Hybrid *Rhodospirillum rubrum* F$_0$F$_1$ ATP Synthases Containing Spinach Chloroplast F$_1$ $\beta$ or $\alpha$ and $\beta$ Subunits Reveal the Essential Role of the $\alpha$ Subunit in ATP Synthesis and Tentoxin Sensitivity*

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Trace amounts (~5%) of the chloroplast $\alpha$ subunit were found to be absolutely required for effective restoration of catalytic function to LiCl-treated chromatophores of *Rhodospirillum rubrum* with the chloroplast $\beta$ subunit (Avital, S., and Gromet-Elhanan, Z. (1991) *J. Biol. Chem.* 266, 7067–7072). To clarify the role of the $\alpha$ subunit in the rebinding of $\beta$, restoration of catalytic function, and conferral of sensitivity to the chloroplast-specific inhibitor tentoxin, LiCl-treated chromatophores were analyzed by immunoblotting before and after reconstitution with mixtures of *R. rubrum* and chloroplast $\alpha$ and $\beta$ subunits. The treated chromatophores were found to have lost, in addition to most of their $\beta$ subunits, approximately a third of the $\alpha$ subunits, and restoration of catalytic activity required re-binding of both subunits. The hybrid reconstituted with the *R. rubrum* $\alpha$ and chloroplast $\beta$ subunits was active in ATP synthesis as well as hydrolysis, and both activities were completely resistant to tentoxin. In contrast, a hybrid reconstituted with both chloroplast $\alpha$ and $\beta$ subunits restored only a MgATPase activity, which was fully inhibited by tentoxin. These results indicate that all three copies of the *R. rubrum* $\alpha$ subunit are required for proton-coupled ATP synthesis, whereas for conferral of tentoxin sensitivity at least one copy of the chloroplast $\alpha$ subunit is required together with the chloroplast $\beta$ subunit. The hybrid system was further used to examine the effects of amino acid substitution at position 83 of the $\beta$ subunit on sensitivity to tentoxin.

The photosynthetic F$_0$F$_1$ ATP synthases found in the thylakoids of chloroplasts and in the cytoplasmic membranes of photosynthetic bacteria couple the movement of protons down an electrochemical proton gradient to the synthesis of ATP during photophosphorylation. The general structure of these ATP synthases is highly conserved, consisting of F$_0$, the membrane-spanning proton channel, and F$_1$, the peripheral membrane sector, which contains the catalytic sites for reversible ATP synthesis. The F$_0$ is composed of five different subunits designated $\alpha$ to $e$ in order of decreasing molecular weight with a stoichiometry of $\alpha_3\beta_3\gamma_2\delta_1\epsilon_1$.

The X-ray crystal structure of bovine heart mitochondrial F$_1$ (MF$_1$) at 2.8-Å resolution (5) defined the three-dimensional structures of alternating $\alpha$ and $\beta$ subunits as forming a closed hexamer having a portion of the $\gamma$ subunit embedded in its central cavity. Among the nucleotide binding sites, which are located one at each of the six $\alpha/\beta$ interfaces, the three catalytic sites, located predominantly on $\beta$ subunits, were found to exist in three different conformational states. This asymmetric feature is compatible with the binding change mechanism, which proposed that the catalytic sites interconvert between three different conformational states during ATP synthesis via energy-dependent affinity changes in substrate binding and product release (6, 7). Several recent studies of isolated (8–10) or membrane-bound (11) F$_1$ have suggested that this is achieved via rotation of the $\gamma$ subunit relative to the $\alpha_3\beta_3$ subassembly. The MF$_1$ crystal structure identified a number of $\alpha$-$\beta$, $\alpha$-$\gamma$, and $\beta$-$\gamma$ contacts, all or some of which may be responsible for dictating the asymmetric properties of each of the catalytic sites during catalysis. The importance of each of these contacts, and the steps involved in changing them, remain to be determined.

Partially dissociated membrane ATP synthase complexes, which can be reassembled by adding isolated $\alpha$ and $\beta$ subunits, can provide suitable tools for identifying and characterizing the interacting protein domains responsible for the binding change process. One such system, where both ATP synthesis and hydrolysis can be followed, was obtained by LiCl treatment of chromatophores isolated from the photosynthetic bacterium *Rhodospirillum rubrum* (12). This treatment was found to release the bulk of their $\beta$ subunits (12, 13) resulting in the loss of over 90% of their ATP synthesis and hydrolysis activities. Both activities could be restored upon reconstituting the treated chromatophores with the released subunits. The treated chromatophores could also be reconstituted with native spinach chloroplast CF$_1$ $\beta$, although the protein preparation was contaminated with trace amounts of CF$_1$ $\alpha$ (14). More recently it was shown that the presence of small amounts of the $\alpha$ subunit was a requirement for the reconstitution of a hybrid ATP synthase with the CF$_1$ $\beta$ subunits (15, 16) or a native enzyme with RfF$_1$ $\beta$ (17, 18), suggesting the possibility that the LiCl treatment also removed some of the $\alpha$ subunit from the $\beta$ subunit.

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*The abbreviations used are: MF$_1$, CF$_1$, EcF$_1$, and RfF$_1$, F$_1$-ATPases from mitochondria, chloroplasts, *E. coli*, and *R. rubrum*, respectively; BChl, bacteriochlorophyll; WT, wild-type; Tricine, $N,N'$-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography.

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chromatophores. The identification of a small amount of an αβ3 dimer in addition to the large amount of β in the LiCl extract (19) has confirmed this possibility. Further analysis (51) has shown that the amount of α subunit, but not of β subunit, released by the LiCl treatment is dependent upon the concentration of the chromatophores during the treatment.

The hybrid RrF1/F1 CFb containing the CFb β subunit with trace amounts of the CFa α subunit was shown to have little, if any, proton-coupled ATP synthesis but 30–40% of the normal MgATPase activity (14). The hybrid MgATPase activity was fully sensitive to tentoxin, a specific CFb inhibitor (20), whereas the control or restored native RrF1/F1 ATP synthesis or hydrolysis activities were completely resistant to tentoxin (14, 21). This result suggested that the β subunit might be responsible for conferring tentoxin sensitivity to the F1 enzyme. An aspartic acid residue at position 83 of CFb1 is essential for tentoxin sensitivity of the chloroplast CF1 ATP synthase (22, 23). However, the observation that the MgATPase activity of membranes isolated from an uncD-deleted E. coli strain complemented with the chloroplast atpB gene was insensitive to tentoxin (24) suggests that additional CFb subunits might also be involved in conferral of this sensitivity.

The fact that the LiCl treatment of R. rubrum chromatophores releases some of the α subunits as well as the β subunits has allowed us to examine the interplay between the α and β subunits, which results in coupled ATP synthesis/hydrolysis and in sensitivity to tentoxin. In this study we folded insoluble recombinant CFb β (25) into a fully functional monomer using the method developed for folding the recombinant CFa1 α subunit (26) and prepared two different hybrid RrF1/F1 enzymes containing RrF1 α and CFb β or CFa1 α and CFb β subunits. The results reveal that (a) the CFb β subunit can restore a significant amount of proton-coupled ATP synthesis to treated chromatophores but only when all three copies of the RrF1 α subunit are present, and (b) the CFa1 α subunit is required, along with the CFb1 β subunit, to confer sensitivity to inhibition by tentoxin as well as high (>8-fold) stimulation by sulfate of the restored MgATPase activity.

EXPERIMENTAL PROCEDURES

Materials—CFb α, β, was isolated as described previously (27). Recombinant RrF1 α (28) and RrF1 β (17) were expressed as inclusion bodies in uncD operon-deleted E. coli DK3 or LM3115 strains, respectively, grown to steady state at 37 °C. The inclusion bodies were solubilized and the protein was folded, concentrated, and subjected to size exclusion HPLC on a Superdex-200 column (Amersham Pharmacia Biotech) as described elsewhere (26). Between 30% and 50% of RrF1 α, and over 90% of RrF1 β subunits eluted as monomers. The respective monomeric peak fractions were pooled, concentrated to 2–3 mg/ml, and stored at −80 °C. LiCl-treated R. rubrum chromatophores were prepared as described by Gromet-Elhanan and Khananshvili (29) with the modifications introduced by Nathanzon and Gromet-Elhanan (51).

Radioactive phosphate was obtained from DuPont. ATP (grade II) and tentoxin were purchased from Sigma. Tentoxin was dissolved in ethanol, diluted to a final concentration of 5 mM, and stored at −80 °C. All other chemicals were the highest quality reagent grade available.

Preparation of CFb β Mutants—All mutations were constructed by enzymatic amplification of the expression plasmid pET3a-βNE3 described previously (25) and modified as described below, using inverse PCR. The primers employed (synthesized and 5′-phosphorylated by Macromolecular Resources, Colorado State University) had abutting 5′-termini allowing for replication of the whole plasmid along with the mutation. The reverse primer was 24 nucleotides long with a base sequence corresponding to bases +238 to +257 of the wild-type atpB sequence and was used for generation of all three codon 83 mutations. The three forward primers had base sequences corresponding to bases +238 to +257. The mutations were generated by substituting the codon for aspartate, GAT, starting at position +247, with GAG, CTT, or GCT to generate codons for glutamate, leucine, or alanine, respectively. Oligonucleotides were generated with the aid of the Primer Design program (SciEd Software). The pET3a-βNE3 plasmid used only for the β mutations contained a modified β subunit gene in which the codon at position 378 (AGG) was modified to UGG, which replaced the arginine with tryptophan. This construct was found to increase the yield of folded monomeric β subunits without affecting any of the catalytic properties of the protein.2 This construct is designated as the wild-type protein in the results presented in Table II.

Plasmid DNA was prepared by ethanol precipitation following phenol/chloroform extraction (30). PCR was carried out in 50 μl of cloned Pfu DNA polymerase reaction buffer containing 4 mM total MgSO4, 20 pmol of each primer, 0.4 mM dNTPs, 60 ng of the pET3a-βNE3 plasmid DNA, and 2.5 units of cloned Pfu DNA polymerase (Stratagene). All PCR reactions were set up on ice and then placed in a GenAmp PCR System 2400 (Perkin-Elmer) pretarmed to 94 °C. The PCR reaction was continued for 25 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 12 min. PCR products were purified by gel electrophoresis followed by electroelution using an ISCO micro trap. The eluted DNA was precipitated with ethanol and circularized by incubating the eluted DNA with 3 units of T4 DNA ligase (Promega) in T4 ligase buffer overnight at room temperature. The plasmid was transformed into competent E. coli XL1-blue cells (24). Cloned plasmid was isolated from the XL1-blue cells using boiling lysis, followed by isopropanol and ethanol precipitation, and transformed into the expression host E. coli BL21(DE3)/pLyS3 (25). The mutations were confirmed by sequencing of the entire β gene using the Applied Biosystems dye deoxy terminator cycle sequencing kit.

Overexpression, Solubilization, and Refolding of WT and Mutant CFb—Recombinant E. coli cells containing either the WT or mutant atpB gene were grown, harvested, and washed as described by Chen et al. (25), except that the washing buffer contained 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and the following protease inhibitors: 1 mM phenylmethylene sulfoxyl fluoride, 2 mM benzamidine, and 10 μg/ml N-tosyl-l-lysine chloroethyl ketone. The cells were lysed by two cycles of freezing and thawing and sonicated to shear the DNA. The inclusion bodies containing the β polypeptide were washed twice, resuspended in 10–12 ml of the above buffer, and stored at −20 °C. The β polypeptide was solubilized and folded using the method developed for the refolding of RrF1 α (26), except that CFb β was solubilized using 4 mM urea and folded at a protein concentration of 0.3 mg/ml.

Reconstitution of LiCl-treated R. rubrum Chromatophores and Assays for Restored Activities—Reconstitution was carried out by incubating, for 1 h at 35 °C, LiCl-treated chromatophores equivalent to 5 μg of BC hollow with the indicated amounts of recombinant or native RF1 and CFb α and β monomers in a final volume of 0.2 ml of reconstitution buffer containing 50 mM Tricine-NaOH (pH 8.0), 25 mM MgCl2, 4 mM ATP, 1 mM EDTA, and 10% glycerol. To assay restoration of Mg ATPase activity, 0.04–0.12 ml of the reconstitution mixture was diluted into a 1-ml assay mixture containing 50 mM Tricine-NaOH (pH 8.0), 5 mM MgCl2, 4 mM sodium phosphate (containing 0.5–1.0 × 10−3 cpm [32P]ATP, 2 mM ATP, 15 mM glucose, 24 units of hexokinase, and 66 μM N-methylphosphonazinethiosulfate. The mixture was incubated in the dark at 35 °C for 3 min before starting the reaction by illumination. The reaction was terminated after 3 min by turning off the lights and adding 0.1 ml of 2 M trichloroacetic acid. The amount of γ-[32P]ATP synthesized was determined as described by Avron (32).

To assay Mg- and Ca-dependent ATP hydrolysis, the reconstituted chromatophores were washed three times with 50 mM Tricine-NaOH (pH 8.0), 50 mM NaCl, and 20% glycerol to remove residual MgCl2 and unbound subunits. Washed chromatophores equivalent to 1–3 μg of BC hollow were incubated for 20 min at 35 °C in a 0.5-m1 reaction mixture containing 50 mM Tricine-NaOH (pH 8.0), 50 mM NaCl, and either 2 mM MgCl2 or 4 mM ATP, or 5 mM CaCl2 and 5 mM ATP. The reactions were stopped by addition of 50 μl of 2 M trichloroacetic acid, and P, release was determined as described by Taussky and Shorr (33).

When assaying for sensitivity to tentoxin, the reconstituted chromatophores were preincubated with 4 μM inhibitor for 20 min at 35 °C in the complete ATP synthesis assay mixture in the dark, or in the MgATPase assay mixture lacking ATP. ATP synthesis was initiated by illumination and ATP hydrolysis by addition of 20 μl of 1 mM ATP.

Other Procedures—Protein concentrations were determined by the method of Lowry et al. (34) or by the method of Bradford (35) using bovine serum albumin as a standard. The BC hollow content of chromatophores was determined at 880 nm using the in vitro extinction coefficient given by Clayton (36). SDS-polycrylamide gel electrophoresis was carried out on 10% polycrylamide gels according to Laemmli (37).
Hybrid ATP Synthases Reveal Functional Roles of $F_1\alpha$ Subunits

RESULTS

Folding and Isolation of Recombinant $CF_1\beta$ Monomers—Size exclusion chromatography was used to assess the differences between $\beta$ polypeptides solubilized and folded from inclusion bodies using an earlier procedure (25) as compared with the method described under “Experimental Procedures.” The earlier method (Fig. 1A) yielded a protein preparation containing approximately equal amounts of monomers and dimers together with a small amount of aggregated protein. This could explain the earlier observation that only about 50% of the folded $CF_1\beta$ was in a conformation capable of binding nucleotides (25). On the other hand, preparations of $CF_1\beta$ solubilized and folded in the presence of 50 mM MgATP by the method developed for $RrF_1\alpha$ (26) was found to contain mainly monomeric $\beta$ subunits together with a small amount of aggregated protein, but no detectable dimers (Fig. 1B). Similar results were obtained for $RrF_1\beta$ (Fig. 1C).

At the protein concentrations used to fold the $\beta$ subunits (~0.3 mg/ml), the $\alpha$ subunit tended to aggregate and less than 20% of the $RrF_1\alpha$ was present in the monomeric form (data not shown). This increased to about 50% at protein concentrations of 50 mg/ml (26). The several mutant $CF_1\beta$ subunits used in this study all folded as well as the wild type protein, as judged by the percentage of monomeric protein present. The monomeric protein fractions of all folded proteins used in subsequent work were collected, pooled, concentrated, and could be stored indefinitely at ~80 °C without loss of reconstitutive activity.

Reconstitution of a Hybrid $RrF_1F_0$ from Recombinant $RrF,\alpha$ and $CF_1\beta$ Subunits—When LiCl-treated chromatophores were incubated with a fixed excess of $CF_1\beta$ and increasing amounts of $RrF_1\alpha$, a significant rate of sulfate-stimulated Mg-dependent ATP hydrolysis activity was restored. At a saturating ratio of added $RrF_1\alpha$ per BChl of about 3, this activity reached nearly 60% of that restored by reconstitution with the $RrF_1\alpha$ and $RrF_1\beta$ monomers. When reconstituted by themselves, neither $RrF_1\beta$ nor $CF_1\beta$ restored any activity. Similarly, the $RrF_1\alpha$ alone did not restore appreciable amounts of activity to the LiCl-treated chromatophores (Fig. 2). The $RrF_1\alpha$ alone or in the presence of either wild-type $CF_1\beta$ or $RrF_1\beta$. LiCl-treated chromatophores, at 5 µg of BChl, were reconstituted with increasing amounts of $RrF_1\alpha$ alone (○) or in the presence of 20 µg of $CF_1\beta$ (○) or $RrF_1\beta$ (○). ATP hydrolysis was measured as described under “Experimental Procedures” in the presence of 20 mM Na$_2$SO$_4$. The residual activity of 80 µmol ATP hydrolyzed/h per mg of BChl of the LiCl-treated chromatophores was subtracted from the values shown.

FIG. 1. Size exclusion HPLC of folded wild-type $CF_1\beta$ and $RrF_1\beta$ subunits and of native $CF_1\alpha$. A, $CF_1\beta$ folded according to Chen et al. (25); B, $CF_1\beta$ folded at 0.3 mg of protein/ml according to Du and Gromet-Elhanan (26); C, $RrF_1\beta$ refolded as in B; D, $CF_1\alpha\beta$ isolated from soluble $CF_1$ according to Gao et al. (27). The refolded subunits were concentrated to approximately 2 mg/ml, and 20 µg of each sample was loaded onto a Superdex-200 column equilibrated and washed with 100 mM sodium phosphate (pH 7.0).

FIG. 2. MgATPase activity can be restored to LiCl-treated $R$. rubrum chromatophores by reconstitution with increasing amounts of $RrF_1\alpha$ in the presence of either wild-type $CF_1\beta$ or $RrF_1\beta$. LiCl-treated chromatophores, at 5 µg of BChl, were reconstituted with increasing amounts of $RrF_1\alpha$ alone (○) or in the presence of 20 µg of $CF_1\beta$ (○) or $RrF_1\beta$ (○). ATP hydrolysis was measured as described under “Experimental Procedures” in the presence of 20 mM Na$_2$SO$_4$. The residual activity of 80 µmol ATP hydrolyzed/h per mg of BChl of the LiCl-treated chromatophores was subtracted from the values shown.

FIG. 3. An active hybrid ATP synthase is formed in LiCl-treated chromatophores reconstituted with increasing amounts of $RrF_1\alpha$ in the presence of added $CF_1\beta$. LiCl-treated chromatophores were reconstituted as described in Fig. 2 with $RrF_1\alpha$ alone (○) or in the presence of 20 µg of $CF_1\beta$ (○). Photophosphorylation was assayed as described under “Experimental Procedures.” The residual activity of 8 µmol of ATP formed/h per mg of BChl of the LiCl-treated chromatophores was subtracted from the values shown.

not restore appreciable amounts of activity to the LiCl-treated chromatophores (Fig. 2). The $RrF_1\alpha/CF_1\beta$ hybrid containing $RrF_1\alpha$ and $CF_1\beta$ also showed a significant rate of restored ATP synthesis, which closely paralleled the restoration of MgATPase activity in its dependence on increasing $\alpha$ subunit concentrations (Fig. 3). The maximal ATP synthesis activity restored at saturating $\alpha$ concentrations amounted to about 30% of the activity restored in the presence of identical ratios of $RrF_1\alpha$ and $\beta$ subunits (Table I).

The $\alpha$ and $\beta$ subunit compositions of chromatophores before and after LiCl treatment and after reconstitution with different mixtures of $\alpha$ and $\beta$ subunits were compared using immunoblots probed with a mixture of antisera produced against the $RrF_1\alpha$ and $\beta$ subunits (Fig. 4). To minimize nonspecific subunit
binding, the reconstitution of all tested native and hybrid complexes was carried out with the minimal amounts of the α and β pairs required to restore maximal activity. The antibody mixture was found to cross-react with CF1 β but not with CF1 α (Fig. 4, lane 1). The LiCl-treated chromatophores appeared to have lost almost all of the β subunits but also a significant amount of the α subunits (Fig. 4, lanes 2 and 3). Reconstitution of the treated chromatophores with a mixture of the refolded RrF1 α and β monomers resulted in their binding to an approximately equimolar ratio (Fig. 4, lane 4). Reconstitution with a mixture of RrF1 α and CF1 β led to the formation of a hybrid complex containing both RrF1 α and CF1 β together with the residual traces of RrF1 β (compare Fig. 4, lanes 4 and 5). Interestingly, when the reconstitution was carried out with either β or α alone, the amount of subunit rebound to the LiCl-treated chromatophores was much lower than when chromatophores were exposed to both subunits together (Fig. 4, compare lanes 4, 6, and 7 and lanes 5 and 8). This suggests that binding of the α and β subunits might be coordinated.

Reconstitution of a Hybrid RrF0F1 Containing CF1 α and β Subunits—An attempt to fold insoluble recombinant CF1 α subunit via the methods described for CF1 β (25) or RrF1 α (26) was unsuccessful, probably due to the rapid aggregation of the α polypeptide upon removal of the solubilizing reagent. It was, therefore, not possible to test whether purified CF1 α can, as RrF1 α, reconstitute hybrids with monomeric RrF1 β or CF1 β. However, since CF1 αβ isolated from LiCl-treated chromatophores was reported to supply the low amounts of CF1 α required for reconstitution of a hybrid RrF0F1 with isolated pure native CF1 β (16), we checked whether it can also form a hybrid with RrF1 β. The CF1 αβ used for this assay was isolated from soluble CF1, which lacked the δ and ε subunits according to Gao et al. (27), and size exclusion HPLC revealed that it was completely dissociated into a mixture of α and β monomers (Fig. 1D). The results of titrating the LiCl-treated chromatophores with increasing amounts of the αβ preparation are shown in Fig. 5 and demonstrate that this mixture could, in the absence of additional added β subunits, restore sulfite stimulated MgATPase activity to nearly the same level obtained with the RrF1 α and β subunits (compare Figs. 2 and 5). Moreover, the reconstitution showed the typical lag in attaining significant rates of ATP hydrolysis, which is associated with the normal reconstitution process (Fig. 2).

When the CF1 αβ titration was carried out in the presence of a fixed amount of the folded CF1 β monomers, there was a marked stimulation of the activity restored at low CF1 αβ concentrations. The stimulation may result from the fact that more than twice as much β than α is required for the reconstitution and so increasing the β to α ratio provides a more optimal condition for reconstitution. A much more surprising result, however, was obtained upon addition of RrF1 β, which completely blocked restoration of the MgATPase activity by the CF1 αβ (Fig. 5).

Table I compares the properties of all of the native and hybrid membrane-bound RrF0F1 complexes examined. The reconstituted native RrF0F1 restored 65–95% of the control, untreated RrF0F1 ATP synthesis and both Ca- and MgATPase activities, whereas the hybrid RrF0F1 containing only CF1 β restored 30–40% of the reconstituted native RrF0F1 Mg-dependent ATP synthesis and hydrolysis, but practically no CaATPase activity. The hybrid containing both CF1 α and β subunits restored only about 30% of the MgATPase activity, but when assayed in the presence of sulfite it reached nearly 100% of the MgATPase activity of the native reconstituted RrF0F1. Sulfite was reported to stimulate the Mg-dependent ATPase activities of control R. rubrum chromatophores (39) as well as of the reconstituted native membrane-bound RrF0F1 (18) by 2–4-fold. A 5–7-fold stimulation of the hybrid enzyme formed with CF1 β containing 5% of CF1 α was reported previously (14). The still greater sulfite stimulation observed for the hybrid enzyme containing similar amounts of CF1 α and β subunits (see Fig. 6, inset, lane 3) might be a specific property of the CF1 αβ interfaces.

Tentoxin Sensitivities of Chromatophores Containing Hybrid F1F1 Enzymes—Control R. rubrum chromatophores (14) or LiCl-treated chromatophores reconstituted with isolated native RrF1 β (14, 21), which was shown to contain ~5% of RrF1 α (17, 40), are completely resistant to inhibition by tentoxin. In contrast, LiCl-treated chromatophores reconstituted either with CF1 β containing ~5% of CF1 α (14) or with CF1 αβ (21) were found to be fully sensitive to tentoxin. However, the hybrid F0F1 enzyme reconstituted with CF1 β plus RrF1 α was unaffected by tentoxin at concentrations sufficient to fully inhibit the enzyme reconstituted with CF1 αβ (Fig. 6). Higher concentrations of tentoxin had no additional effect (data not shown). This result inferred that the α subunit also plays a role in conferring tentoxin sensitivity. To confirm that the CF1 α does indeed bind to LiCl-treated chromatophores along with the CF1 β, the membranes were solubilized and probed with antibodies raised against purified CF1 αβ (Fig. 6, inset). Chromatophores reconstituted with RrF1 α and β subunits (Fig. 6, inset, lane 1) did not bind detectable amounts of the CF1 antibodies, indicating a lack of cross-reactivity for the antibodies.
However, in chromatophores reconstituted with RrF₁ α and CF₁ β, or CF₁ αβ (Fig. 6, inset, lanes 2 and 3), similar amounts of the CF₁ β were bound in each case. Furthermore, the chromatophores incubated with CF₁ αβ bound a significant amount of the CF₁ α subunit in addition to the β subunit (Fig. 6, inset, lane 3), thus confirming that sensitivity to tentoxin does indeed correlate with the binding of both CF₁ α and β subunits to the treated R. rubrum chromatophores. The absence of any cross-reaction between the CF₁ α antibodies and RrF₁ α prevented an evaluation of the ratio of bound CF₁ α to the remaining RrF₁ α.

**Mutation of CF₁ β-Asp<sup>83</sup> Block Tentoxin Inhibition**—It was proposed earlier (22) that Asp<sup>83</sup> on the β subunit plays a critical role in conferring tentoxin sensitivity to CF₁, perhaps via a direct interaction with the toxin. We have used the chromatophore reconstitution system to test this hypothesis. Three mutant CF₁ β subunits were prepared in which Asp<sup>83</sup> was substituted for the larger acidic side chain of Glu (β-D83E), for Ala (β-D83A) with a less bulky side chain, and for Leu (β-D83L) with a bulky but uncharged side chain. The capacity of these mutants to stimulate the restoration of MgATPase activity was examined by reconstituting LiCl-treated chromatophores with a small amount of CF₁ αβ, which, by itself, restored only a low MgATPase activity, in the presence of a 100-fold excess of monomeric mutant or WT CF₁ β, which restored a 3-fold higher MgATPase activity (Table II). The hybrid chromatophores reconstituted with CF₁ αβ minus or plus additional WT CF₁ β were fully inhibited by tentoxin. In contrast, all three of the hybrid enzymes reconstituted with CF₁ αβ and the three mutant CF₁ β subunits were insensitive to tentoxin. Their complete insensitivity to tentoxin can be explained by the fact that the tentoxin-sensitive native CF₁ β monomer present in the small amount of CF₁ αβ used as a source of the α subunit (Table II) was diluted 100-fold with the added mutant CF₁ β monomers.

**DISCUSSION**

Previous work has shown that the MgATPase activity of LiCl-treated chromatophores could be restored by their reconstitution either with CF₁ β containing trace amounts (~5%) of CF₁ α (14), or with a preparation of CF₁ αβ containing an equimolar ratio of both subunits but not with a highly purified preparation of CF₁ β (15). High rates of MgATPase activity could, however, be restored to the treated chromatophores when the purified CF₁ β was supplemented with small amounts of the CF₁ αβ subunit preparation (16). We have shown here that LiCl treatment of the R. rubrum chromatophores releases a significant amount of the α subunit along with the β subunits, thus explaining the requirement for small amounts of α in addition to β subunits for restoration of ATP synthesis and hydrolysis in the treated chromatophores. In an accompanying paper (51), it is further shown that the amount of α released increases with decreasing chromatophore concentration during the LiCl treatment, whereas the bulk of the β subunit is released at all tested concentrations. It is, therefore, also possible in this system to vary the ratio of the released α and β subunits.

The preferential release of RrF₁ β, which has been demonstrated by immunoblotting of the LiCl supernatant (51) as well as of the treated chromatophores (Fig. 4, lanes 2 and 3) is not readily explained by examining the crystal structure of MF₁ in which the αββ hexamer appears to be stabilized by multiple contacts with the γ subunit (5). It can be better explained by the recently proposed structural models of the F<sub>0</sub>F<sub>1</sub> ATP synthase, which suggest the presence of two connections between the F<sub>0</sub> and F<sub>1</sub> parts of the enzyme (7, 41–43). One connection involves the γ, ε, and c subunits and is proposed to function as the rotating portion of the enzyme. The second connection contains the δ subunit and the two b subunits and is suggested to act as a stator, which binds the F<sub>1</sub> αββ hexamer to the F<sub>0</sub>
sector allowing the smaller subunits to rotate with respect to the hexamer. Earlier biochemical evidence has shown that a readily formed disulfide link between the EcF1δ and one of the α subunits did not inhibit ATPase activity (44, 45). Furthermore, with bifunctional cross-linking reagents, α-δ dimers and α-α-δ trimers, but not β-δ dimers, were identified in EcF1 (46) and TF1 (47). The formation of such dimers and trimers as well as b-δ and b-b dimers have recently been observed by cross-linking of introduced cysteine residues in EcF1F1α, δ, and b subunits (48, 49). The specific interactions between these subunits could stabilize a portion of the RrFα in the R. rubrum membranes during the LiCl treatment, thus resulting in the retention of at least one and possibly two α subunits per RrF1F1 complex while nearly all of the RrFβ subunits are removed.

The treated R. rubrum chromatophores enabled us to form two types of RrF0F1/CF1 hybrids. One contained mostly CF1β and exclusively RrFα, while the other also contained CF1α, which replaced the released portion of RrF1α. A comparison of the activities of these two hybrids with control RrF1F1 demonstrated that F1α plays an essential role in a number of catalytic properties. Two specific CF1α functions were documented; it was absolutely required, together with CF1β, for obtaining full inhibition of the restored MgATPase activity by tentoxin, as well as for the high stimulation of this restored activity by sulfite (Fig. 6 and Table I).

Earlier reports demonstrated that the binding of 1 mol of tentoxin/mol of heat-activated CF1α was sufficient for obtaining full inhibition of its ATPase activity (20). So, although the absence of any cross-reaction between RrF1α antibodies and CF1α (Fig. 4, lane 1) or vice versa (Fig. 6, inset) did not enable the determination of the ratio of the bound CF1α to the remaining RrF1α, the full inhibition of the hybrid containing both CF1α and β by tentoxin sets a lower limit of 1 mol CF1α bound/mol of reconstituted RrF0F1/CF1α. CF1β-Asp83 was shown to be required for the inhibitory action of tentoxin and residues of different charge, such as lysine, or different spacer length such as glutamate, could not replace it (22, 23). Table II illustrates that this aspartate is indeed essential for obtaining full inhibition by tentoxin in the presence of CF1α. It could not be replaced even by the noncharged leucine or by the smaller alanine, thus suggesting the importance of the CF1β-Asp83 charge and spacer length for tentoxin binding and/or inhibition.

The mitochondrial equivalent of CF1β-Asp83, MF1β-Glu67, is located at an α/β interface in the bovine heart MF1 structure (5). If the CF1β-Asp83 forms part of the tentoxin binding site, then CF1α residues located near the CF1β-Asp83 may also contribute to the binding of tentoxin. Comparison of known amino acid sequences of α subunits from both tentoxin-sensitive and -insensitive species revealed that, while all α subunits share considerable homology, a stretch of amino acid residues between 121 and 133 (chloroplast numbering) shows conservation only among the sensitive α subunits of spinach, pea, and C. reinhardtii. This stretch of amino acids is very divergent in the resistant α subunits of RrF1 and EcF1 with several amino acids differing in charge and size from the consensus sequence of sensitive CF1α subunits. In addition, several of the residues in the equivalent stretch of amino acids in MF1 are located within 10 Å of Glu67β on the β subunit. Two such residues in CF1α Ser131 and Pro132, are good candidates for having involvement in tentoxin binding. We are currently mutating these residues into RrF1α to test this possibility.

The MgATPase activity of treated chromatophores reconstituted with CF1β containing ~5% CF1α were stimulated 5–7-fold by sulfite as compared with the 2–3-fold stimulation obtained with chromatophores reconstituted with RrF1β (14). These results could not specify whether the CF1α or β or both were responsible for the extra high sulfite stimulation. The results presented in Table I indicate that, in the hybrid containing only CF1β, the stimulation is very similar to the one obtained with the control R. rubrum chromatophores, but when CF1α is also present, an 8-fold stimulation is obtained. This high stimulation was found to raise the level of the MgATPase activity restored in the hybrid enzyme formed with CF1αβ to the activity restored with RrF1α and β. The lower initial MgATPase activity of the hybrid containing CF1α and β subunits may in part reflect the overall latency of the ATPase activity of CF1α, which is considered necessary to limit wasteful ATP hydrolysis by CF1αβ, in the dark (1–3).

CF1α exerts a negative effect on the restoration of proton-coupled ATP synthesis, which was obtained only when the treated chromatophores were reconstituted with RrF1α in the presence of either RrF1β or CF1β (Table I). These results suggest that the chromatophores containing bound CF1α are not properly coupled. In contrast, the hybrid chromatophores containing CF1β in the presence of RrF1α showed significant rates of ATP synthesis, indicating that CF1β could replace at least some of the important energy coupling interactions of RrF1β. The formation of hybrid RrF0F1/CF1 with chimeric CF1α/CF1α and CF1β/RrF1β, whose preparation is now under way, should help to identify the F1α and/or β domains that are essential for the tight protein-protein interactions required for efficient proton-coupled ATP synthesis or hydrolysis.

Interestingly, although CaATPase activity is not coupled to proton translocation (50) it was not restored by any hybrid RrF0F1/CF1 (Ref. 14 and see Table I). Recent results obtained with an RrF1β-T159S mutant (51) have demonstrated that substitution of serine for threonine in the active site blocks the restoration of Ca-dependent ATPase activity while enabling full restoration of both Mg-dependent ATP synthesis and hydrolysis. These two sets of results likely demonstrate differences in the geometry of the active sites on CF1β and RrF1β, as well as on RrF1β-T159S when occupied by CaATP as compared with MgATP.
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