted and the EDTA was removed by passing the resuspended protein through two 3 mL Sephadex G-50 centrifuge columns equilibrated with 50 mM Tris-HCl (pH 8.0), 50 mM NaCl. To eliminate precipitation after thermal denaturation, the calorimetry was performed in 10 mM Hepes-NaOH (pH 7.5) and 200 mM sucrose (Wang et al., 1993). CF_1 was transferred to this buffer by passage through a 3 mL Sephadex G-50 centrifuge column equilibrated with the Hepes/sucrose mixture. The protein samples, at 1.5 mg/mL, were scanned at 1 °C/min using a microcalorimeter built by V. Plotnikov and P. Privalov at Johns Hopkins University from a DASM-1M prototype (Carra et al., 1994).

To test the cold lability of different forms of $CF_1(-\epsilon)$, the loss of Ca^{2+}-ATPase activity in the cold was monitored. Before use, the protein was passed through two 3 mL Sephadex G-50 centrifuge columns equilibrated with 50 mM Tris-HCl (pH 8.0). The protein was diluted to 15 μg/mL (7.2−9.6 mL total) with 50 mM Tris-HCl (pH 8.0 at 20 °C) and allowed to sit at 25 °C for 5 min. Half of the protein solution was then transferred to a prechilled 12 × 75 mm borosilicate glass test tube on ice. Aliquots (200 μL) were removed for activity assays at selected times up to 60 min. The aliquots were added to 300 μL of reaction mix at 25 °C so that the final assay concentrations were 5 mM ATP, 5 mM $CaCl_2$, 40−50 mM Tris-HCl (pH 8.0), and 3 μg of protein. After 3 min at 25 °C, the tubes were removed, 500 μL of 0.5 N trichloroacetic acid was added, and the tubes were placed on ice. P_i was determined colorimetrically using a malachite green assay.

Modifications of published green procedures (Lanzetta et al., 1979; van Veldhoven & Mannaerts, 1987) were kindly provided by Dr. A. T. Jagendorf. Two stock solutions, Solution A and Solution B, were made in advance and stored at room temperature.

Solution A: Polyvinyl alcohol (0.55% w/v final concentration) was dissolved in 80 °C water. After the solution cooled to room temperature, malachite green was added to 0.055% (w/v) final concentration and the volume was brought up with water.

Solution B: 2.5% ammonium molybdate tetrahydrate (w/v) dissolved in 4 N HCl.

Solution C: 1 part of Solution A was mixed with 1 part of Solution B and 4 mg of *p*-methylaminophenol sulfate was added per mL. After mixing, the solution stood at room temperature for 30 min before use. The solution was prepared fresh daily.

To a 500 μL sample in water containing 1−10 nmol of P_i was added 500 μL of Solution C, and the solution was vortexed. After 2 min, 500 μL of 10% citric acid (w/v) was added and the solution was mixed well. The absorbance was determined at 600 nm after at least 30 min.

The N-terminal sequencing of trypsin-modified β was performed by the Analytical Chemistry and Peptide/DNA Synthesis Facility at Cornell University. The trypsin-truncated β subunit was transferred from a 10% SDS−polyacrylamide gel to a polyvinylidene difluoride membrane according to manufacturer specifications (Immobilon Tech Protocol #TP006).

CF_1 concentrations were determined by absorbance at 277 nm using an extinction coefficient of 0.483 cm^2/mg (Bruist & Hammes, 1981) or by published procedures (Lowry et al., 1951). The molecular mass of CF_1 is 400 kDa (Moroney et al., 1983). Nucleotide analysis by high-performance liquid chromatography (HPLC) was performed as described in Soteropoulos et al. (1994) and Moal et al. (1989) using a Chromega chromegabond mc18/54.6 mm × 10 cm reversed-phase column on a Beckman 342 gradient liquid chromatograph.

Table 1: Thermal Denaturation of Modified CF_1 Preparations

subunit composition of preparation	T_m (°C)[a]	
	EDTA-treated	Mg^{2+}-ATP-loaded
$\alpha_3\beta_3\gamma\delta\epsilon$	56.6	64.0
$\alpha_3\beta_3\gamma\delta$	55.8	62.4
$\alpha_3\beta_3\gamma\delta$, reduced[b]	55.8	61.4
$\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$[c]	56.6	60.3

[a] T_m denotes the temperature at the maximum excess heat capacity. [b] The γ disulfide was reduced, and the cysteine residues were alkylated with iodoacetic acid. [c] The γ was cleaved into fragments of 8000, 14 850, and 10 000 molecular weight ($\gamma_{(8,15,10)}$). The N-terminal 16 amino acids had been removed from α (α^t), and the C-terminus of β had been truncated (β^t).

Aquacide was purchased from Calbiochem (San Diego, CA), and the polyvinylidene difluoride membranes were purchased from Millipore (Bedford, MA).

RESULTS

Thermal Denaturation of CF_1. To determine the implications of trypsin cleavage of the γ subunit on the structural stability of CF_1, modified forms of CF_1 were subjected to differential scanning calorimetry. Incubation of CF with EDTA in the presence of ammonium sulfate removes bound ATP, leaving 1.0−1.5 mol of ADP bound per mol of CF_1. Reduction of the γ disulfide or cleavage of the γ subunit into three fragments that remain tightly associated with the $\alpha_3\beta_3$ hexamer (Hightower & McCarty, 1996a) does not destabilize the EDTA-treated protein (Table 1). There is no significant difference in the T_m values for any of the different forms of CF_1. Because the trypsin-treated form of CF_1, $\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$, also has truncated α and β subunits, it can be surmised that these modifications do not affect the thermal stability of the complex. As previously determined, removal of the δ and ϵ subunits does not destabilize the complex during heating (Wang et al., 1993). However, occupancy of the nucleotide binding sites is very important for maintaining the structural integrity of the CF_1 (Wang et al., 1993). Loading the enzyme with Mg^{2+}-ATP did protect all forms of CF_1 from denaturation. It was previously shown (Shapiro et al., 1991) that incubation of CF_1 with Mg^{2+}-ATP results in the loading of tightly bound Mg^{2+}-ATP to two noncatalytic sites. Close to 2 mol of ADP per mol of enzyme are also bound. The stabilization from Mg^{2+}-ATP loading is less for the trypsin-treated enzyme, T_m of 60.3 °C versus 56.6 °C, than it is for the other forms, T_m of 64.0 °C versus 56.6 °C for CF_1. Mg^{2+}-ATP may not be as tightly bound in the trypsin-treated enzyme. Wang et al. (1993) reported that trypsin-treated $CF_1(-\epsilon)$ was more thermolabile than $CF_1(-\epsilon)$. In these experiments, thermolability was determined by loss of ATPase activity upon heating. However, the procedures used previously to cleave the enzyme did not completely remove the trypsin from the CF_1.

Cold Inactivation of $CF_1(-\epsilon)$. The cold lability of different forms of $CF_1(-\epsilon)$ was tested to study the influence of different subunits on dissociation of the complex. With the exception of the CF_1 treated with trypsin on the thylakoid membranes, the other forms of protein were made from the same preparation of CF_1. All protein samples were stored as ammonium sulfate precipitates in the presence of 1 mM ATP and 2 mM EDTA to remove tightly bound Mg^{2+}-ATP. By HPLC analysis, the ADP content of the EDTA-treated protein ranged from 1.1 to 1.5 mol of ADP per mol of enzyme. Modification of the enzyme by trypsin treatment or subunit removal did not significantly change the amount of bound nucleotide.

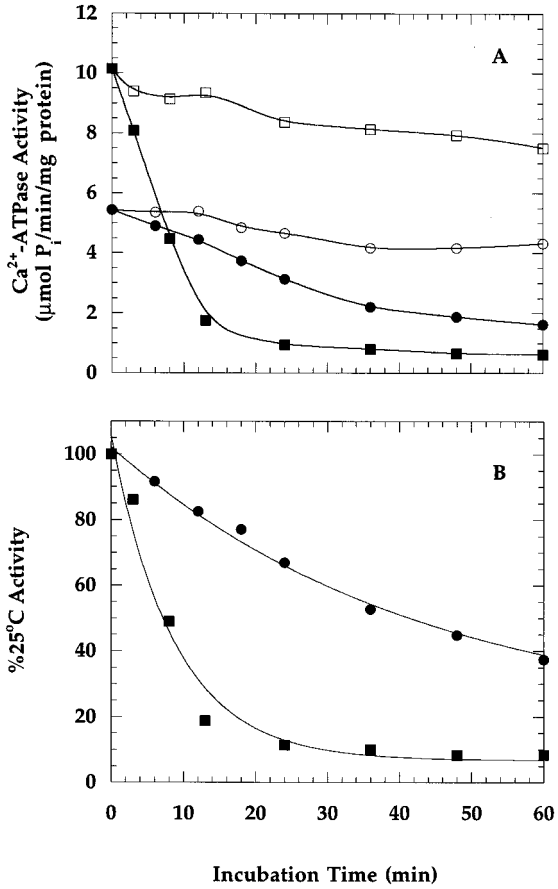

FIGURE 1: Cold inactivation of the Ca^{2+}-ATPase activity of $\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$ and $\alpha_3\beta_3\gamma\delta$. A. The Ca^{2+}-ATPase activity of the preparations, diluted to 15 μg/mL, was determined for the samples incubated at 25 °C (□, $\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$; ○, $\alpha_3\beta_3\gamma\delta$) and in the cold (■, $\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$; ●, $\alpha_3\beta_3\gamma\delta$). Maximum activities were 10.1 and 5.4 μmol of P_i/min/mg of protein for $\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$ and $\alpha_3\beta_3\gamma\delta$, respectively. B. Replot of the data in A as % 25 °C activity values. The data were fitted to eq 1. The $t_{50\%}$ values were 7.2 min for $\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$ (■) and 42.0 min for $\alpha_3\beta_3\gamma\delta$ (●).

Enzyme preparations were diluted to 15 μg/mL prior to cold treatment to minimize any stabilization by free nucleotide after dissociation, and the ATPase assays were performed at 25 °C to minimize the possibility of reconstitution of the complex. The decrease in activity was not due to dilution of the enzyme. The activity of the protein kept at 25 °C decreased slightly over time but not to the same extent as the protein incubated in the cold (Figure 1A). Previous work with mitochondrial F_1 (Penefsky & Warner, 1965) and CF_1 (McCarty & Racker, 1965; R. E. McCarty, unpublished results) showed that cold inactivation is associated with dissociation of the enzyme.

To compare the cold lability of different forms of $CF_1(-\epsilon)$, the % 25 °C activity, the activity of the protein incubated in the cold divided by the activity of the protein kept at 25 °C, was determined for each time point. Figure 1B shows a representative graph for the inactivation of $\alpha_3\beta_3\gamma\delta$ and $\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$. The data were fitted to the equation

$$P(t) = \Delta P_{max} \exp(-kt) + P_{min} \quad (1)$$

where P is the % 25 °C activity at time t, ΔP_{max} is the maximum chnge in the % 25 °C activity, and P_{min} is the minimum % 25 °C activity. The curve fits, which were

Table 2: Effect of γ Modification on Cold Stability.

subunit composition of preparation[a]	form of γ	n[b]	$t_{50\%}$ (min)[c]
$\alpha_3\beta_3\gamma\delta$	intact, oxidized	4	38.7 ± 3.2
	intact, reduced[d]	3	30.8 ± 6.4[e]
$\alpha_3\beta^t{}_3\gamma\delta$	intact, oxidized	4	20.2 ± 1.9
	intact, reduced	4	17.5 ± 2.0
$\alpha_3\beta^t{}_3\gamma_{(27,10)}\delta$	γ27 and γ10, oxidized	3	16.8 ± 0.7
	γ27 and γ10, reduced	3	17.4 ± 2.6
$\alpha^t{}_3\beta^t{}_3\gamma$	intact, oxidized	4	9.0 ± 1.8
$\alpha^t{}_3\beta^t{}_3\gamma_{(8,15,10)}$	γ8, γ15, γ10	5	8.1 ± 0.9

[a] Truncation of the α and β subunits is denoted by the superscript "t". [b] n is the number of cold inactivations for each type of protein. [c] $t_{50\%}$ is the time for loss of 50% of the ATPase activity of samples incubated on ice versus samples kept at 25 °C. The Ca^{2+}-ATPase activity assays were performed at 25 °C for 3 min with 3 μg of protein. [d] For all forms of reduced protein, the γ disulfide was reduced with DTT and then blocked by alkylation of the cysteines with iodoacetic acid. [e] Over time, the reduced form of the enzyme appeared to be proteolyzed. The proteolysis may account for the variation in the $t_{50\%}$ values.

Table 3: Effect of β Truncation on Cold Stability

subunit composition of preparation[a]	n[b]	$t_{50\%}$ (min)[c]
$\alpha_3\beta_3\gamma\delta$	4	38.7 ± 3.2
$\alpha_3\beta^t{}_3\gamma\delta$	3	19.8 ± 1.4
$\alpha_3\beta^t{}_3\gamma$	3	12.3 ± 0.8
$\alpha^t{}_3\beta^t{}_3\gamma$	4	9.0 ± 1.8

[a] Truncation of the α and β subunits is denoted by the superscript "t". [b] n is the number of cold inactivations for each type of protein. [c] $t_{50\%}$ is the time for loss of 50% of the ATPase activity of samples incubated on ice versus samples kept at 25 °C. The Ca^{2+}-ATPase activity assays were performed at 25 °C for 3 min with 3 μg of protein.

Table 4: Effect of δ Removal on Cold Stability

subunit composition of preparation[a]	n[b]	$t_{50\%}$ (min)[c]
$\alpha_3\beta_3\gamma\delta$	4	38.7 ± 3.2
$\alpha_3\beta_3\gamma$	5	15.2 ± 2.1
$\alpha_3\beta_3\gamma$ reconstituted with δ[d]	3	37.0 ± 3.7
$\alpha_3\beta^t{}_3\gamma\delta$	3	19.8 ± 1.4
$\alpha_3\beta^t{}_3\gamma$	3	12.3 ± 0.8
$\alpha_3\beta^t{}_3\gamma$ reconstituted with δ[d]	3	17.3 ± 2.2

[a] Truncation of the α and β subunits in denoted by the superscript "t". [b] n is the number of cold inactivations for each type of protein. [c] $t_{50\%}$ is the time for the loss of 50% of the ATPase activity of samples incubated on ice versus samples kept at 25 °C. The Ca^{2+}-ATPase activity assays were performed at 25 °C for 3 min with 3 μg of protein. [d] Reconstitution was performed with 2 mol of δ:1 mol of $CF_1(-\delta)$ for 10 min at room temperature. Excess δ was removed by G-50 Sephadex centrifuge columns and ammonium sulfate precipitation.

performed by KaleidaGraph 3.0, have R values ranging from 0.979 to 0.999. The time point on this curve corresponding to a loss of 50% activity in the cold was taken as the $t_{50\%}$ value. Approximately 10% of the activity remained after incubation of various forms of CF_1 in the cold. It is possible that nucleotides released during dissociation of the protein are able to bind to and protect a small population of the complexes and that partial $\alpha_n\beta_n\gamma$ or $\alpha_n\beta_n\delta$ complexes may retain some activity.

Effect of Modifying the γ Subunit on Cold Stability. The γ subunit of CF_1 can be modified by reduction of the disulfide bond and trypsin cleavage. Trypsin cleavage results in the formation of two large fragments (γ27 and γ10) when oxidized CF_1 is incubated with trypsin on the thylakoid membranes during illumination (Schumann et al., 1985; Hightower & McCarty, 1996b) or three large fragments (γ8, γ15, and γ10) when reduced $CF_1(-\epsilon)$ is treated with trypsin in solution (Hightower & McCarty, 1996a). Regardless of whether the enzyme was trypsinized on the membrane or in solution, cleavage of the γ subunit has little or no significant effect on the stability of the complex in the cold (Table 2). The $t_{50\%}$ values for $\alpha_3\beta^t{}_3\gamma\delta$ with intact γ (20.2 ± 1.9 min) and $\alpha_3\beta^t{}_3\gamma_{(27,10)}\delta$ with cleaved γ (γ27 and γ10) (16.8 ± 0.7 min) are similar. In addition, there is no significant difference in the cold lability of $\alpha^t{}_3\beta^t{}_3\gamma_{(8,15,10)}$, $t_{50\%}$ of 8.1 ± 0.9 min, where the γ subunit was reduced and fully cleaved (γ8, γ15, and γ10) and the similar protein, $\alpha^t{}_3\beta^t{}_3\gamma$, $t_{50\%}$ of 9.0 ± 1.8 min, with intact γ. Reduction of the γ disulfide also does not seem to greatly affect the cold stability of any form of the complex. The large error for the reduced control was probably due to proteolysis of the enzyme over a period of several months.

Effect of Truncating the β Subunit on Cold Stability. Trypsin treatment of CF_1 on the membranes or in solution results in modification of the β subunit. On polyacrylamide gels, the trypsinized β runs slightly slower than the unmodified β (Moroney & McCarty, 1982a,b). The N-terminal sequencing of the truncated β resulted in a sequence of FRINPTTSDP. The deduced amino acid sequence for the β subunit of spinach CF_1 is MRINPTTSDP (Zurawski et al., 1982), indicating that the N-terminus of trypsinized β is intact. This finding suggests that the trypsin modification is at the C-terminus of β. Even though this cleavage does not have much effect on the rate of ATP hydrolysis (Moroney & McCarty, 1982a), it does cause destabilization of the protein in the cold (Table 3). The protein with truncated β, $t_{50\%}$ of 19.8 ± 1.4 min, loses its ATPase activity twice as fast as the protein with intact β, $t_{50\%}$ of 38.7 ± 3.2 min. Cleavage of 16 amino acids from the N-terminus of α (M. L. Richter, personal communication) does not seem to destabilize the protein very much. This form of CF_1 is lacking the δ subunit and has truncated β and is, therefore, already extremely unstable. At this time, it is not possible to make CF_1 that only has truncated α.

Effect of δ Removal on Cold Stability. Removal of the δ subunit from CF_1 causes an increase in the rate of inactivation in the cold (Table 4). Fifty percent of the ATPase activity is lost in 15.2 ± 2.1 min for the protein lacking the δ subunit, $\alpha_3\beta_3\gamma$, in contrast with 38.7 ± 3.2 min when the δ is present, $\alpha_3\beta_3\gamma\delta$. Reconstituting the δ deficient enzyme with purified δ restores the structural stability of the complex, $t_{50\%}$ of 37.0 $\pm$ 3.7 min. The same trends are apparent for $CF_1(-\epsilon)$ with truncated β. Because truncating the β subunit destabilizes the complex, the effect of removing the δ is not as severe. The δ subunit was not able to significantly stabilize the $\alpha^t{}_3\beta^t{}_3\gamma$, $t_{50\%}$ of 11.9 min for the reconstituted enzyme versus 9.0 ± 1.8 min for $\alpha^t{}_3\beta^t{}_3\gamma$. SDS−polyacrylamide gel electrophoresis revealed that the $\alpha^t{}_3\beta^t{}_3\gamma$ did not bind as much δ as either the $\alpha_3\beta_3\gamma$ or the $\alpha_3\beta^t{}_3\gamma$.

The purified δ subunit used for the reconstitutions was functionally active. Maximal light dependent proton uptake by CF_1 bound to NaBr-treated thylakoid membranes is only observed when both the δ and ϵ subunits are present (Patrie & McCarty, 1984). The $\alpha_3\beta_3\gamma$ reconstituted with δ used for the cold inactivation experiments showed quenching of ACMA fluorescence after reconstitution with the thylakoid membranes and addition of purified ϵ. Quenching was also

observed when purified δ was added to a mixture of NaBr particles and CF_1 lacking the δ subunit (data not shown).

Influence of Nucleotides on Cold Stability. As previously reported (McCarty & Racker, 1966; Posorske & Jagendorf, 1976), the addition of nucleotides to the incubation medium stabilizes the enzyme in the cold. Incubating $\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$ in 50 mM Tris-HCl (pH 8.0 at 20 °C), 200 mM NaCl, 100 μM ATP led to a $t_{50\%}$ of 24.2 min. In contrast, incubation of $\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$ in the salt solution without ATP resulted in a $t_{50\%}$ value of 3.9 min. The presence of ATP in the medium protected the enzyme from dissociating in the cold. Preloading the $\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$ with Mg^{2+} did not seem to affect the cold lability of the enzyme ($t_{50\%}$ of 7.7 min). It was not possible to study the effect of Mg^{2+}-ATP on the cold lability of CF_1. Loading the noncatalytic sites with Mg^{2+}-ATP inhibits the Ca^{2+}-ATPase activity. The ATPase activity of the protein kept at 25 °C increased as the Mg^{2+}-ATP was released from the enzyme during the incubation.

DISCUSSION

F_1-ATPases are large structures that consist of nine subunits, $\alpha_3\beta_3\gamma\delta\epsilon$. Even though it is possible to isolate complexes composed of just the α and β subunits, either $\alpha\beta$ or $\alpha_3\beta_3$, these complexes have minimal ATPase activity and are not as stable as the native enzyme (Kagawa et al., 1989; Miwa and Yoshida, 1989; Harada et al., 1991; Avital & Gromet-Elhanan, 1991; Gromet-Elhanan & Avital, 1992; Andralojc & Harris, 1992; Gao et al., 1995). The γ subunit, which is required for high ATPase activity and structural stability, is located in the central cavity of the $\alpha_3\beta_3$ hexamer and seems to be associated with one $\alpha\beta$ pair (Gogol et al., 1989; Boekema et al., 1990; Boekema & Böttcher, 1992; Wilkens & Capaldi, 1992; Abrahams et al., 1994). Removal of full-length γ or the tryptic fragments requires harsh conditions that dissociate the complex (Gao et al. 1995; Hightower & McCarty, 1996a), implying that the interaction of the γ subunit with the α and β subunits is very strong. It is logical to assume that the γ subunit plays a major role in stabilizing the enzyme complex.

Modification of the γ subunit by either reduction or proteolysis does not affect the structural integrity of CF_1 during thermal denaturation or cold inactivation. CF_1 in which the γ subunit has been cleaved into three pieces is no more cold labile than the similar form of CF_1 in which γ is intact. In addition, the thermal stability of $\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$ is the same as full-subunit CF_1 when both forms have been treated with EDTA to remove ATP bound to noncvatalytic sites. Even though the γ is cut in $\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$, almost 92% of the subunit is still firmly attached to the hexamer. Reduction and trypsin cleavage mainly affect the extra domain (Hightower & McCarty, 1996a) that is only found in the γ subunit of photosynthetic organisms. This region of the γ subunit is not well conserved among different organisms. There is some evidence that the conserved C-terminus, which is present in all forms of the enzyme tested here, is necessary for the formation of functional complexes (Miki et al., 1986; Iwamoto et al., 1990; Paul et al., 1994). Inter- and intrasubunit contacts must be very important for maintaining the structural integrity of the complex.

Truncation of the C-terminus of β has been observed for spinach CF_1 and CF_1CF_o (Moroney & McCarty, 1982a,b) and *E. coli* F_1 and F_1F_o (Bragg & Hou, 1987; Gavilanes-Ruiz et al., 1988). The small shift in the mobility of the subunit on SDS−polyacrylamide gels suggests that only a few residues are being removed. From the deduced sequence of CF_1 β (Zurawski et al., 1982), it is most likely that 1, 3, or 12 residues are removed since the next closest cleavage site is 43 amino acids in from the C-terminus. Truncation of the C-terminus of β does not significantly increase the ATPase activity of CF_1 (Moroney & McCarty, 1982a) or affect binding of the ϵ subunit (Soteropoulos et al., 1992). In the partial crystal structure of the bovine heart mitochondrial F_1, this region of β is located at the bottom of the $\alpha_3\beta_3$ hexamer. Unfortunately, the C-terminal eight residues of β are not resolved (Abrahams et al., 1994). Why this region of the β subunit plays such an important role in stabilizing the enzyme in the cold it is not known. Interactions of the C-termini of the β subunits with part of the α or γ subunits may help keep the core structure of the enzyme from dissociating in the cold.

The function of the δ subunit is not well understood. In *E. coli*, δ is required to bind F_1 to F_o (Sternweis & Smith, 1977; Dunn & Futai, 1980). In the chloroplast system, the δ subunit is not required for binding but it is required for functional binding to CF_o (Andreo et al., 1982; Patrie & McCarty, 1984; Xiao & McCarty, 1989). Previous studies have suggested that δ interacts with CF_o subunits (Beckers et al., 1992). The δ subunit of *E. coli* is protected from trypsin cleavage when F_1 is bound to F_o (Gavilanes-Ruiz et al., 1988). Other evidence suggests that δ interacts with the N-terminus of α. Both α and δ are protected from proteolysis when the CF_1 is bound to CF_o (Moroney & McCarty, 1982b; Patrie & McCarty, 1984, Wetzel & McCarty, 1993) probably by interaction with at least one CF_o subunit. F_1 with truncated α subunits does not rebind to membranes presumably because the F_1 can not bind the δ subunit (Abrams et al., 1976; Dunn et al., 1980, Andreo et al., 1982; Patrie & McCarty, 1984). It is not known if the α and δ subunits interact directly or if there is a change in the structure of the enzyme upon removal of the residues from the N-terminus of α that prohibits binding of δ.

We have now shown that the δ subunit provides structural integrity to the soluble enzyme complex. Removal of the δ subunit, like truncation of the C-terminus of β, does not affect the ATPase activity of CF_1 (Patrie & McCarty, 1984; Yu & McCarty, 1985). However, the presence of the δ subunit has a dramatic effect on the cold lability of CF_1. The only other evidence for a stabilizing role of the δ subunit is the ability to reconstitute $\alpha_3\beta_3\delta$ complexes for the thermophilic bacterium PS3 (Yoshida et al., 1977; Yokoyama et al., 1989).

It was previously reported that the cold inactivation of CF_1 is accelerated by trypsin treatment (Deters et al., 1975). At the time, it was proposed that, in addition to digestion of δ and ϵ, trypsin also removed the γ subunit. Cleavage of the α and β subunits was only surmised. It is now clear that the increased cold lability of this enzyme, which did contain fragments of the γ subunit and is similar to $\alpha^t_3\beta^t_3\gamma_{(8,15,10)}$, was due to cleavage of the δ subunit and the C-terminus of β.

Occupancy of the nucleotide binding sites provides stability in both the heat and the cold. However, the 1−1.5 mol of ADP that remains tightly bound to the enzyme after EDTA treatment may not be a major stabilizing factor. Preliminary results with nucleotide-depleted $CF_1(-\epsilon)$ have shown that it is almost as stable as $CF_1(-\epsilon)$ in the cold (J. Digel, unpublished results). We have shown that reduction of the γ-disulfide or cleavage of the γ subunit into several large

fragments does not destabilize the core complex. Seemingly benign modifications like truncating the extreme C-terminus of the β subunit or removal of the δ subunit greatly destabilize the enzyme in the cold. The main stabilizing forces in CF_1 are probably interactions among the subunits. Interaction of the γ subunit with α/β may provide a basal level of structural integrity to the complex. Modification of the structure of the γ subunit without major loss of intersubunit contacts would, as we have shown, not greatly affect the stability. The β and δ subunits, as well as nucleotides, provide additional stabilization to the CF_1.

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