Journal of Bioenergetics and Biomembranes, Vol. 26, No. 5, 1994

Tight Nucleotide Binding Sites and ATPase Activities of the *Rhodospirillum rubrum* RrF_1-ATPase as Compared to Spinach Chloroplast CF_1-ATPase

Sara Weiss,[1] Richard E. McCarty,[2] and Zippora Gromet-Elhanan[1]

Received March 28, 1994; revised May 19, 1994

Solubilized *Rhodospirillum rubrum* RrF_1-ATPase, depleted of loosely bound nucleotides, retains 2.6 mol of tightly bound ATP and ADP/mol of enzyme. Incubation of the depleted RrF_1 with Mg^{2+}-ATP or Mg^{2+}-AMP-PNP, followed by passage through two successive Sephadex centrifuge columns, results in retention of a maximal number of 4 mol of tightly bound nucleotides/mol of RrF_1. They include 1.5 mol of nonexchangeable ATP, whereas all tightly bound ADP is fully exchangeable. A similar retention of only four out of the six nucleotide binding sites present on CF_1 has been observed after its passage through one or two centrifuge columns. These results indicate that the photosynthetic, unlike the respiratory, F_1-ATPases have faster k_{off} constants for two of the Mg-dependent nucleotide binding sites. This could be the reason for the tenfold lower Mg^{2+} than Ca^{2+}-ATPase activity observed with native RrF_1, as with ϵ-depleted, activated CF_1. An almost complete conversion of both RrF_1 and CF_1 from Ca^{2+}- to Mg^{2+}-dependent ATPases is obtained upon addition of octylglucoside, at concentrations below its CMC, to the ATPase assay medium. Thus, octylglucoside seems to affect directly the RrF_1 and CF_1 divalent cation binding site(s), in addition to its proposed role in relieving their inhibition by free Mg^{2+} ions. The RrF_1-ATPase activity is 30-fold more sensitive than CF_1 to efrapeptin, and completely resistant to either inhibition or stimulation by the CF_1 effector, tentoxin. Octylglucoside decreases the inhibition by efrapeptin and tentoxin, but exposes on CF_1 a low-affinity, stimulatory site for tentoxin.

KEY WORDS: *Rhodospirillum rubrum* RrF_1-ATPase; chloroplast CF_1; nucleotide binding sites; Ca^{2+}- and Mg^{2+}-ATPase activity; octylglucoside; F_1-ATPase inhibitors; efrapeptin; tentoxin.

INTRODUCTION

All respiratory and photosynthetic energy-coupling membranes contain a multisubunit F_0F_1 ATPsynthase-ATPase that couples the synthesis and hydrolysis of ATP to transmembrane proton transport. Although F_0F_1 complexes from various sources are structurally and functionally similar, some important differences, especially in rates of ATP hydrolysis and number of tight nucleotide binding sites, have been observed between the respiratory mitochondrial and *Escherichia coli* enzymes and those of chloroplasts. The respiratory membranes and their isolated F_1 complexes show rapid rates of Mg^{2+}-ATPase activity (Penefsky, 1979; Dunn and Heppel, 1981) as compared to the extremely low rates of ATP hydrolysis reported for thylakoid membrane-bound and isolated chloroplast

[1] Department of Biochemistry, The Weizmann Institute of Science, Rehovot, 76100 Israel.

[2] Department of Biology, The Johns Hopkins University, Baltimore, Maryland, 21218-2685.

[3] Abbreviations: CF_1, EcF_1, MF_1, RrF_1, and TF_1, the soluble F_1-ATPase from chloroplasts, *E. coli*, mitochondria, *R. rubrum*, and the thermophilic bacterium PS3, respectively: AMP-PNP, adenylyl-β,γ-imidodiphosphate; CMC, critical micellar concentration; DTT, dithiothreitol, LDAO, lauryl dimethylamine oxide.

[4] Dedicated to Professor Achim Trebst in honor of this 65th birthday.

0145-479X/94/1000-0573$07.00/0

CF_1 (McCarty and Moroney, 1985).[3] Activation of the latent CF_1 into a Ca^{2+}-ATPase is obtained by various treatments that remove its ϵ-subunit and reduce a disulfide bond which is specific to the CF_1 γ-subunit. These treatments reveal, however, only a very low CF_1 Mg^{2+}-ATPase activity. The maximum number of Mg-dependent tight nucleotide binding sites retained on MF_1 and EcF_1 after passage through gel filtration centrifuge columns is six (Cross and Nalin, 1982; Wise *et al.*, 1983). Six sites have recently been observed also on CF_1, but only under strict equilibrium conditions (Girault *et al.*, 1988; Shapiro *et al.*, 1991b). After passage through one (Xue *et al.*, 1987) or two (Shapiro *et al.*, 1991a) centrifuge columns, only four nucleotides were retained on CF_1. Interestingly, the rat liver MF_1 retained also only 4–5 mol nucleotides/mole when assayed in the presence of either Mg^{2+} or Co^{2+} (Williams *et al.*, 1987).

The F_0F_1 ATP synthase of the photosynthetic bacterium *Rhodospirillum rubrum* occupies an intermediate position between the respiratory and chloroplast enzymes with regard to its ATPase activity. When bound to chromatophore membranes it is an active ATPase exhibiting, as in *E. coli* (Kanazawa *et al.*, 1980; Dunn and Heppel, 1981), both Mg^{2+} and Ca^{2+}-dependent activities (Johansson *et al.*, 1972) at a ratio of 3:1 (Oren and Gromet-Elhanan, 1977). But, whereas the EcF_0F_1 and EcF_1 solubilized from *E. coli* retain both activities (Kanazawa *et al.*, 1980; Wise *et al.*, 1983), the solubilized *R. rubrum* F_0F_1 and F_1 complexes lose the Mg^{2+}-ATPase activity. Detergent solubilized RrF_0F_1 catalyzes a twofold faster ATP hydrolysis in the presence of Ca^{2+} than of Mg^{2+} (Bengis-Garber and Gromet-Elhanan, 1979; Schneider *et al.*, 1979), and isolated RrF_1 is predominantly a Ca^{2+}-ATPase (Johansson *et al.*, 1973). The RrF_1 ATPase activity is thus similar to that of the Ca^{2+}-ATPase of preactivated, ϵ-depleted CF_1, except that with RrF_1 preactivation leading to dissociation of the ϵ-subunit is not required. In this respect RrF_1 behaves rather similarly to EcF_1, where in contrast to CF_1 the inhibitory, but loosely bound ϵ subunit, dissociates upon dilution of the EcF_1 complex into assay buffers (Dunn and Heppel, 1981).

In view of the much lower Mg^{2+}- than Ca^{2+}-ATPase activity of both native RrF_1 and ϵ-depleted CF_1 it is interesting to find out whether the nucleotide binding properties of RrF_1 are similar to those of EcF_1 and MF_1 or to CF_1. No measurements of nucleotide binding to isolated RrF_1 have been reported up to now. There are only two published determinations of tightly bound nucleotides in coupled *R. rubrum* chromatophores, which were assumed to be bound to RrF_1. Harris and Baltscheffsky (1979) found 12 and 8.3 mmol of ATP and ADP, respectively, tightly bound per mole of bacteriochlorophyll, whereas Andralojc and Harris (1993) found only 1.76 and 1.38 mmol of ATP and ADP bound/per mole of bacteriochlorophyll. The reason for these very different numbers is not clear, since in both cases there was no information on the ratios of total protein and/or RrF_1 to bacteriochlorophyll. A direct examination of nucleotide binding to isolated RrF_1 is therefore required.

In both RrF_1 (Johansson *et al.*, 1973; Oren and Gromet-Elhanan, 1979) and activated CF_1 (Hochman *et al.*, 1976) free Mg^{2+} was a potent inhibitor of ATPase activity, and unmasking of their Mg^{2+}-ATPase has been obtained by addition of certain anions (Nelson *et al.*, 1972; Webster *et al.*, 1977) or detergents (Soe *et al.*, 1978; Pick and Bassilian, 1982) to the assay medium. A further detailed investigation on activation of native CF_1-ATPase by detergents has, however, revealed that they do also remove the ϵ-subunit from latent CF_1 (Feng and McCarty, 1985), thus suggesting that expression of CF_1Mg^{2+}-ATPase requires both removal of the ϵ-subunit and relief of inhibition by free Mg^{2+}. Since RrF_1 is an active ATPase in the presence of its ϵ-subunit, it offers a simpler assay system for studying the correlation between relief of inhibition by free Mg^{2+} and expression of a Mg^{2+}-ATPase.

In this paper we determine the number and properties of nucleotide binding sites on RrF_1 and compare them with those observed in latent CF_1. We also examine in detail the effect of octylglucoside on the RrF_1 and CF_1Ca^{2+}- and Mg^{2+}-ATPase activities and on their sensitivity to F_1-ATPase inhibitors.

EXPERIMENTAL PROCEDURE

Materials

Spinach CF_1 was prepared as described previously (Shapiro and McCarty, 1990). When used for nucleotide binding studies it was freed from contaminating traces of ribulose biphosphate carboxylase/oxygenase

(rubisco) by affinity chromatography (Soteropoulus *et al.*, 1992). CF_1 was stored at 4°C as the ammonium sulfate (50% W/V) precipitate, or in liquid nitrogen in buffer containing 50 mM Na-tricine (pH 8.0), 50 mM NaCl, 4 mM ATP, and 10% glycerol. RrF_1 was removed from *R. rubrum* chromatophores according to Norling *et al* (1988), purified, and stored as described by Khananshvili and Gromet-Elhanan (1983). The storage buffer contained 50 mM Na-tricine (pH 8.0), 2 mM ATP, 1 mM EDTA, 1 mM DTT, and 10% glycerol.

ATP was of the highest purity available from Sigma and contained no measurable quantity of ADP by HPLC analysis. AMP-PNP was purchased from ICN Biochemical, and octylglucoside, quercetin, NBf-Cl, and tentoxin from Sigma. Efrapeptin was a gift of Dr. R. L. Hamill of Eli Lilly Co. Stock solutions of quercetin and NBf-Cl were prepared in DMSO and of tentoxin and efrapeptin in water.

Nucleotide Binding Studies

Prior to each experiment, ammonium sulfate-precipitated CF_1 was dissolved in 50 mM Tris-HCl (pH 8.0) and 50 mM NaCl (TN buffer). This latent CF_1 and the native RrF_1 were freed from all loosely bound nucleotides by three successive filtrations on fine grade Sephadex G-50 columns (Penefsky, 1977) equilibrated with TN buffer. The eluted enzymes, designated as depleted CF_1 and RrF_1, were incubated either with no additions or with added Mg^{2+}-ATP and Mg^{2+}-AMP-PNP as described in the text. Two successive Sephadex centrifuge columns were used to remove excess and loosely bound nucleotides. For analysis of the remaining tightly bound nucleotides, 100-μl aliquots of the final eluants, containing between 15 and 20 μM enzyme, were precipitated in the cold with 50 μl of 1.2 M $HClO_4$. After 5 min on ice the mixture was neutralized and $KClO_4$ precipitated with 25 μl of 1.3 M K_2CO_3. Each sample was centrifuged two times: once for 5 min at 8700 x g and the supernatant was recentrifuged in a Beckman microfuge for 10 min. The final supernatants were either stored at −20°C or immediately subjected to ion-pairing high-pressure liquid chromatography (HPLC) as described by Shapiro and McCarty (1990). The concentrations of nucleotides in the tested samples were determined by comparing the obtained integrated peak areas with those of nucleotide solutions of known concentration.

Preactivation by Octylglucoside

Both native RrF_1 and latent CF_1 were preactivated by incubation for 20 min at 35°C in a solution containing 50 mM Na-tricine (pH 8.0), 4 mM ATP, 5 mM DTT, 40 mM octylglucoside, and 0.5–1 mg of protein/ml. Aliquots of all preactivated enzymes were diluted 100-fold into the ATPase assay medium.

Assay Procedures

ATPase activity was assayed as follows: native untreated or preactivated RrF_1 and CF_1 (3–10 μg of protein) were preincubated for 5 min at 35°C in a reaction mixture that contained 50 mM Na-tricine (pH 8.0) with or without the stated concentrations of octylglucoside and/or inhibitors. The assay was started by addition of a mixture of 4 mM ATP and either 8 mM $CaCl_2$ or 2 mM $MgCl_2$. After 5 min at 35°C Pi release was determined according to Taussky and Shorr (1953). Protein concentrations were determined by the method of Lowry *et al.* (1951).

RESULTS

Tight Nucleotide Binding Sites on RrF_1 and CF_1

CF_1, resuspended from an ammonium sulfate precipitate and depleted of loosely bound nucleotides has been shown to contain between 1.3 and 1.7 mol of tightly bound ADP/mol of enzyme, but no tightly bound ATP (Shapiro *et al.*, 1991a). An identical pattern was observed here with latent depleted CF_1, whereas a similarly depleted native RrF_1 retained around 2.6 mol of tightly bound nucleotides/mol of enzyme, which were distributed between ATP and ADP (Table I). No bound AMP was detected on either CF_1 or RrF_1.

Incubation with Mg^{2+}-ATP leads to binding of about two additional moles of ATP/mol of CF_1, but only one additional mole of ATP/mol of RrF_1, thus raising the total number of tightly bound nucleotides to about four per mole of either enzyme (Table I). Incubation of both enzymes with saturating concentrations of Mg^{2+}-AMP-PNP results also in detection of up to 4 mol of tightly bound nucleotides/mol of CF_1 or RrF_1 (Table I and Fig. 1). In both enzymes about 1 mol of ADP exchanges with AMP-PNP, but the tightly bound ATP in RrF_1 does not exchange with medium AMP-PNP. This leads to an overall

Table I. Mg-Dependent Tight-Binding Sites for ADP, ATP, and AMP-PNP on RrF_1 as Compared to Latent CF_1[a]

Enzymes and additions		mol bound nucleotide/mol F_1[b]			
		ATP	ADP	AMP-PNP	Total
RrF_1	None	1.40 ± 0.05	1.20 ± 0.12	—	2.60
RrF_1	Mg^{2+}-ATP	2.10 ± 0.15	1.30 ± 0.06	—	3.40
RrF_1	Mg^{2+}-AMP-PNP	1.35 ± 0.10	0.28 ± 0.17	1.95 ± 0.10	3.58
CF_1	None	0.10 ± 0.12	1.62 ± 0.20	—	1.72
CF_1	Mg^{2+}-ATP	2.05 ± 0.07	1.60 ± 0.15	—	3.65
CF_1	Mg^{2+}-AMP-PNP	0.10 ± 0.10	0.54 ± 0.20	3.03 ± 0.20	3.67

[a] Depleted RrF_1 and CF_1 were incubated for 1.5 h at room temperature in TN buffer with 2 mM $MgCl_2$ and either 5 mM ATP or 5 mM AMP-PNP. One sample was incubated with buffer only. Excess and loosely bound nucleotides were removed, and concentrations of protein and tightly bound nucleotides were measured as described under Experimental Procedures.
[b] Values shown are means ± S.D.; $n = 3$.

incorporation of about 2 mol of AMP-PNP/mol of RrF_1 as compared to 3 mol/mol of latent CF_1 (Table I) or activated CF_1 (Shapiro *et al.*, 1991a).

Tight binding of saturating concentrations of Mg^{2+}-AMP-PNP to RrF_1 is very rapid at 35°C. The time dependence shown in Fig. 1 reveals that the first mole of AMP-PNP/RrF_1 binds within 1 min, probably to an empty nucleotide binding site, since tightly bound ATP does not decrease and ADP decreases by less than 15%. Further binding of AMP-PNP correlates closely with the disappearance of all tightly bound ADP, so that after 30 min at 35°C only 0.1 mol of ADP, but about 1.5 mol of ATP, remain tightly bound per mole of RrF_1, and up to 2.5 mol of AMP-PNP are incorporated per mole of RrF_1.

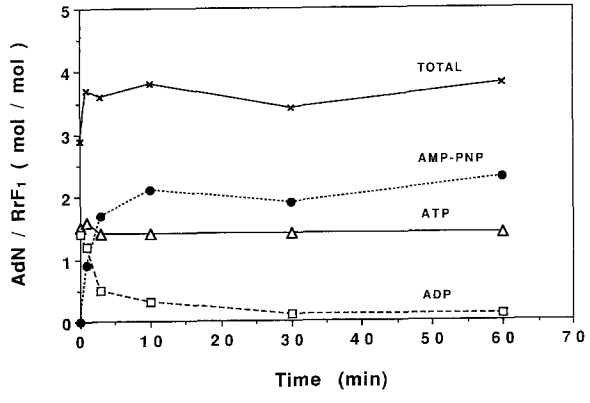

Fig. 1. Time course of Mg^{2+}-AMP-PNP tight binding to RrF_1 and exchange with tightly bound ADP. Depleted RrF_1 was incubated at 35°C in TN buffer containing 2 mM $MgCl_2$ and 5 mM AMP-PNP. Incubations were stopped at the indicated time points by application of the sample to the first centrifuge column. Further treatment of the samples and analysis of bound nucleotides are described under Experimental Procedures. The 0-min sample was incubated without Mg^{2+}-AMP-PNP.

Effect of Octylglucoside on various RrF_1 and CF_1 ATPase Activities

Figures 2 and 3 illustrates major differences in ATPase activities of CF_1 and RrF_1. Untreated native CF_1 shows no detectable Ca^{2+}- or Mg^{2+}-ATPase activity, whereas untreated native RrF_1 is an active Ca^{2+}-ATPase (Fig. 2), and a 7- to 10-fold slower Mg^{2+}-ATPase (Fig. 3). These results were obtained

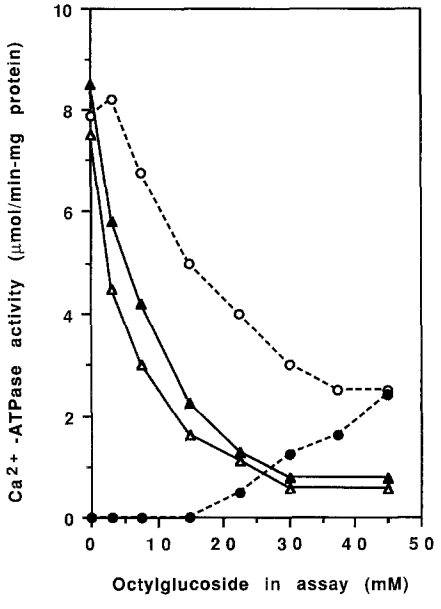

Fig. 2. Increasing concentrations of octylglucoside in assay inhibit the Ca^{2+}-ATPase of RrF_1 and preactivated CF_1 but stimulate the Ca^{2+}-ATPase of latent CF_1. Conditions of preactivation, dilution, incubation with the indicated concentrations of octylglucoside, and ATPase assay are described under Experimental Procedures. ATPase activity was followed after addition of a mixture containing 4 mM ATP and 8 mM $CaCl_2$. ●, latent, untreated CF_1; ○, preactivated CF_1; ▲, native, untreated RrF_1; △, preactivated RrF_1.

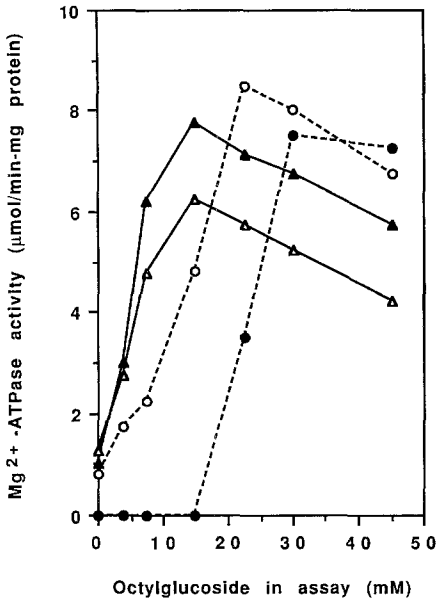

Fig. 3. Increasing concentrations of octylglucoside in assay stimulate RrF_1 and CF_1 Mg^{2+}-ATPase activity. Treatments, procedures, and symbols of assayed enzymes are as described in Fig. 2, except that ATPase activity was followed after addition of a mixture containing 4 mM ATP and 2 mM $MgCl_2$.

with no additions using a Ca^{2+}/ATP ratio of 2 and a Mg^{2+}/ATP ratio of 0.5, which were found to be optimal for activated CF_1 (Hochman *et al.*, 1976; Pick and Bassilian, 1982; Feng and McCarty, 1985) as well as for RrF_1 (not shown).

Preactivation of the native RrF_1 containing all five F_1 polypeptide subunits by octylglucoside followed by a 100-fold dilution into an assay with no added octylglucoside did not change its active Ca^{2+}-ATPase (Fig. 2) or slow Mg^{2+}-ATPase (Fig. 3). In native latent CF_1 such preactivation induced both Ca^{2+}- and Mg^{2+}-ATPase activities but to a different extent: The CF_1 Ca^{2+}-ATPase was much more activated (Fig. 2) than the CF_1 Mg^{2+}-ATPase (Fig. 3). This preactivation, which has been shown to release at least some of the inhibitory ϵ subunit from latent CF_1 (Feng and McCarty, 1985), thus raised the ϵ-depleted CF_1 Ca^{2+}- and Mg^{2+}-ATPase activities to the level obtained in the untreated, ϵ-containing RrF_1 (Figs. 2 and 3).

A different and much more complicated effect was observed upon addition of increasing concentrations of octylglucoside to the ATPase assay medium. The Ca^{2+}-ATPase activity of either native or pretreated RrF_1 was inhibited (Fig. 2), whereas the very slow Mg^{2+}-ATPase activity of both types of RrF_1 is stimulated (Fig. 3). These opposite effects show a very similar dependence on octylglucoside concentration. A 50% inhibition or stimulation is already obtained at 5 mM octylglucoside, a concentration well below the octylglucoside CMC, and ineffective in releasing the CF_1 ϵ-subunit (Feng and McCarty, 1985). Addition of octylglucoside to the assay medium converted preactivated CF_1 from a Ca^{2+}- to a Mg^{2+}-dependent ATPase, except that 15 mM octylglucoside was required for a 50% effect (Figs. 2 and 3).

With latent CF_1 the presence of octylglucoside in the assay exhibited a much more complicated effect. The latent CF_1 Ca^{2+}-ATPase is rather induced by high concentrations of octylglucoside that release the inhibitory CF_1 ϵ-subunit (Feng and McCarty, 1985), so in the presence of 45 mM octylglucoside in the assay an identical rate of ATP hydrolysis was obtained in both latent and preactivated CF_1 Ca^{2+} ATPases (Fig. 2). The latent CF_1 Mg^{2+}-ATPase activity, as the RrF_1 and preactivated CF_1 Mg^{2+}-ATPases, was markedly stimulated by addition of octylglucoside into the assay (Fig. 3).

These results indicate that the effect of octylglucoside on RrF_1 is much simpler than on CF_1 because the presence of the ϵ subunit does not inhibit expression of the RrF_1-ATPase activity.

Inhibitors of RrF_1 and CF_1-ATPase Activities

The similarities and differences in nucleotide binding properties and response to activation processes obtained with RrF_1 and CF_1 prompted us to compare also the effect of various F_1 inhibitors on both enzymes. For two inhibitors, tentoxin and efrapeptin, RrF_1 and CF_1 exhibit markedly different sensitivities (Figs. 4 and 5).

Tentoxin is produced by a plant pathogenic fungus and functions as a species-specific potent effector of CF_1 (Steele *et al.*, 1976; Selmand and Durbin, 1978). Heat- or trypsin-activated CF_1 Ca^{2+}-ATPases from sensitive plants, such as lettuce or spinach, were inhibited 50% by as little as 10–30 nM tentoxin (Selman and Durbin, 1978). On the other hand, CF_1 from plants insensitive to the pathogen, such as radish or various tobacco strains, required 20 to 1000 times higher concentrations of tentoxin for 50% inhibition (Steele *et al.*, 1976; Conrad *et al.*, 1981). Lettuce and spinach CF_1 have, in addition to the high-affinity, inhibitory site for tentoxin, also a low-affinity site, which at 50–1000 μM tentoxin stimulates up to 4-fold their Ca^{2+}-ATPase activity (Steele *et al.*, 1978; and Fig. 4).

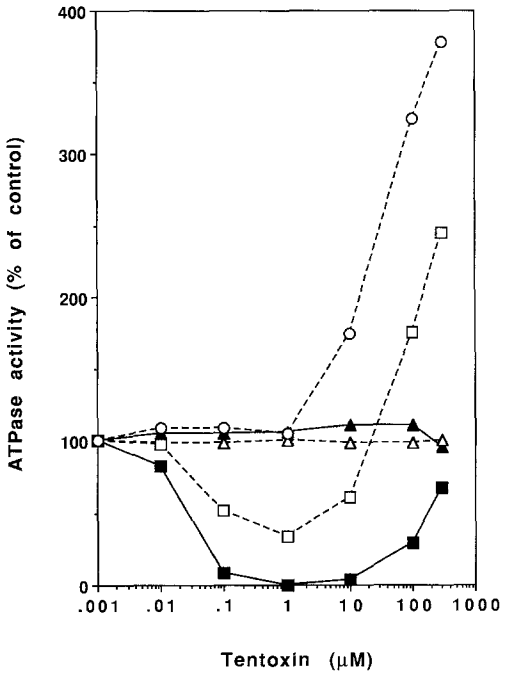

Fig. 4. Effect of tentoxin on RrF_1 and CF_1 ATPase activities. Preactivated RrF_1 and CF_1 were prepared and assayed as described under Experimental Procedures. The Ca^{2+}-ATPase activities of RrF_1(▲) and CF_1(■) were assayed with no added octylglucoside. The Mg^{2+}-ATPase activities of RrF_1(△) and CF_1(□) were assayed in the presence of 16 mM octylglucoside. The CF_1 Mg^{2+}-ATPase was also assayed in the presence of 40 mM octylglucoside (○). Control Ca^{2+}-ATPase activities in μmol Pi released/min per mg of protein were: 7.1 for RrF_1 and 10.2 for CF_1. Control Mg^{2+}-ATPase activities were 8.2 for RrF_1 and 5.6 for CF_1 with 16 mM octylglucoside, and 8.6 for CF_1 with 40 mM octylglucoside.

Figure 4 illustrates that, in contrast to spinach CF_1, the RrF_1 Ca^{2+}-ATPase activity is neither inhibited nor stimulated by tentoxin concentrations ranging between 1 nM to 300 μM. So the RrF_1 Ca^{2+}-ATPase is fully resistant to tentoxin, being uneffected even by concentrations that inhibit the CF_1 Ca^{2+}-ATPase activity of various insensitive plants.

Unlike with the Ca^{2+}-ATPase, the reported effects of tentoxin on CF_1Mg^{2+}-ATPase activity are very variable, even for CF_1 complexes isolated from sensitive plants. In some cases very little or no inhibition was obtained by up to 10 μM tentoxin (Pick and Bassilian, 1982; Pick *et al.*, 1982), whereas in another case an identical pattern of tentoxin inhibition of both Ca^{2+} and Mg^{2+}-dependent CF_1-ATPase activities was reported (Hu *et al.*, 1993). These very different effects of tentoxin were obtained when the CF_1 Mg^{2+}-ATPase activity was unmasked

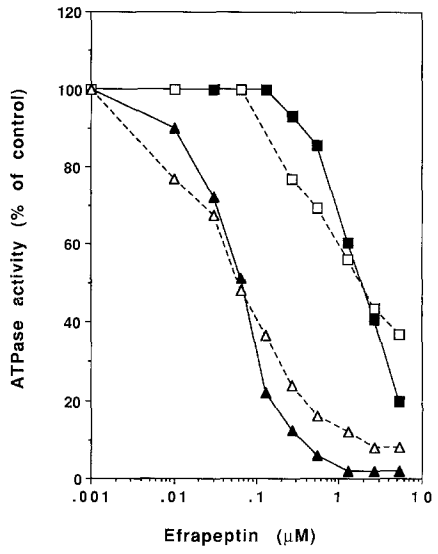

Fig. 5. Efrapeptin inhibition of RrF_1 and CF1 ATPase activities. Preactivated RrF_1 and CF_1 were prepared and assayed as described under Experimental Procedures. Symbols of assayed enzymes and activities are as described in Fig. 4.

by addition of sulfite (Hu *et al.*, 1993) as compared to octylglucoside (Pick and Bassilian, 1982).

The results summarized in Fig. 4 indicate that the presence of octylglucoside in the assay does indeed decrease the inhibition by tentoxin, while increasing its stimulatory effect. Thus, with 16 mM octylglucoside in the assay there is a pronounced, although not complete, inhibition of the CF_1 Mg^{2+}-ATPase by 100 nM to 10 μM tentoxin, and a 2.5 fold stimulation by 300 μM tentoxin. But in the presence of 40 mM octylglucoside, the concentration used in earlier investigations (Pick and Bassilian, 1982; Pick *et al.*, 1982), no inhibition is observed even with up to 1 μM tentoxin. Furthermore the stimulatory effect of tentoxin starts already at 10 μM and amounts to about 4-fold at 300 μM tentoxin (Fig. 4). The Mg^{2+}-ATPase activity of RrF_1 is not affected at all by tentoxin even when assayed in the presence of 16 mM octylglucoside (Fig. 4). It is also completely insensitive to tentoxin in the presence of sulfite (not shown). So in RrF_1, unlike in CF_1, both Ca^{2+}- and Mg^{2+}-dependent ATPase activities are completely unaffected by tentoxin under all assay conditions used.

Efrapeptin has been reported to inhibit completely isolated RrF_1 (Webster *et al.*, 1977). When tested under identical conditions it inhibits both RrF_1 and CF_1, but the latter is much less sensitive (Fig. 5). Half maximal inhibition of both RrF_1

Ca^{2+}- and Mg^{2+}-ATPase activities is obtained at a ratio of 1.6 mol per mole of RrF_1, whereas for CF_1 a ratio of 50 mol of efrapeptin per mole of enzyme is required. RrF_1 is thus as sensitive to efrapeptin as MF_1 (Cross and Kohlbrenner, 1978), while the lower sensitivity of CF_1 is similar to that reported for EcF_1 (Wise *et al.*, 1983). The inhibition of RrF_1 by efrapeptin, as that of CF_1 by tentoxin, is decreased by increasing concentrations of octylglucoside in the assay. The similar effect of efrapeptin on Ca^{2+}- and Mg^{2+} ATPase activities has been observed with 16 mM octylglucoside in the Mg^{2+} ATPase assay (Fig. 5), whereas with 40 mM octylglucoside a 5-fold higher concentration of efrapeptin is required.

Among other tested general F_1 inhibitors, quercetin and NBf-Cl have been found to inhibit RrF_1 and CF_1 to a similar extent and were not affected by the presence of octylglucoside in the assay medium, whereas the inhibition of both enzymes by azide has decreased by 6- to 10-fold upon increasing the concentration of octylglucoside in the assay from 16 to 40 mM (not shown).

DISCUSSION

A total number of four Mg-dependent tightly bound nucleotides are retained on RrF_1 (Table I and Fig. 1) as well as on latent or activated CF_1 (Xue *et al.*, 1987; Shapiro *et al.*, 1991a), after passage through one or two successive centrifuge columns. The photosynthetic F_1-ATPases thus form a separate group from most respiratory F_1 ATPases, which retain six nucleotide binding sites after passage through a centrifuge column (Cross and Nalin, 1982; Wise *et al.*, 1983). However, when assayed under strict equilibrium conditions, six nucleotide binding sites have been observed also on CF_1 (Girault *et al.*, 1988; Shapiro *et al.*, 1991b), and it is therefore generally accepted that all F_1-ATPases have six nucleotide binding sites (Penefsky and Cross, 1991). The lower number of tightly bound nucleotides retained on CF_1 and RrF_1 does indicate that two of their bound nucleotides have faster k_{off} constants.

An additional difference between the two groups of F_1-ATPases is illustrated in Figs. 2 and 3. The photosynthetic enzymes used in the nucleotide binding assays are mainly Ca^{2+}-ATPases showing either no or a very low Mg^{2+}-ATPase activity, whereas both MF_1 and EcF_1 are very active Mg^{2+}-ATPases (Penefsky, 1979; Dunn and Heppel, 1981). The much lower Mg-ATPase activity of RrF_1 and CF_1 could be due to the faster K_{off} constants of two of their Mg-dependent nucleotide binding sites. Binding or incorporation of a maximal number of nucleotides has been found to depend on the presence of optimal concentrations of $MgCl_2$ in various F_1 complexes (Cross and Nalin, 1982; Shapiro *et al.*, 1991a) as well as in the isolated RrF_1 β subunit (Gromet-Elhanan and Khananshvili, 1984; Khananshvili and Gromet-Elhanan, 1984).

The very slow Mg^{2+}-ATPase activities of activated, ϵ-depleted CF_1 and native, ϵ-containing RrF_1 can be stimulated by addition of either anions, such as maleate, bicarbonate, or sulfite (Nelson *et al.*, 1972; Webster *et al.*, 1977), or detergents such as octylglucoside (Soe *et al.*, 1978; Pick and Bassilian, 1982). The anions have been reported to stimulate the Mg^{2+}-ATPase of activated CF_1 (Nelson *et al.*, 1972) or native RrF_1 (Webster *et al.*, 1977; Norling *et al.*, 1988) while showing no significant effect on their respective Ca^{2+}-ATPase activities. But the stimulation by octylglucoside is accompanied in both enzymes by a parallel inhibition of the Ca^{2+}-dependent ATP hydrolysis (Figs. 2 and 3). The effect of these two types of activating agents must therefore be very different. The effect of anions is restricted to relief of inhibition by free Mg^{2+} ions. Octylglucoside, on the other hand, exerts an additional, more direct effect on the specificity of the divalent cation binding site(s) of RrF_1 and activated CF_1 toward Ca^{2+} and Mg^{2+}. A very similar effect, of almost complete conversion of RrF_1 from Ca^{2+}- to Mg^{2+}-dependent ATPase, has been observed when ATP was replaced by the hydrolyzable analog $1,N^6$-etheno ATP (Schafer *et al.*, 1980).

An indication that changes in specificity of an F_1-ATPase toward Mg^{2+} can result from a single point mutation in one essential amino acid residue, serine 174, on the $F_1\beta$ subunit has been reported in EcF_1 (Noumi *et al.*, 1984). In the mutant this serine 174, which is fully conserved in all tested F_1-ATPases (Walker *et al.*, 1985), is changed to phenylalanine. This change results in a large decrease in the Mg^{2+}-ATPase activity but very little change in the Ca^{2+}-ATPase activity of the mutated EcF_1 (Kanazawa *et al.*, 1980; Noumi *et al.*, 1984). The neutral detergent, LDAO, has been reported to stimulate by 5- to 6-fold the EcF_1Mg^{2+}-ATPase activity, but its effect on the EcF_1 Ca^{2+}-ATPase has not been tested (Lotscher *et al.*, 1984). It would be

very interesting to compare the effect of LDAO on both Ca^{2+}- and Mg^{2+}-ATPase activities of the wild type EcF_1 and its ser 174 → Phe mutant. LDAO has also been shown to stimulate by up to 4-fold the TF_1 Mg^{2+}-ATPase (Paik *et al.*, 1993).

In addition to stimulating the Mg^{2+}-ATPase activity of various F_1-ATPases, octylglucoside and LDAO have also been shown to exert a very interesting dual effect on the action of various F_1 inhibitors. We show here that the presence of 40 mM octylglucoside decreases the inhibitory action of azide, efrapeptine, and tentoxin on RrF_1 and CF_1. Moreover, in parallel to the inactivation of the high-affinity, inhibitory tentoxin site on CF_1, octylglucoside also unmasked or activated the CF_1 low-affinity, stimulatory tentoxin site (Fig. 4).Using stimulating concentrations of LDAO, Paik *et al.*, (1993) have observed that the stimulation of the TF_1Mg^{2+}-ATPase by a high-affinity rhodamine 6G site was masked, while a low-affinity, inhibitory site for the dye was exposed. Interestingly, for both rhodamine 6G (Paik *et al.*, 1993) and tentoxin (Gromet-Elhanan and Avital, 1992) the effect of the high-affinity site has been suggested to require the interaction of the γ subunit with the α and β subunits, whereas for the low-affinity site only the α and β subunits are required.

ACKNOWLEDGMENTS

This work was supported by a grant from the United States–Israel Binational Science Foundation (BSF), Jerusalem, Israel and from the Minerva Willstätter Center for Research in Photosynthesis, Rehovot, Israel (to Z.G-E.) and by a grant DMB 91-04742 from the National Science foundation (to R.E.M.).

REFERENCES

Andralojc, P. J., and Harris, D. A. (1993). *Biochim. Biophys. Acta* **1143**, 51–61.

Bengis-Garber, C., and Gromet-Elhanan, Z. (1979). *Biochemistry* **18**, 3577–3581.

Conrad, P. L., Conrad, J. M., and Durbin, R. D. (1981). In *Energy Coupling Photosynthesis* (Selman, B. R., and Selman-Reimer, S., eds.), Elsevier/North Holland, New York, pp. 175–178.

Cross, R. L., and Kohlbrenner, W. E. (1978). *J. Biol. Chem.* **253**, 4865–4873.

Cross, R. L., and Nalin, C. M. (1982). *J. Biol. Chem.* **257**, 2874–2881.

Dunn, S. D., and Heppel, L. A. (1981). *Arch. Biochem. Biophys.* **210**, 421–436.

Feng, Y., and McCarty, R. E. (1985). *Arch. Biochem. Biophys.* **238**, 61–68.

Girault, G., Berger, G., Galmiche, J.-M., and Andre, F. (1988). *J. Biol. Chem.* **263**, 14690–14695.

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

Gromet-Elhanan, Z., and Khananshvili, D. (1984). *Biochemistry* **23**, 1022-1028.

Harris, D. A., and Baltscheffsky, M. (1979). *Biochem. Biophys. Res. Commun.* **86**, 1248–1255.

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

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

Johansson, B. C., Baltscheffsky, M., and Baltscheffsky, H. (1972). In *Proceedings of the IInd International Congress on Photosynthesis Research* (Forti, G., Avron, M., and Melandri, B., eds.), Vol. 2, Dr. W. Junk N. V., The Hague, pp. 1203–1209.

Johansson, B. C., Baltscheffsky, M., and Baltscheffsky, H. (1973). *Eur. J. Biochem.* **40**, 109–117.

Kanazawa, H., Horiuchi, Y., Takagi, M., Ishino, Y., and Futai, M. (1980). *J. Biochem.* **88**, 695–703.

Khanashvili, D., and Gromet-Elhanan, Z. (1983). *J. Biol. Chem.* **258**, 3714–3719.

Khananshvili, D., and Gromet-Elhanan, Z. (1984). *FEBS Lett.* **178**, 10–14.

Lotscher, H. R., de Jong, C., and Capaldi, R. A. (1984). *Biochemistry* **23**, 4140–4143.

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

McCarty, R. E., and Moroney, J. V. (1985). In *The Enzymes of Biological Membranes* (Martonosi, A., ed.), 2nd edn., Plenum Press, New York, pp. 383–413.

Nelson, N., Nelson, H., and Racker, E. (1972). *J. Biol. Chem.* **247**, 6506–6510.

Norling, B., Strid, A., and Nyren, P. (1988). *Biochim. Biophys. Acta* **935**, 123–129.

Noumi, T., Mosher, M. E., Nator, S., Futai, M., and Kanazawa, H. (1984). *J. Biol. Chem.* **259**, 10071–10075.

Oren, R., and Gromet-Elhanan, Z. (1977). *FEBS Lett.* **79**, 147–150.

Oren, R., and Gromet-Elhanan, Z. (1979). *Biochim. Biophys. Acta* **548**, 106–118.

Paik, S. R., Yokoyama, K., Yoshida, M., Ohta, T., Kagana, Y., and Allison, W. S. (1993). *J. Bioenerg. Biomembr.* **25**, 679–684.

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

Penefsky, H. S. (1979). *Adv. Enzymol.* **49**, 223–280.

Penefsky, H. S., and Cross, R. L. (1991). *Adv. Enzymol.* **64**, 173–214.

Pick, U., and Bassilian, S. (1982). *Biochemistry* **24**, 6144–6152.

Pick, U., Conrad, P. L., Conrad, J. M., Durbin, R. D., and Selman, B. R. (1982). *Biochim. Biophys. Acta* **682**, 55–58.

Schafer, H. J., Muller, H. W., and Dose, K. (1980). *Biochem. Biophys. Res. Commun.* **95**, 1113–1118.

Schneider, E., Muller, H. W., Rittinghaus, K., Thiele, V., Schwulera, V., and Dose, K. (1979). *Eur. J. Biochem.* **97**, 511–517.

Selman, B. R., and Durbin, R. D. (1978). *Biochim. Biophys. Acta* **502**, 29–37.

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

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

Shapiro, A. B., Gibson, K. D., Scheraga, H. A., and McCarty, R. E. (1991b). *J. Biol. Chem.* **266**, 17276–17285.

Soe, G., Nishi, N. Kakono, T., and Yamashita, J. (1978). *J. Biochem.* **84**, 805–814.

Soteropoulos, P., Süss, K.-H., and McCarty, R. E. (1992). *J. Biol. Chem.* **267**, 10348–10354.
Steele, J. A., Uchytil, T. F., and Durbin, R. D., Bhatnagar, P., and Rich, D. H. (1976). *Proc. Natl. Acad. Sci. USA* **73**, 2245–2248.
Steele, J. A., Uchytil, T. F., and Durbin, R. D. (1978). *Biochim. Biophys. Acta* **504**, 136–141.
Taussky, H. H., and Shorr, E. (1953). *J. Biol. Chem.* **202**, 675–685.
Walker, J. E., Fearnly, I. M., Gay, N. J., Gibson, B. W., Northrop, F. D., Powell, S. J., Runswick, M. J., Saraste, M., and Tybulewicz, V. L. J. (1985). *J. Mol. Biol.* **184**, 677–700.
Webster, G.-D., Edwards, P. A., and Jackson, J. B. (1977). *FEBS Lett.* **76**, 29–35.
Williams, N., Hullihen, J., and Pedersen, P. L. (1987). *Biochemistry* **26**, 162–169.
Wise, J. G., Duncan, T. M., Latchney, L. R., Cox, D. W., and Senior, A. E. (1983). *Biochem. J.* **215**, 343–350.
Xue, Z., Zhou, J.-M., Melese, T., Cross, R. L., and Boyer, P. D. (1987). *Biochemistry* **26**, 3749–3753.