Spinach Chloroplast Coupling Factor CF_1-$\alpha_3\beta_3$ Core Complex: Structure, Stability, and Catalytic Properties†

Maxim Sokolov and Zippora Gromet-Elhanan*

Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

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ABSTRACT: A minimal chloroplast coupling factor CF_1 core complex, containing only α and β subunits, has been isolated from spinach thylakoids [Avital, S., & Gromet-Elhanan, Z. (1991) *J. Biol. Chem. 266,* 7067−7072]. This $CF_1(\alpha\beta)$ exhibited a low MgATPase activity, which was stimulated but not inhibited by low concentrations of the species-specific CF_1 effector tentoxin. As is reported here, the structure of $CF_1(\alpha\beta)$ could not be determined due to its instability. However, its pretreatment with high tentoxin concentrations resulted in a remarkable 50-fold stimulation of the MgATPase activity as well as stabilization of its hexameric structure, thus enabling the isolation of a more active CF_1-$\alpha_3\beta_3$ complex by size-exclusion chromatography. A detailed characterization of the MgATPase activity of this tentoxin-stabilized CF_1-$\alpha_3\beta_3$ hexamer, as compared to the activity of a CF_1 complex lacking the ϵ subunit, revealed similar apparent K_m values and a similar stimulation by the presence of 100 μM tentoxin in the assay medium, but drastic differences in all other tested assays. Most pronounced were their different temperature profiles and different responses to all added inhibitors and stimulators of the CF_1 MgATPase activity and to excess free Mg^{2+} ions. The specific properties of the stable CF_1-$\alpha_3\beta_3$ hexamer are identical to those earlier reported for its parent-unstable $CF_1(\alpha\beta)$. These results indicate that, although the CF_1 γ subunit is not required for the low $CF_1(\alpha\beta)$ ATPase activity nor for the higher activity of the tentoxin-stabilized CF_1-$\alpha_3\beta_3$, it plays a central role in obtaining the typical functional properties of the CF_1-ATPase. Kinetic cooperativity could not be critically tested as yet with any F_1-$\alpha_3\beta_3$. However, tentoxin, as azide, has been shown to inhibit multisite but not unisite catalysis. Therefore, the observation that CF_1-$\alpha_3\beta_3$ is only stimulated by tentoxin suggests that the required presence of CF_1-γ for obtaining *inhibition* by tentoxin reflects the role of this subunit in cooperative interactions between the catalytic sites.

The water-soluble CF_1-ATPase[1] is the catalytic sector of the chloroplast CF_oF_1 ATP synthase−ATPase complex. CF_1, like all other F_1-ATPases, is composed of five kinds of subunits designated α−ϵ and has a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (McCarty & Moroney, 1985; Nalin & Nelson, 1987; Jagendorf *et al*. 1991). The CF_1-ATPase activity does not require the presence of the δ and ϵ subunits, since their complete removal (Patrie & McCarty, 1984) was found to leave a fully active $CF_1(-\delta-\epsilon)$-ATPase (Mitra & Hammes, 1988). This CF_1-$\alpha_3\beta_3\gamma$ has also been found to retain the sensitivity of the whole CF_1 to its specific inhibitor, tentoxin (Hu *et al*., 1993). Fully active F_1-$\alpha_3\beta_3\gamma$ complexes were even assembled from 3:3:1 mixtures of isolated α, β, and γ subunits of the TF_1-ATPase from the thermophilic bacterium PS3 (Yoshida *et al*., 1977) and of the EcF_1-ATPase from *Escherichia coli* (Futai, 1977; Dunn & Futai, 1980). A similar assembly of such an active CF_1 complex has recently been reported (Chen & Jagendorf, 1994). In this case, a 3:3:1 mixture of overexpressed urea-denatured CF_1 α, β, and γ subunits was used, and their assembly required the presence of several chloroplast molecular chaperones. The exact structure of this $CF_1(\alpha\beta\gamma)$ complex has not been determined.

A smaller catalytic $CF_1(\alpha\beta)$ complex, composed only of equimolar ratios of α and β subunits, has been isolated from a membrane-bound CF_oF_1 by a procedure that involves extraction of coupled spinach thylakoids with 2 M LiCl in the presence of MgATP (Avital & Gromet-Ethanan, 1990), and fractionation of the resulting extract by ion-exchange HPLC (Avital & Gromet-Elhanan, 1991). This minimal CF_1-$(\alpha\beta)$ core complex showed, however, a much lower MgATPase activity than the activated CF_1-ATPase and was not inhibited by the specific CF_1 inhibitor tentoxin nor by the general F_1 inhibitor azide (Gromet-Elhanan & Avital, 1992; Sokolov *et al*., 1992).

Active F_1-ATPase complexes containing only F_1 α and β subunits have also been assembled from a 1:1 mixture of overexpressed α and β subunits of the TF_1-ATPase (Kagawa *et al*., 1989; Miwa & Yoshida, 1989). Assembly in the absence of nucleotides and Mg^{2+} ions led to formation of a TF_1-$\alpha_3\beta_3$ hexamer that dissociated into a TF_1-$\alpha_1\beta_1$ dimer upon addition of MgAT(D)P (Ohta *et al*., 1990; Harada *et al*., 1991). A much lower MgATPase activity than that of the whole parent F_1 complex, and insensitivity to inhibition by azide, was also reported with these TF_1 core complexes (Kagawa *et al*., 1989; Miwa & Yoshida, 1989), as well as with an RrF_1-$\alpha_1\beta_1$ complex isolated from a LiCl extract of *Rhodospirillum rubrum* chromatophores (Andralojc & Harris,

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* Author to whom correspondence should be addressed.

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[1] Abbreviations: CF_1, RrF_1, and TF_1, soluble F_1-ATPase from chloroplasts, *R. rubrum*, and the thermophilic bacterium PS3, respectively; $CF_1(\alpha\beta)$, complex containing equal amounts of α and β subunits of CF_1; $CF_1(-\epsilon)$, CF_1 lacking the ϵ subunit; Rubisco, ribulose-bisphosphate carboxylase/oxygenase; HPLC, high-performance liquid chromatography; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; Nbf-Cl, 7-chloro-4-nitrobenzofurazan.

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1992). A chaperone-assisted assembly of urea-denatured overexpressed CF_1 α and β subunits has recently been suggested (Chen & Jagendorf, 1994), but no information concerning the structure and/or function of such an assembled $CF_1(\alpha\beta)$ complex was presented.

The reported isolation and/or assembly of active $F_1(\alpha\beta)$ complexes from various sources suggests that even the F_1-γ subunit is not required for assembly of these core complexes nor for their activity. But their very low rates of catalysis, and resistance to inhibition by azide, indicate that the presence of the F_1-γ subunit leads not only to a change in the symmetric structure of the $\alpha_3\beta_3$ hexamer but also to changes in its catalytic properties. A stable F_1-$\alpha_3\beta_3$ complex, that retains its structure under assay conditions, would provide an excellent tool for identifying all typical functional properties of F_1-ATPases which depend on the presence of their γ subunits.

We describe here the isolation of a stable CF_1-$\alpha_3\beta_3$ hexamer from the $CF(\alpha\beta)$ complex released by LiCl extraction of spinach chloroplasts. A detailed characterization of its catalytic properties reveals that they are very different from those of a $CF_1(-\epsilon)$ complex released by the same LiCl extraction procedure.

MATERIALS AND METHODS

Preparations. The $CF_1(\alpha\beta)$ and $CF_1(-\epsilon)$ complexes were isolated from a 2 M LiCl extract of thoroughly washed spinach thylakoids as described by Avital and Gromet-Elhanan (1990, 1991) and Sokolov *et al*. (1992), except that a cocktail of the following protease inhibitors was added to all stages of isolation and ion-exchange HPLC: 1.5 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, and 3 nM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone. Small amounts of Rubisco, which remain in these thylakoids even after five rounds of washings, accompany rather specifically the CF_1-$(\alpha\beta)$ complex (Sokolov *et al*., 1992). This contaminating Rubisco was removed by affinity chromatography on an anti-Rubisco column as described by Soteropoulos *et al*. (1992), except that the column was preequilibrated at 4 °C in TMANG buffer containing 50 mM Tricine−NaOH, pH 8.0, 4 mM $MgCl_2$, 4 mM ATP, 180 mM NaCl, and 10% glycerol. The $CF_1(\alpha\beta)$ complex was loaded and eluted in the same buffer and finally concentrated with a Centricon 10 (Amicon) at 4 °C.

For large-scale isolation of a stable CF_1-$\alpha_3\beta_3$ hexamer, the purified $CF_1(\alpha\beta)$ complex, at 6 mg/mL, was incubated with 0.2 mM tentoxin in TMANG buffer for 5 min at 35 °C, loaded on a size-exclusion HPLC column (Superdex 200 HR 10/30, Pharmacia), and eluted at room temperature with the same buffer at a flow rate of 0.5 mL/min. All eluted fractions were kept on ice, and their protein concentration was monitored by Bradford (1976). The $\alpha_3\beta_3$ complex, eluting at 9.4 mL (see Figure 2), was concentrated to about 4 mg/mL with a Centricon 10 (Amicon) at 4 °C and stored in liquid nitrogen.

Assays. Mg^{2+}-dependent ATP hydrolysis was measured in 0.5 mL of 50 mM Tricine−NaOH, pH 8.0, and MgATP containing [γ-^{32}P]ATP. The time, temperature, and MgATP concentrations are described in the text. The reaction was started by the addition of 5−50 μg of protein and stopped by 0.5 mL of 0.5 M trichloroacetic acid. The released [^{32}P]-P_i was estimated as described by Shahak (1982). One unit of activity is defined as the amount of protein that will hydrolyze 1 μmol of ATP per minute. The substrate−velocity data were analyzed as a simple Michaelis−Menten type reaction without cooperativity. The assumption of the simple Michaelis−Menten type is valid with the MgATP concentrations employed in the present study. The apparent kinetic parameters were determined by a linear regression method for Lineweaver−Burk plots.

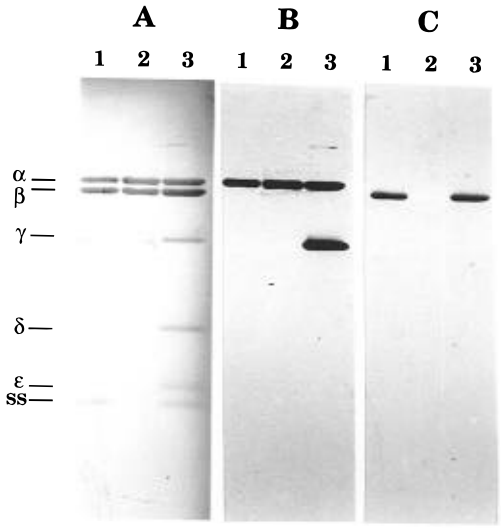

FIGURE 1: SDS−PAGE profile (A) and Western immunoblotting (B and C) of the $CF_1(\alpha\beta)$ complex purified by affinity chromatography. 4 μg of protein was applied to each lane, separated on a 13.5% SDS−PAGE, and transfered to nitrocellulose. Part A was stained with Amido Black. Part B was probed with a mixture of antisera produced against the CF_1 α and γ subunits, and part C was probed with an antiserum against the Rubisco large subunit. Lanes 1 and 2, $CF_1(\alpha\beta)$ before and after passage through an anti-Rubisco column; lane 3, CF_1 containing traces of Rubisco.

Protein concentration was determined by a modification of Lowry method (Lowry *et al*., 1951), or with the Pierce BCA protein assay, using bovine serum albumin as the standard. SDS−PAGE on 13.5% polyacrylamide gels was run according to the Laemmli procedure (Laemmli, 1970), but with a high concentration of Tris (Fling & Gregerson, 1986), and stained by Coomassie brilliant blue R250. Western immunoblot analysis was carried out as described by Gershoni (1988) in triplicate. One strip of the nitrocellulose paper was stained with Amido Black (Kuno & Kihara, 1967). The second and third strips were probed with rabbit polyclonal antibodies produced against the CF_1 α and γ subunits and the Rubisco large subunit. Antigen−antibody complexes were visualized by the Amersham ECL detection system.

Materials. [γ-^{32}P]ATP was purchased from Amersham. Octyl glucoside, Nbf-Cl, tentoxin, and the protease inhibitors were obtained from Sigma. Efrapeptin was a gift of Dr. R. L. Hamill of Eli Lilly Co. The anti-Rubisco affinity column was a gift of Drs. P. Soteropoulus and R. E. McCarty, Department of Biology, The Johns Hopkins University.

RESULTS

Structural Studies. All preparations of $CF_1(\alpha\beta)$, obtained from 2 M LiCl extracts of thoroughly washed thylokoids by ion-exchange HPLC, were found to have two trace contaminants. One ran on SDS−PAGE slightly ahead of the CF_1 γ subunit; the other ran below the CF_1 ϵ subunit (compare Figure 1A, lanes 1 and 3). A Western immunoblot, probed with a mixture of antibodies raised against CF_1 α and γ

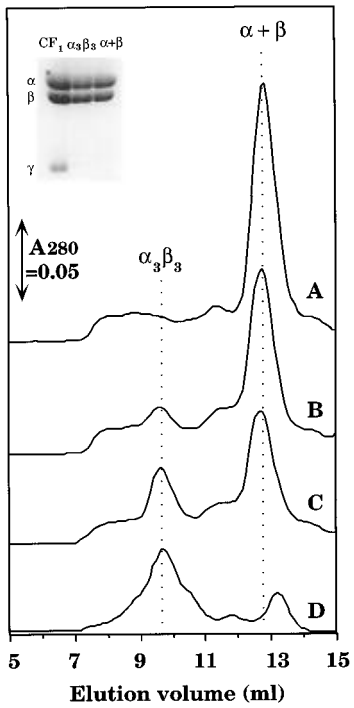

FIGURE 2: Size-exclusion HPLC elution profiles of purified CF_1-($\alpha\beta$) before and after preincubation with tentoxin. 0.1 mL of CF_1-($\alpha\beta$) at 6 mg/mL in TMANG buffer was applied at room temperature on a Superdex 200 HR 10/30 column, equilibrated, and eluted at a flow rate of 0.5 mL/min with 50 mM Tricine−NaOH, pH 8.0, containing 4 mM $MgCl_2$, 180 mM NaCl, and 10% glycerol. The position of the $\alpha_3\beta_3$ complex and the mixture of dissociated $\alpha+\beta$ was determined by calibration with protein markers. (A) Untreated $CF_1(\alpha\beta)$; (B) $CF_1(\alpha\beta)$ preincubated with 0.2 mM tentoxin for 10 min on ice; (C) some as (B), but the preincubation was for 5 min at 35 °C; (D) the $\alpha_3\beta_3$ peak pooled from three runs of curve C was concentrated and rechromatographed. Inset: SDS−PAGE profile of the peak fractions of curve C together with a sample of CF_1 showing the positions of the α, β, and γ subunits.

subunits, established the complete absence of CF_1-γ from the $CF_1(\alpha\beta)$ complex (Figure 1B, lanes 1 and 3). It also indicated that small amounts of Rubisco contaminate the CF_1-($\alpha\beta$) complex as well as the whole CF_1 used here as a protein marker, since both of them reacted with an antibody against the Rubisco large subunit (Figure 1C, lanes 1 and 3). This subunit is not seen on the stained strip (Figure 1A), because it runs on SDS−PAGE together with the CF_1 β subunit (McCarty & Moroney, 1985). But the band that runs below the CF_1 ϵ subunit in both $CF_1(\alpha\beta)$ and the whole CF_1 is the small subunit (SS) of Rubisco (Figure 1A, lanes 1 and 3). Since even these small amounts of Rubisco might interfere with structural examinations of the $CF_1(\alpha\beta)$ complex, they were removed by affinity chromatography on an anti-Rubisco column (compare Figure 1A and C, lanes 1 and 2). Removal of Rubisco did not change the specific properties of the CF_1-($\alpha\beta$) ATPase activity reported by Gromet-Elhanan and Avital (1992) and Sokolov *et al*. (1992).

All our attempts to define the structure of this purified $CF_1(\alpha\beta)$ by size-exclusion HPLC were unsuccessful, because it was found to dissociate into a mixture of the individual α and β subunits (Figure 2A), that exhibit no ATPase activity (Gromet-Elhanan, 1992). Full dissociation of the $CF_1(\alpha\beta)$ complex, unlike the $TF_1(\alpha\beta)$ complexes (Kagawa *et al*., 1989; Miwa & Yoshida, 1989; Ohta *et al*., 1990; Harada *et al*., 1991), was obtained both in the absence (Figure 2A) and in the presence of ATP (not shown). Also, all other

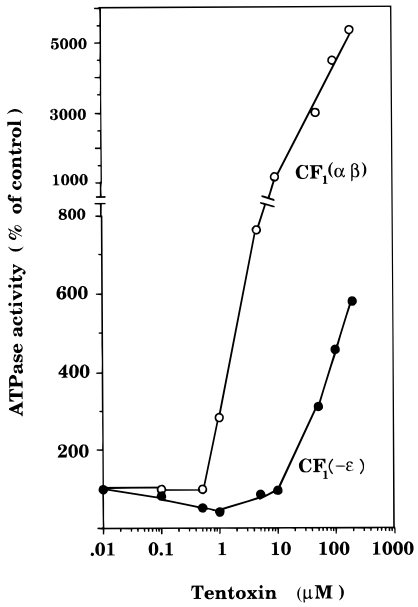

FIGURE 3: Effect of tentoxin on the MgATPase activity of purified $CF_1(\alpha\beta)$ and $CF_1(-\epsilon)$ complexes. The ATPase activity of CF_1-($\alpha\beta$) (○) and $CF_1(-\epsilon)$ (●) was assayed as described under Materials and Methods, for 5 min at 35 °C with 1 mM $MgCl_2$, 1 mM ATP, and the indicated concentrations of tentoxin.

conditions reported to decrease the dissociation of the TF_1-($\alpha\beta$) complexes and facilitate the isolation of a TF_1-$\alpha_3\beta_3$ hexamer (Kagawa *et al*., 1989; Miwa & Yoshida, 1989) were completely ineffective with $CF_1(\alpha\beta)$. They include an increase in the concentration of the applied $CF_1(\alpha\beta)$ complex from 1 to 8 mg/mL, and/or a 5-fold increase in the total amount of the loaded material as well as exchanging the NaCl present in the elution buffer with Na_2SO_4. These results indicate that $CF_1(\alpha\beta)$ is much more labile than $TF_1(\alpha\beta)$.

A long search for a stabilizing agent or condition yielded one that was found to stabilize the structure while dramatically increasing the ATPase activity of the $CF_1(\alpha\beta)$ complex. This double effect was obtained in the presence of tentoxin, which is a very specific effector of various CF_1-ATPases (Steele *et al*., 1976, 1978a). CF_1 from sensitive plant species, such as lettuce or spinach, is inhibited by 0.1−10 μM tentoxin. But, when present at higher concentrations, tentoxin has been found to stimulate both the trypsin-activated CaATPase (Steele *et al*., 1978a) and the octyl glucoside-activated MgATPase (Pick *et al*., 1982) of all sensitive CF_1 complexes.

Earlier tests carried out with the Rubisco-contaminated $CF_1(\alpha\beta)$ have revealed that, although its MgATPase activity was resistant to inhibition by tentoxin, it did bind tentoxin rather tightly, and was even somewhat stimulated by tentoxin at concentrations that inhibit the parent CF_1-ATPase (Gromet-Elhanan & Avital, 1992). The effect of tentoxin on the MgATPase activity of the purified $CF_1(\alpha\beta)$ as compared to a $CF_1(-\epsilon)$ was therefore tested at a wide range of concentrations, including those known to stimulate the CF_1-ATPase activity (Figure 3). The $CF_1(-\epsilon)$ utilized for this study was released in trace amounts from spinach membrane-bound CF_oF_1 by the same 2 M LiCl extraction that released the $CF_1(\alpha\beta)$ complex (Avital & Gromet-Elhanan, 1990, 1991; Sokolov *et al*., 1992). Unlike the whole CF_1, this $CF_1(-\epsilon)$ exhibits some MgATPase activity even in the absence of added activators, such as octyl glucoside or sulfite, and can therefore be compared with the $CF_1(\alpha\beta)$ MgATPase activity under identical assay conditions. Figure 3 demonstrates that

Table 1: Effect of Various Inhibitors and Activators on the MgATPase Activity of $CF_1(-\epsilon)$ and the Tentoxin-Stabilized CF_1-$\alpha_3\beta_3$[a]

	MgATPase activity [μmol min^{-1} (mg of protein)$^{-1}$] assayed			
	without tentoxin		with tentoxin	
added effectors	CF_1-$\alpha_3\beta_3$	$CF_1(-\epsilon)$	CF_1-$\alpha_3\beta_3$	$CF_1(-\epsilon)$
none	2.8 (100)	1.6 (100)	6.4 (100)	4.7 (100)
octyl glucoside	1.9 (68)	11.9 (744)	2.0 (31)	7.8 (166)
sulfite	2.5 (89)	11.6 (725)	3.3 (52)	11.0 (234)
azide	2.3 (82)	0.0 (0)	5.0 (78)	0.5 (11)
efrapeptin	2.8 (100)	0.1 (6)	6.1 (95)	0.5 (11)
Nbf-Cl	2.8 (100)	0.1 (6)	ND[b]	ND

[a] MgATPase activity was assayed as described under Materials and Methods, for 1.5 min at 35 °C with 0.5 mM $MgCl_2$ and 1 mM ATP. The reaction was started by addition of 7 μg of the protein into the assay medium. When stated, 0.1 mM tentoxin, 1 mM sodium azide, 10 μM efrapeptin, 40 mM octyl glucoside, and 25 mM sodium sulfite were added into the assay medium. For testing the effect of Nbf-Cl, both complexes were incubated, at 3.5 mg of protein/mL, with 0.5 mM Nbf-Cl for 3 h on ice before dilution into the assay medium. The numbers in parentheses are the percent of control. [b] ND, not determined.

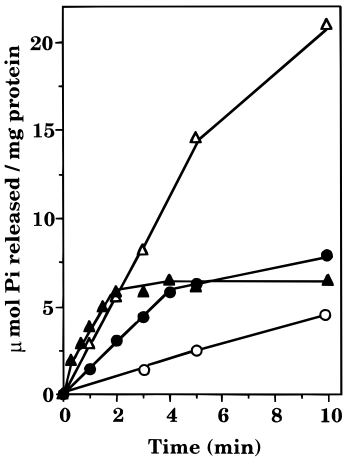

FIGURE 4: Time and temperature dependence of ATP hydrolysis by the tentoxin-stabilized CF_1-$\alpha_3\beta_3$ and the $CF_1(-\epsilon)$ complex. The ATPase activity was assayed as described under Materials and Methods, with 0.5 mM $MgCl_2$ and 1 mM ATP at 24 °C (open symbols) or 35 °C (closed symbols). (△, ▲) CF_1-$\alpha_3\beta_3$; (○, ●) $CF_1(-\epsilon)$.

the purified $CF_1(\alpha\beta)$ ATPase, that is not inhibited at all by tentoxin, is already stimulated by 3-fold at 1 μM tentoxin, and up to 50-fold at 200 μM tentoxin. On the other hand, $CF_1(-\epsilon)$, which is inhibited by 0.1−1 μM tentoxin, and stimulated back to its original low activity by 10 μM tentoxin, shows only a 6-fold further stimulation by 200 μM tentoxin.

The unusually high degree of stimulation of the $CF_1(\alpha\beta)$ MgATPase activity in the presence of 200 μM tentoxin was found to be accompanied by a dramatic stabilization of the $CF_1(\alpha\beta)$ complex, that enabled the isolation of a CF_1-$\alpha_3\beta_3$ hexamer. As is illustrated in Figure 2B, pretreatment of a concentrated sample of $CF_1(\alpha\beta)$ with 200 μM tentoxin for 10 min on ice, before application on the size-exclusion HPLC column, led already to the appearance of a small amount of a CF_1-$\alpha_3\beta_3$ hexamer. The ratio of $\alpha_3\beta_3/(\alpha+\beta)$ increased to about 0.7 when the pretreatment was carried out for 5 min at 35 °C (Figure 2C). Longer periods of pretreatment did not increase this ratio. But rechromatography of a pool of the tentoxin-stabilized CF_1-$\alpha_3\beta_3$, to which ATP was added at the 4 mM concentration present in the application buffer, resulted in an $\alpha_3\beta_3/(\alpha+\beta)$ ratio of >4.0 (Figure 2D). This ratio, which reflects the stability of the $\alpha_3\beta_3$ hexamer, was further increased when ATP was present together with $MgCl_2$ not only in the application buffer but also throughout the size-exclusion HPLC run. Large-scale preparations of stable CF_1-$\alpha_3\beta_3$ were therefore carried out in the presence of 4 mM $MgCl_2$ and 4 mM ATP, as described under Materials and Methods.

An SDS−PAGE profile of the isolated CF_1-$\alpha_3\beta_3$ revealed a completely pure complex, containing only the α and β subunits (see inset to Figure 2). The contaminant band running near the CF_1 γ subunit, which appeared even in the Rubisco-less $CF_1(\alpha\beta)$ preparation (Figure 1A, lane 2), was removed from the $\alpha_3\beta_3$ hexamer during the size-exclusion chromatography. The parent $CF_1(\alpha\beta)$ preparation is probably composed mainly of this $\alpha_3\beta_3$ hexamer, since trace amounts of a putative $\alpha_1\beta_1$ dimer, which appear during its dissociation in the absence of tentoxin (Figure 2A), do not increase by pretreatment with tentoxin (see Figure 2B,C) nor after rechromatography of the $\alpha_3\beta_3$ hexamer (Figure 2D).

Catalytic Properties of the Tentoxin-Stabilized CF_1-$\alpha_3\beta_3$ Core Complex. This isolated pure CF_1-$\alpha_3\beta_3$ had a relatively high MgATPase activity (Table 1). Since the $CF_1(\alpha\beta)$ from which it was isolated after pretreatment with 200 μM tentoxin has earlier been shown to bind tentoxin quite tightly (Gromet-Elhanan & Avital, 1992), it is possible that this bound tentoxin is retained also on the pure $\alpha_3\beta_3$ hexamer obtained by size-exclusion chromatography. We have therefore examined whether addition of tentoxin to the ATPase assay medium could lead to any further stimulation of this CF_1-$\alpha_3\beta_3$ activity. As is illustrated in Table 1, the presence of 100 μM tentoxin during the ATPase assay resulted in a 2−3-fold stimulation of the CF_1-$\alpha_3\beta_3$ MgATPase activity. A similar stimulation was observed with $CF_1(-\epsilon)$, which did not undergo any preactivation and showed therefore an even lower MgATPase activity than CF_1-$\alpha_3\beta_3$. The response of these two complexes to various other effectors of the CF_1-ATPase activity was, however, very different. Thus, both octyl glucoside and sulfite enhanced the $CF_1(-\epsilon)$ MgATPase activity by about 7-fold in the absence of added tentoxin, and by 2−3-fold in the presence of 100 μM tentoxin, whereas the CF_1-$\alpha_3\beta_3$ activity was either not affected or even somewhat inhibited. The $\alpha_3\beta_3$ hexamer was also fully resistant to azide, as well as to other tested CF_1 inhibitors, such as efrapeptin and Nbf-Cl, all of which block completely the $CF_1(-\epsilon)$ ATPase activity (Table 1).

Azide was earlier found to inhibit the CF_1-ATPase by increasing the binding of inhibitory MgADP (Murataliev *et al.*, 1991). Zhang and Jagendorf (1995) have shown that azide inhibits even unisite ATP hydrolysis by spinach thylakoid-bound CF_1 as long as this CF_1 contains some bound ADP. It seemed therefore possible that in the presence of ADP azide will inhibit also the MgATPase activity of CF_1-$\alpha_3\beta_3$. However, even after a long pretreatment with millimolar concentrations of MgADP, the CF_1-$\alpha_3\beta_3$ complex retained its relatively high rate of MgATPase activity and resistance to inhibition by azide. The response of CF_1-$\alpha_3\beta_3$ to azide as well as various other CF_1-ATPase inhibitors is thus very different from the recorded responses of both activated membrane-bound and soluble CF_1-ATPases.

The MgATPase activity of CF_1-$\alpha_3\beta_3$ differs from that of $CF_1(-\epsilon)$ in many additional aspects. The $\alpha_3\beta_3$ hexamer exhibited an identical activity at 24 and 35 °C, whereas the $CF_1(-\epsilon)$ ATPase was much faster at 35 °C than at 24 °C (Figure 4). However, in both complexes the MgATPase activity decreased quite rapidly at 35 °C, whereas at 24 °C

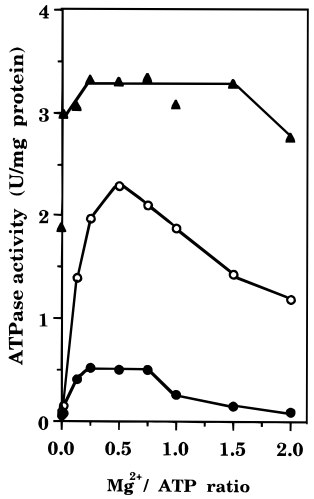

FIGURE 5: Mg^{2+} requirement of ATP hydrolysis by the tentoxin-stabilized CF_1-$\alpha_3\beta_3$ and the $CF_1(-\epsilon)$ complex. The ATPase activity of the $\alpha_3\beta_3$ hexamer was assayed for 2 min, and of the $CF_1(-\epsilon)$ for 15 min, at 24 °C, with 1 mM ATP and $MgCl_2$ to give the indicated Mg^{2+}/ATP ratios. The reaction was started by the addition of 2 μL of each protein complex at 3.6 mg/mL in TMANG buffer, thus introducing 16 μM $MgCl_2$ into the reaction mixture. The assays at zero Mg^{2+}/ATP, which contained 16 μM $MgCl_2$ and 1 mM ATP, were therefore conducted in the presence of 55 μM EDTA. (▲) CF_1-$\alpha_3\beta_3$; (●) $CF_1(-\epsilon)$; (○) $CF_1(-\epsilon)$ assayed in the prescence of 0.1 mM tentoxin.

it remained linear for much longer periods. All further assays were therefore carried out at 24 °C.

The Mg^{2+} ion requirement for ATP hydrolysis by both complexes is also very different (Figure 5). When assayed under linear conditions at 24 °C, the $CF_1(-\epsilon)$ ATPase activity, as that of the whole activated CF_1 (Hochman *et al*., 1976), was fully dependent on the presence of Mg^{2+} ions, but became progressively inhibited by excess free Mg^{2+}. Maximal ATPase activity was obtained at Mg^{2+}/ATP ratios of 0.3−0.7, whereas at ratios of 1 and 2, 50−85% inhibition was recorded. High concentrations of tentoxin in the assay medium, which stimulate the $CF_1(-\epsilon)$ ATPase activity by 4−5-fold, were found to decrease the inhibition by free Mg^{2+} ions, so that at a Mg^{2+}/ATP ratio of 1 only 10% inhibition was observed and even at a ratio of 2 over 60% of the CF_1-$(-\epsilon)$ ATPase activity was still retained (Figure 5). A very similar overall stimulation of the MgATPase activity coupled with a drastic decrease in its inhibition by free Mg^{2+} ions has been recorded in lettuce CF_1 in the presence of octyl glucoside (Pick & Bassilian, 1982).

At 24 °C, the CF_1-$\alpha_3\beta_3$ ATPase activity in the absence of any added tentoxin was even higher than the tentoxin-stimulated $CF_1(-\epsilon)$ ATPase activity (Figure 5). Furthermore, the activity of this $\alpha_3\beta_3$ hexamer was not affected by wide changes in the Mg^{2+}/ATP ratio. This $\alpha_3\beta_3$ hexamer was isolated in a pure, stable form in the presence of 4 mM $MgCl_2$ and 4 mM ATP. So even without any exogenously added $MgCl_2$, the CF_1-$\alpha_3\beta_3$ ATPase assay medium contained 16 μM $MgCl_2$, which was introduced together with the protein. Under these conditions, an ATPase activity of 2.9 units/mg of protein was obtained, and no further significant change in activity was observed with up to 2 mM added $MgCl_2$, which gives a final Mg^{2+}/ATP ratio of 2. In order to reduce the Mg^{2+}/ATP ratio down to zero, 55 μM EDTA was added into the assay mixture, and an ATPase activity of 1.9 units/mg of protein was detected (Figure 5). This very high ATPase activity suggests that some Mg^{2+} ions remain tightly

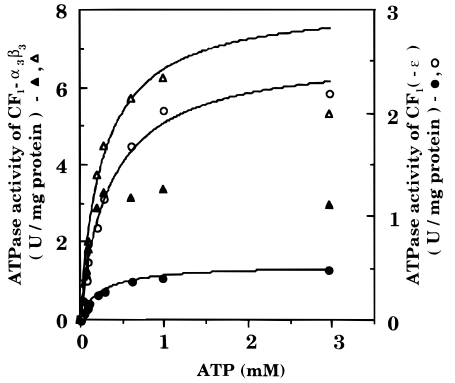

FIGURE 6: Dependence of the MgATPase activity of the tentoxin-stabilized CF_1-$\alpha_3\beta_3$ and the $CF_1(-\epsilon)$ complex on substrate concentration. The rates of ATP hydrolysis were measured as described under Materials and Methods, at 24 °C, with a Mg^{2+}/ATP ratio of 0.5 and in the absence (closed symbols) or presence (open symbols) of 0.1 mM tentoxin in the assay medium. The activity of CF_1-$\alpha_3\beta_3$ was assayed for 2 min and of $CF_1(-\epsilon)$ for 10 min. The reaction was started by the addition of 3 μL of each protein complex at 3.6 mg/mL in TMANG buffer, thus introducing 25 μM MgATP into the reaction mixture. The points are experimental, and the lines are the calculated best-fit curves using the parameters shown in Table 2. (▲, △) CF_1-$\alpha_3\beta_3$; (●, ○) $CF_1(-\epsilon)$.

Table 2: ATP Hydrolysis by the Tentoxin-Stabilized CF-$\alpha_3\beta_3$ and the $CF_1(-\epsilon)$ Complex[a]

complex	K_m (mM)	V_{max} (units/mg of protein)
$CF_1(-\epsilon)$	0.25 ± 0.01	0.52 ± 0.04
$CF_1(-\epsilon)$ + tentoxin	0.36 ± 0.04	2.58 ± 0.10
CF_1-$\alpha_3\beta_3$	0.30 ± 0.05	6.85 ± 0.62
CF_1-$\alpha_3\beta_3$ + tentoxin	0.27 ± 0.03	8.20 ± 0.42

[a] The data were analyzed as a simple Michaelis−Menten type reaction without cooperativity. The details of the analyses are given under Materials and Methods. ATPase activity was assayed at 24 °C. K_m and V_{max} were calculated with CF_1-$\alpha_3\beta_3$ for [ATP] ≤ 0.3 mM and with CF_1-$\alpha_3\beta_3$ + tentoxin for [ATP] ≤ 1.0 mM.

bound to the $\alpha_3\beta_3$ hexamer and are not removed even by adding a 3-fold excess of EDTA. Characterization of the divalent cation specificity of this $\alpha_3\beta_3$ complex must therefore await the establishment of conditions for the complete removal of all bound Mg^{2+} from this complex without causing its dissociation into individual α and β subunits.

Figure 6 illustrates the ATPase activity of CF_1-$\alpha_3\beta_3$ and $CF_1(-\epsilon)$ complexes measured as a function of the ATP concentration. The data for $CF_1(-\epsilon)$ represent, both in the absence and in the presence of 100 μM tentoxin in the assay medium, a single Michaelis−Menten type reaction with no cooperativity, when assayed here at ATP concentrations from 0.025 to 3.0 mM. With CF_1-$\alpha_3\beta_3$ in the presence of added tentoxin, a similar profile was observed only up to 1 mM ATP, as the rate of hydrolysis obtained at 3 mM was lower than at 1 mM ATP (Figure 6). With CF_1-$\alpha_3\beta_3$ in the absence of added tentoxin, but in the presence of its tightly bound tentoxin, the deviation from Michaelis−Menten kinetics was observed already above 0.3 mM ATP, because there was no further increase in the rate of ATP hydrolysis at the higher ATP concentrations. The kinetic parameters listed in Table 2 indicate that the apparent K_m values of $CF_1(-\epsilon)$ and CF_1-$\alpha_3\beta_3$ are rather similar and not affected by the presence of tentoxin in the assay medium, whereas the stimulatory effect of tentoxin on the MgATPase activity of $CF_1(-\epsilon)$ is mainly due to a 5-fold increase in the apparent V_{max} value. The earlier observed stimulation of the CF_1-CaATPase by high

tentoxin concentrations (Steele *et al*., 1978a) has, on the other hand, been found to involve both a decrease in K_m from 1 to 0.3 mM and a 2.7-fold increase in V_{max}. The much higher apparent V_{max} value obtained, even in the absence of added tentoxin, with CF_1-$\alpha_3\beta_3$, as compared to $CF_1(-\epsilon)$, is due to its higher activity at 24 °C (see Figure 4) as well as to the presence of tightly bound tentoxin in the $\alpha_3\beta_3$ hexamer and the absence of the down-regulation of the rate of ATP hydrolysis by the disulfide bond of the CF_1 γ subunit (McCarty & Moroney, 1985; Nalin & Nelson, 1987; Jagendorf *et al*., 1991).

DISCUSSION

The structure of the catalytic $CF_1(\alpha\beta)$ core complex earlier isolated from spinach chloroplasts (Avital & Gromet-Elhanan, 1991; Gromet-Elhanan & Avital, 1992; Sokolov *et al*., 1992) has not been elucidated because of its high lability. This study has shown that high concentrations of tentoxin, which lead to a 50-fold stimulation of the CF_1-$(\alpha\beta)$ MgATPase activity, cause also a remarkable stabilization of its $\alpha_3\beta_3$ hexameric structure (Figures 2 and 3). This stabilization must depend on the presence of bound tentoxin since the isolated $\alpha_3\beta_3$ hexamer remains stable and exhibits a high MgATPase activity after removal of all the free tentoxin during size-exclusion chromatography.

The MgATPase activity of the tentoxin-stabilized CF_1-$\alpha_3\beta_3$ hexamer exhibits very similar properties to those reported for its labile parent $CF_1(\alpha\beta)$ complex (Gromet-Elhanan & Avital, 1992), which has been isolated and purified in the complete absence of tentoxin (Avital & Gromet-Elhanan, 1991; Sokolov *et al*., 1992). The activities of both complexes are not affected by the presence of octyl glucoside, sulfite, azide, or excess free Mg^{2+} ions in their ATPase assay medium. These properties are very different from those recorded for the ATPase activities of larger CF_1 complexes, including an isolated native CF_1-$\alpha_3\beta_3\gamma$ (Hu *et al*., 1993), the recently assembled $CF_1(\alpha\beta\gamma)$ complex (Chen & Jagendorf, 1994), and also the $CF_1(-\epsilon)$ complex (see Table 1 and Figure 5). The γ-containing CF_1 complexes are markedly stimulated by methanol and/or octyl glucoside as well as sulfite, and inhibited by azide, excess free Mg^{2+} ions, and low concentrations of tentoxin. It has also been reported that the removal of the δ- and ϵ-polypeptides from CF_1 does not cause significant changes in the structure, kinetics, and nucleotide binding sites of the enzyme (Mitra & Hammes, 1988). Our observations that further removal of the γ-polypeptide leads to destabilization of the hexameric structure of the remaining $CF_1(\alpha\beta)$ complex, and to drastic changes in its catalytic properties, indicate that the CF_1 γ subunit is most important for maintaining the structural and functional properties of the CF_1-ATPase.

A summary of earlier as well as our present investigations with tentoxin suggests that the CF_1 γ subunit exerts its effect via the β subunit. Thus, Steele *et al*. (1976, 1978b), using a synthesized tritium-labeled tentoxin with a high specific radioactivity (47 mCi/mmol), have demonstrated that inhibition of the lettuce CF_1 CaATPase is due to its binding to a single site with an affinity constant of 2×10^8 M^{-1}. They also suggested that the stimulatory effect of 0.01−1 mM tentoxin, showing an apparent K_a of 6.3×10^3 M^{-1}, could arise through binding of tentoxin to a second, low-affinity site (Steele *et al*., 1978a). The stimulation of the CF_1-ATPase activity by tentoxin was, however, found to be reversible, and therefore no direct assays of its binding to the second site have been reported. Using a ^{14}C-labeled tentoxin, isolated from a culture of the tentoxin-producing fungus *Alternaria alternata* grown in the presence of [^{14}C]-leucine, Dahse *et al*. (1994) have recently assayed the binding of tentoxin to a spinach $CF_1(-\epsilon)$ complex. Their results led them to suggest that dissociation of the CF_1 ϵ subunit might correlate with an increased binding of tentoxin to the second, low-affinity site. They have also demonstrated some direct binding of tentoxin to an isolated CF_1 β subunit. But the 1000-fold lower specific radioactivity of their [^{14}C]tentoxin as compared to [^3H]tentoxin (Steele *et al*., 1976) did not enable any further characterization of this site.

Unlike all γ-containing CF_1 complexes, the $CF_1(\alpha\beta)$ complex isolated in the complete absence of tentoxin is absolutely resistant to inhibition by tentoxin, although it is markedly stimulated by tentoxin concentrations above 1 μM (Gromet-Elhanan & Avital, 1992; and Figure 3). These strange results cannot be interpreted by suggesting that the high-affinity tentoxin inhibitory site is located on the γ subunit, since there is strong evidence that the CF_1 β subunit forms at least part of the inhibitory tentoxin binding site (Richter *et al*., 1986; Avni *et al*., 1992; Hu *et al*., 1993; Chen & Jagendorf, 1994). We have tried to examine the direct binding of labeled tentoxin to the $CF_1(\alpha\beta)$ complex. But unfortunately the highly labeled [^3H]tentoxin is no more available (R. D. Durbin, personal communication), and the specific radioactivity of the [^{14}C]tentoxin was too low to enable any clear characterization of tentoxin binding to the $CF_1(\alpha\beta)$ complex (Avital and Gromet-Elhanan, unpublished observations). By using Sephadex centrifuge columns, it was, however, demonstrated that the $CF_1(\alpha\beta)$ complex binds tentoxin rather tightly (Gromet-Elhanan & Avital, 1992).

A possible explanation for the observed strange correlation between the presence of the CF_1 γ subunit and the appearance of *inhibition* by tentoxin is that γ induces a conformational change in one or more of the β subunits in the unstable CF_1-$(\alpha\beta)$ complex. This change in conformation could stabilize both the hexameric structure of $CF_1(\alpha\beta)$ as well as the binding of tentoxin to its high-affinity inhibitory site. Several experimental findings have indicated that the inhibitory effect of tentoxin can also be induced by the presence of a fragmented CF_1 γ subunit. Thus, the smallest CF_1-ATPase which has been found to be inhibited by tentoxin and to bind it very similarly to whole CF_1 is a trypsin-activated enzyme (Steele *et al*., 1977; Hu *et al*., 1993) consisting of CF_1 α and β subunits (Deters *et al*., 1975) together with fragments of the CF_1 γ subunit (Moroney & McCarty, 1982; Hu *et al*., 1993).

The results reported here with the tentoxin-stabilized CF_1-$\alpha_3\beta_3$ stress our earlier conclusion based on the properties of the $CF_1(\alpha\beta)$-MgATPase (Gromet-Elhanan & Avital, 1992) that the CF_1 γ subunit is absolutely necessary for obtaining the normal catalytic properties of CF_1-ATPase. One very important property of all F_1-ATPases, which could not as yet be tested under optimal conditions with any F_1-$\alpha_3\beta_3$ hexamer, is their catalytic site cooperativity. Thus, the TF_1-$\alpha_3\beta_3$ hexamer was found to dissociate into $\alpha_1\beta_1$ dimers in the presence of even micromolar concentrations of MgATP (Harada *et al*., 1991). Because the dimers were also active ATPases, their presence together with the hexamers complicated the interpretation of earlier measurements of ATP hydrolysis by the TF_1-$\alpha_3\beta_3$ (Miwa & Yoshida, 1989). The tentoxin-stabilized CF_1-$\alpha_3\beta_3$ has, on the other hand, been

isolated only in the presence of millimolar concentrations of MgATP, so the effect of ATP concentration on its MgATPase activity could be assayed only above the 25 μM ATP introduced with the protein. With CF_1 activated by heat, octyl glucoside, or tentoxin, cooperative interactions have been demonstrated already below 250 μM ATP, but became most pronounced below 20 μM ATP (Kohlbrenner & Boyer, 1983). It is therefore not surprising that no catalytic cooperativity could be detected with CF_1-$\alpha_3\beta_3$ (Figure 6).

Experiments with the general F_1 inhibitor azide and especially with the CF_1 inhibitor tentoxin provide additional information on the problem of catalytic site cooperativity. Both azide (Futai *et al*., 1989) and tentoxin (Fromme *et al*., 1992) were reported to inhibit multisite, but not unisite, F_1 ATP hydrolysis and were therefore suggested to block catalytic cooperativity. However, all isolated $F_1(\alpha\beta)$ complexes, including the $\alpha_3\beta_3$ hexamers, were found to be completely resistant to inhibition by tentoxin (Avital & Gromet-Elhanan, 1992; Gromet-Elhanan & Sokolov, 1995) and/or azide (Miwa & Yoshida, 1989; Andralojc & Harris, 1992; Avital & Gromet-Elhanan, 1992; and see Table 1). At least in the case of tentoxin both $CF_1(\alpha\beta)$ and CF_1-$\alpha_3\beta_3$ bind it and are stimulated by it. So their resistance to inhibition by tentoxin can indeed reflect the absence of cooperative interactions between their catalytic sites.

The tentoxin-stabilized, CF_1-$\alpha_3\beta_3$ hexamer and its parent $CF_1(\alpha\beta)$, whose ATPase activities are different from those of CF_1-$\alpha_3\beta_3\gamma$, provide very promising tools for following changes in their catalytic properties induced by addition of the CF_1 γ subunit. Such tests might now be possible by using the recently isolated, overexpressed, urea-denatured, and chaperone-refolded CF_1 γ subunit (Chen & Jagendorf, 1994). When this paper was ready for submission, Gao *et al*. (1995) reported an additional method for isolation of the native CF_1 γ subunit together with an assembled labile CF_1-$\alpha_3\beta_3$ from soluble CF_1. Both types of CF_1 γ could reconstitute active $CF_1(\alpha\beta\gamma)$ complexes. They can therefore enable us to define which fragments of the CF_1 γ subunit (Moroney & McCarty, 1982) play a role in expression of the typical properties of the CF_1-ATPase.

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REFERENCES

Andralojc, P. J., & Harris, D. A. (1992) *FEBS Lett. 310*, 187−192.

Avital, S., & Gromet-Elhanan, Z. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. III, pp 45−48, Kluwer Academic Publishers, Dordrecht.

Avital, S., & Gromet-Elhanan, Z. (1991) *J. Biol. Chem. 266*, 7067−7072.

Avni, A., Anderson, J. D., Holland, N., Rochaix, J.-D., Gromet-Elhanan, Z., & Edelman, M. (1992) *Science 257*, 1245−1247.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Chen, G. G., & Jagendorf, A. T. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11497−11501.

Dahse, I., Pezennec, S., Girault, G., Berger, G., Andre, F., & Liebermann, B. (1994) *J. Plant Physiol. 143*, 615−620.

Deters, D. W., Racker, E., Nelson, N., & Nelson, H. (1975) *J. Biol. Chem. 250*, 1041−1047.

Dunn, S. D., & Futai, M. (1980) *J. Biol. Chem. 255*, 113−118.

Fling, S. P., & Gregerson, D. S. (1986) *Anal. Biochem. 155*, 83−88.

Fromme, P., Dahse, I., & Graber, P. (1992) *Z. Naturforsch 47C*, 239−244.

Futai, M. (1977) *Biochem. Biophys. Res. Commun. 79*, 1231−1237.

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

Gao, F., Lipscomb, B., Wu, I., & Richter, M. L. (1995) *J. Biol. Chem. 270*, 9763−9769.

Gershoni, J. M. (1988) *Methods Biochem. Anal. 33*, 1−58.

Gromet-Elhanan, Z. (1992) *J. Bioenerg. Biomembr. 24*, 447−452.

Gromet-Elhanan, Z., & Avital, S. (1992) *Biochim. Biophys. Acta 1102*, 379−385.

Gromet-Elhanan, Z., & Sokolov, M. (1995) *Photosyn. Res. 46*, 79−86.

Harada, M., Ohta, S., Sato, M., Ito, Y., Kabayashi, N., Sone, N., Ohta, T., & Kagawa, Y. (1991) *Biochim. Biophys. Acta 1056*, 279−284.

Hochman, Y., Lanir, A., & Carmeli, C. (1976) *FEBS Lett. 61*, 255−259.

Hu, N., Mills, D. A., Huchzermeyer, B., & Richter, M. L. (1993) *J. Biol. Chem. 268*, 8536−8540.

Jagendorf, A. T., McCarty, R. E., & Roberson, D. (1991) *Cell Cult. Somatic Cell Genet. Plants 7B*, 225−254.

Kagawa, Y., Ohta, S., & Otawara-Hamamoto, Y. (1989) *FEBS Lett. 249*, 67−69.

Kohlbrenner, W. E., & Boyer, P. D. (1983) *J. Biol. Chem. 258*, 10881−10886.

Kuno, H., & Kihara, H. K. (1967) *Nature 215*, 974−975.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

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

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

Mitra, B., & Hammes, G. G. (1988) *Biochemistry 27*, 245−250.

Miwa, K., & Yoshida, M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6484−6487.

Moroney, J. V., & McCarty, R. E. (1982) *J. Biol. Chem. 257*, 5910−5914.

Murataliev, M. B., Milgrom, Y. M., & Boyer, P. D. (1991) *Biochemistry 30*, 8305−8310.

Nalin, C. M., & Nelson, N. (1987) *Curr. Top. Bioenerg. 15*, 273−294.

Ohta, S., Harada, M., Ito, Y., Kobayashi, Y., Sone, N., & Kagawa, Y. (1990) *Biochem. Biophys. Res. Commun. 171*, 1258−1263.

Patrie, W. J., & McCarty, R. E. (1984) *J. Biol. Chem. 259*, 11121−11128.

Pick, U., & Bassilian, S. (1982) *Biochemistry 21*, 6144−6152.

Pick, U., Conrad, P. L., Conrad, J. M., Dunkin, R. D., & Selman, B. R. (1982) *Biochim. Biophys. Acta 682*, 55−58.

Richter, M. L., Gromet-Elhanan, Z., & McCarty, R. E. (1986) *J. Biol. Chem. 261*, 12109−12113.

Shahak, Y. (1982) *Plant Physiol. 70*, 87−91.

Sokolov, M., Avital, S., & Gromet-Elhanan, Z. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) Vol. II, pp 653−659, Kluwer Academic Publishers, Dordrecht.

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

Steele, J. A., Uchytil, T. F., Durbin, R. D., Bhatnagar, P., & Rich, D. H. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2245−2248.

Steele, J. A., Uchytil, T. F., & Durbin, R. D. (1977) *Biochim. Biophys. Acta 459*, 347−350.

Steele, J. A., Uchytil, T. F., & Durbin, R. D. (1978a) *Biochim. Biophys. Acta 504*, 136−141.

Steele, J. A., Durbin, R. D., Uchytil, T. F., & Rich, D. H. (1978b) *Biochim. Biophys. Acta 501*, 72−82.

Yoshida, M., Sone, N., Hirata, H., & Kagawa, Y. (1977) *J. Biol. Chem. 252*, 3480−3485.

Zhang, S., & Jagendorf, A. T. (1995) *J. Biol. Chem. 270*, 6607−6614.

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