Assembled F$_1$-(αβ) and Hybrid F$_1$-α$_3$β$_3$γ-ATPases from Rhodospirillum rubrum α, Wild Type or Mutant β, and Chloroplast γ Subunits

DEMONSTRATION OF Mg$^{2+}$ VERSUS Ca$^{2+}$-INDUCED DIFFERENCES IN Catalytic Site Structure and Function*

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Refolding together the expressed α and β subunits of the Rhodospirillum rubrum F$_1$ (RrF$_1$)-ATPase led to assembly of only α$_3$β$_3$ dimers, showing a stable low MgATPase activity. When incubated in the presence of AlCl$_3$, NaF and either MgAD(T)P or CaAD(T)P, all dimers associated into closed α$_3$β$_3$ hexamers, which also gained a low CaATPase activity. Both hexamer ATPase activities exhibited identical rates and properties to the open dimer MgATPase. These results indicate that: a) the hexamer, as the dimer, has no catalytic cooperativity; b) aluminium fluoride does not inhibit their MgATPase activity; and c) it does enable the assembly of RrF$_1$-α$_3$β$_3$ hexamers by stabilizing their noncatalytic αβ interfaces. Refolding of the RrF$_1$-α and β subunits together with the spinach chloroplast F$_1$ (CF$_1$)-γ enabled a simple one-step assembly of two different hybrid RrF$_1$-α$_3$β$_3$/CF$_1$-γ complexes, containing either wild type RrF$_1$-β or the catalytic site mutant RrF$_1$-βT159S. They exhibited over 100-fold higher CaATPase and MgATPase activities than the stabilized hexamers and showed very different catalytic properties. The hybrid wild type MgATPase activity was, as that of RrF$_1$ and CF$_1$, and unlike its higher CaATPase activity, regulated by excess free Mg$^{2+}$ ions, stimulated by sulfite, and inhibited by azide. The hybrid mutant had on the other hand a low CaATPase but an exceptionally high MgATPase activity, which was much less sensitive to the specific MgATPase effectors. All these very different ATPase activities were regulated by thiol modulation of the hybrid unique CF$_1$-γ disulfide bond. These hybrid complexes can provide information on the as yet unknown factors that couple ATP binding and hydrolysis to both thiol modulation and rotational motion of their CF$_1$-γ subunit.

The F$_0$F$_1$-ATP synthases catalyze the synthesis of ATP from ADP and P$_i$ at the expense of a transmembrane electrochemical proton gradient generated by the respiratory or photosynthetic electron transport chains. Its membrane-bound F$_0$ sector functions as the proton pathway and has, in bacterial and photosynthetic cells, a subunit composition of a$_1$β$_1$ε$_3$ and a$_1$β$_1$γ$_3$ respectively. The catalytic F$_1$ sector, which functions as a soluble ATPase, has in all cells a subunit composition of a$_3$β$_3$ε$_3$, and six nucleotide-binding sites located on the α and β subunits. Its three catalytic β sites show high negative cooperativity in substrate binding and strong positive cooperativity in catalysis (1–5). The minimal F$_1$ subcomplex, which resembles the whole F$_0$-ATPase in its catalytic properties, is the F$_1$-α$_3$β$_3$γ-ATPase. Such subcomplexes were reconstituted from either native (6, 7) or recombinant (8, 9) individual subunits of the respiratory TF$_1$ and EcF$_1$-ATPases. Similar highly active pure photosynthetic subcomplexes have been assembled up to now only by incubating an isolated native CF$_1$-(αβ) complex with a native (10) or recombinant (11) γ subunit.

A high resolution x-ray structure of the bovine heart mitochondrial MF$_1$ (12) demonstrated the alternating arrangement of the α and β subunits in a closed hexamer, with the resolved N- and C-terminal α-helices of the γ subunit embedded in its central cavity and all six nucleotide-binding sites residing at alternating αβ interfaces. The three catalytic sites, located mainly on the β subunit, appeared in three different conformational states that, in association with the unique resolved part of the γ subunit, imposed an asymmetric structure on the α$_3$β$_3$ hexamer (12). This MF$_1$-α$_3$β$_3$γ structure is compatible with the binding change mechanism (3, 13), which proposed that ATP synthesis involves transitions between different but interacting catalytic sites, via energy-dependent affinity changes in substrate binding and product release. Such transitions were first suggested to occur via movement or rotation of a cluster of the catalytic α$_3$β$_3$ subunits around a core of the single copy γ or ε subunit (14). The reversible proton-transporting ATP hydrolysis was later proposed to generate rotation of the F$_1$-γ subunit which, when transmitted to F$_0$, could result in pumping of protons back across the membrane (15), possibly via coupled rotation of the F$_1$-γ subunit with the F$_0$-c subunits (3, 16). MgATPase-induced rotation of γ within immobilized α$_3$β$_3$ hexamers was observed in genetically engineered respiratory TF$_1$- and EcF$_1$-α$_3$β$_3$γ complexes (17, 18). But its further coupling with F$_0$-c rotation, which has been tested in some recent reports, did not yield clear results (19–21). So there is at

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1 The abbreviations used are: CF$_1$, EcF$_1$, MF$_1$, RrF$_1$, and TF$_1$, F$_1$-ATPases from chloroplasts, E. coli, mitochondria, R. rubrum, and thermophilic Bacillus PS8, respectively; α$^1$ and β$^1$, the α and β subunits of the RrF$_1$-ATPase; γ$^3$, the γ subunit of spinach chloroplast CF$_1$-ATPase; DTT, dithiothreitol; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; Tricine, N,N,N′-tris(hydroxymethyl)methylglycine; WT, wild type.
present no direct correlation between γ rotation and proton-coupled ATP synthesis and hydrolysis.

Full elucidation of the detailed mechanism of action of the F_{0}F_{1}-ATP synthase will depend on the identification of the specific domains that participate in its proton-coupled ATP synthesis and hydrolysis as well as in the regulation of these reversible activities and their possible correlation with the γ or γ-c rotation. Tight regulation of ATP hydrolysis is especially important in photosynthetic cells, where it prevents the depletion of essential cellular ATP pools in the dark (1, 4, 5). Plant chloroplasts and bacterial chromatophores have a number of such regulatory pathways, which operate in their membrane-bound F_{0}F_{1} as well as the solubilized F_{1}-ATPases. Both chloroplasts (22) and chromatophores (23) show a high sensitivity to inhibition by excess free Mg^{2+} ions, which results in a drastic decrease of their MgATPase activities at Mg/ATP ratios above 0.5. A unique chloroplast regulatory system, termed thiol modulation, involves reduction-oxidation of a disulfide bond (24) formed between Cys^{199} and Cys^{205} in a region of its γ subunit that does not appear in any other F_{1}-γ subunits (25). But there are as yet no assembled CF_{1}-αβγ or similar photosynthetic complexes that can be engineered for studies aimed at elucidating the molecular mechanism of their regulatory systems and the possible ATPase-induced rotation of their γ subunit.

In this investigation, we have used our earlier developed procedure for refolding and assembly of the α and β subunits of the photosynthetic bacterium Rhodospirillum rubrum into α^{R}\beta^{R} dimers (26) to follow their further association into α^{R}\beta^{R} hexamers, and with the recombinant chloroplast γ (11), into hybrid α^{R}\beta^{R}γ^{C}-ATPases. Two types of such highly active hybrids were assembled, containing WT or the catalytic site mutant β^{R}-T159S (27), and both retained the specific γ^{C} redox regulation. These hybrid complexes provide the first photosynthetic F_{1} assemblies that can be genetically engineered for probing rotational catalysis. Both hybrids show, besides a very high MgATPase also a CaATPase activity, that in the hybrid WT α^{R}\beta^{R}γ^{C} is much higher than the MgATPase of the hybrid mutant α^{R}\beta^{R}γ^{C}—was prepared by the molecular mechanism of their regulatory systems and the possible ATPase-induced rotation of their γ subunit.

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**EXPERIMENTAL PROCEDURES**

**Materials**—The recombinant α^{R} (26, 28), WT β^{R} (29), and the mutant β^{R}-T159S (27) subunits were expressed in insoluble inclusion bodies and solubilized by urea, as described by Du and Gromet-Elhanan (26). Recombinant γ^{C} was expressed and refolded as described by Sokolov et al. (11). RrF_{1} was prepared from R. rubrum chromatophores according to Weiss et al. (30). All other reagents were of the highest purity available.

**Assembly and Isolation of α^{R}\β^{R} Dimers**—The dimers were assembled by refolding the urea-solubilized α^{R} and β^{R} together according to the procedure developed for their optimal refolding into functional monomers (26). The refolded mixture was concentrated to about 1 mg/ml by Centriprep-10 (Amicon), precipitated with 60% saturated (NH_{4})_{2}SO_{4}, and resuspended in TGN buffer containing 50 mM Tricine-NaOH, pH 8.0, 20% glycerol, and 50 mM NaCl. The remaining insoluble aggregates were removed by centrifugation, and the refolded mixture was loaded on the size-exclusion HPLC Superdex-200 column (Amersham Pharmacia Biotech) and eluted with 100 mM NaFp, pH 7.0, containing 10% glycerol at a flow rate of 0.5 ml/min. The pooled α,β dimer peak was concentrated, transferred to TGN buffer by elution-centrifugation through Sephadex G-50 columns, and stored at −80 °C. Assembly of α^{R}\β^{R} Hexamers—The isolated α^{R}\β^{R} dimers could be fully converted into the α^{R}\β^{R} hexamers only when incubated, at >1 mg of protein/ml for 1 h at 22 °C, in TGN buffer containing also 10 mM NaFp and 0.5 mM AlCl_{3}, in the presence of either 1 mM of CaCl_{2} or MgCl_{2} and 1 mM ATP or ADP (see Fig. 1D). Their activities could therefore be assayed directly by diluting samples assembled in the presence of each cation and ADP into the same cation-ATPase assay mixtures.

**Assembly and Isolation of the Hybrid WT and Mutant α^{R}\β^{R}γ^{C}-Hybrids**—The hybrid WT α^{R}\β^{R}γ^{C} was assembled by two procedures as follows: 1) incubation of the isolated α^{R}\β^{R} dimers with refolded γ^{C} for 1 h at 22 °C in TGN buffer in the presence of 1 mM MgCl_{2} or CaCl_{2} and 1 mM ADP which in this mixture, as in the hexamer assembly mixture, is as effective as ATP. Each incubated sample could therefore be assayed directly for its ATPase activity as well as its ATP-dependent HPLC. 2) Refolding the urea-solubilized recombinant α^{R} and β^{R} each at 50 μg/ml, together with urea-solubilized γ^{C} at 20 μg/ml, according to the procedure developed above for assembly of α^{R}\β^{R} dimers. The assembled hybrid complex was isolated by size-exclusion HPLC as described for the dimers, except that the hybrids were eluted with buffer containing 100 mM Tricine-NaOH, 50 mM NaCl and 10% glycerol. The peak containing the hybrid complex was pooled, concentrated, exchanged into TGN buffer as described for the dimers, and stored at −80 °C. The hybrid mutant α^{R}\β^{R}γ^{C} complex was assembled and isolated as described for the hybrid WT.

**Assays of ATPase Activities**—The activities of RrF_{1} and both hybrid α^{R}\β^{R}γ^{C} and WT and mutant complexes were measured with 4–20 μg of protein for 5 min at 35 °C in 0.5 ml of an assay mixture containing 50 mM Tricine-NaOH, pH 8.0, 50 mM NaCl, 4 mM ATP, and 2 mM of either MgCl_{2} or MnCl_{2} or 4 mM CaCl_{2}. The activities of the isolated α^{R} and β^{R} dimers were measured with 30 μg of protein for 30 min under the conditions described above. To compare the ATPase activities of the α^{R}\β^{R} and hexamers with those of RrF_{1}, the hexamers were first assembled by incubating the dimers and γ (described above, and Ref. 26) under identical conditions. The ATPase activities of this RrF_{1}, and the freshly assembled α^{R}\β^{R} hexamers were measured by dialuting each incubation mixture into the relevant assay mixtures, which also contained 10 mM NaF and 0.5 mM AlCl_{3}. All ATPase activity assays were started by adding the protein complexes and stopped by adding 50 μl of 2 × trichloroacetic acid, and the released Pi was measured as described by Tauskys and Shorr (31). The effect of reduction or oxidation on the ATPase activities of RrF_{1}, and the hybrid complexes was tested by their preincubation for 1 h at 35 °C in TGN buffer containing either 10 mM DTT or 100 μM CuCl_{2}, followed by dilution into the relevant assay mixtures.

**Other Procedures**—SDS-PAGE was carried out on the Nuva Precast Tris-glycine gradient gels. The protein bands were visualized by staining with Coomassie Brilliant Blue R-250. Protein concentrations were determined by the Bradford method (32) or according to Lowry et al. (33), using bovine serum albumin as a standard.

**RESULTS**

**Stepwise Assembly of RrF_{1}-α^{R}\β^{R} Dimers and α^{R}\β^{R}γ^{C} Hexamers**—Active dimers, but no hexamers, have been assembled by incubation of the isolated α^{R} and β^{R} monomers for 5 min at 35 °C in the presence of MgATP (26). These dimers showed a maximal MgATPase rate of 0.14 units/mg of protein, which remained linear for at least 1 h at 35 °C. In search for conditions that might enable the assembly of α^{R}\β^{R}γ^{C} hexamers, the urea-solubilized α^{R} and β^{R} subunits (26) were refolded together as described under “Experimental Procedures.” They did indeed assemble directly, but again only into α^{R}\β^{R}γ^{C} dimers with no indication for the appearance of any larger complexes (Fig. 1A). This simple one-step refolding/assembly procedure enabled the isolation of large amounts of the pooled concentrated dimer peak, which remained very stable in TGN buffer at protein concentrations above 1 mg/ml, even when incubated for 1 h at 22 °C (Fig. 1B). However, when diluted to the 10–20-fold lower protein concentrations used for ATPase activity assays, they remained stable only in presence of either MgADP or MgATP or even CaADP (not shown).

The closed αβ̄ hexamer, resolved in the x-ray crystallographic structure of bovine mitochondrial MF_{1} (12), has all six F_{1} nucleotide-binding sites arranged at alternating catalytic and noncatalytic α/β interfaces. The isolated α^{R}\β^{R} dimers have therefore either of the two catalytic interfaces. One fact that their MgATPase activity (26) is similar to that of CF_{1}-αβ̄ (10, 34) indicates that these dimers contain the catalytic nucleotide-binding site at their αβ interface. Their inability to associate into an α^{R}\β^{R}γ^{C} hexamer therefore seems to reflect a very specific lower stability of the noncatalytic RrF_{1} αβ interface. Indeed from the R. rubrum chromatophore-bound
Refolding of the RrF1-αβ3 dimers and their further association into stable α3β3 hexamers in the presence of aluminum fluoride, allows the urea-solubilized α and β to be folded together, and 15 μg of the concentrated product were loaded on a Superdex-200 column and eluted as described under "Experimental Procedures"; B–D, 15 μg of the pooled α3β3 dimer peak, concentrated up to 1.5 mg of protein/ml as described under "Experimental Procedures," were incubated in TGN buffer for 1 h at 22 °C with the following additions: B, none; C, 10 mM NaF and 0.5 mM AlCl3; D, as C plus 1 mM MgAD(T)P or CaAD(T)P; E and F were incubated as in D, diluted by 10-fold into TGN buffer, and further incubated at 1 h at 22 °C without (E) or with (F) 10 mM NaF, 0.5 mM AlCl3, and 1 mM MgAD(T)P or CaAD(T)P. All incubated samples were loaded on the column and eluted as described for A.

RrF1, only native dimers have been isolated (35). From chloroplast, on the other hand, only unstable α3β3 hexamers could be obtained, and they readily dissociated into mixtures of their respective α and β monomers (10, 36).

A search for compounds or conditions that can stabilize the noncatalytic RrF1, αβ interfaces and enable the association of the α3β3 dimers into hexamers has yielded the results demonstrated in Fig. 1, C and D. Incubation of the concentrated stable dimers (Fig. 1B) with NaF and AlCl3 that form aluminum fluoride (AlF3) transition state analog of F1 nucleotide-binding sites (37–40), resulted in their partial conversion into the closed α3β3 hexamers (Fig. 1C). Full association of these dimers into hexamers was obtained by their incubation with both NaF and AlCl3 in the presence of either Mg2+ or Ca2+ and either ADP or ATP at a cation/nucleotide ratio of 1 (Fig. 1D). AIF3 was reported earlier to inhibit various F1-MgATPases but only after their very specific stepwise preincubation first with low ADP and high MgCl2 concentrations, then with NaF, and finally with AlCl3. The structure of bovine MF1, fully inhibited by this procedure, has recently been resolved (41). It shows that both aluminum trifluoride and Mg2+-ADP were bound, in a quasi-reversible manner, to the catalytic nucleotide-binding site of the MF1-βDP subunit. However, under our very different one-step incubation conditions, which associated practically all the α3β3 dimers into α3β3 hexamers (compare Fig. 1, B and D), the RrF1-MgATPase activity was hardly affected by AIF3 (see Fig. 5). Furthermore, these α3β3 hexamers remained fully stable only in the presence of AIF3 and Mg- or CaAD(T)P at cation/AD(T)P ratios of 1 (Fig. 1, E and F). So under our association conditions AIF3 does not bind irreversibly to the catalytic αβ interface of the dimers and does not inhibit their MgATPase activity. It rather seems to bind in a reversible manner to the open noncatalytic nucleotide-binding site on the dimer α3 subunit and facilitate its association with the β3 of another dimer, leading to their assembly into the closed hexameric structure.

Assembly of Hybrid WT and Mutant F1-α3β3γ Complexes—Incubation of the α3β3γ dimers with an F1-γ subunit instead of AIF3 resulted in their assembly into a stable, highly active α3β3γ complex (Fig. 2). A recombinant CF1-γ subunit was used for these studies since there is as yet no available native or recombinant γ. This γ was found to assemble with the native unstable CF1-α3β3γ into a stable highly active α3β3γ complex (11), and its incubation with the isolated α3β3γ dimers resulted in their assembly into a hybrid α3β3γ complex (Fig. 2, A and B). This assembly could also be followed by the dramatic 100-fold increase (Fig. 3) of the low 0.01–0.15 units/mg MgATPase activity of the α3β3γ dimers (26). But unlike the fast assembly of the α3 and β3 monomers into dimers, which reached their maximal MgATPase activity after a 5-min incubation at 22 °C (26), this assembly was slow, requiring about 60 min for completion. A very similar time dependency was reported for the assembly of a CF1-(αβγ) complex from isolated native CF1-(αβ) and a CF1-γ complex (10). The increase in activity during the assembly of the α3β3γ complex was also fully dependent on the amount of γ, saturating at a molar...
of a hybrid mutant complex containing the γC subunit were incubated at 22 °C in 50 μl of TGN buffer without (Fig. 3, inset). The relatively high amount of γC required for obtaining this saturated activity was due to the tendency of both native and refolded γC to aggregate, since they remained soluble only when stored in a buffer containing 0.3 M LiCl at pH 9.5 (10, 11). So when γC was diluted into the incubation buffer with the αRβRγC complex (Fig. 2C), it partially precipitated out during their slow assembly into the hybrid αRβRγC complex. The time- and γC-dependent increase in the MgATPase activity during the assembly is therefore presented in units/mg αβ (Fig. 3).

The hybrid αRβRγC was also assembled by refolding together all three urea-solubilized subunits under the conditions developed for refolding the αRβRγC dimers (see Fig. 1A). This much simpler one-step refolding procedure, which resulted in direct assembly of the αRβRγC complex (Fig. 2C), enabled the isolation of large amounts of a pure, fully stable hybrid WT complex (Fig. 2D and inset). It was also used for the assembly of a hybrid mutant complex containing the βR-T159S catalytic site mutant (27). This mutant ββ subunit was shown to bind in the presence of small amounts of monomeric αR into a β-less chromatophore membrane-bound RrF1βF, which also lacked about 20% of its α subunit and lost all ATP synthesis and hydrolysis activities. The reconstituted chromatophores regained all their Mg2+-dependent activity but none of the Ca2+-dependent activities (27). Modulation of the Hybrid WT and Mutant F1αβγ/CF1γ Complexes by Their γC Oxidation/Reduction—A unique feature of the chloroplast CF1 ATPase activity is its high regulation by the reduction/oxidation of the disulfide bond formed between Cys199 and Cys205 in its γC subunit (24). The region containing these cysteine residues is completely missing from respiratory F1γ subunits, as well as from the γ subunit of cyanobacteria and purple photosynthetic bacteria, including the RrF1γ (25). All ATPase activities of RrF1 showed indeed no response to either reduction by DTT or oxidation by CuCl2 (Fig. 4A). However, both γC-containing hybrid WT and mutant F1-
Specific Properties of Assembled $RrF_1\alpha_\beta_\gamma$-CF$_1\gamma$ Hybrids

The properties of the CaATPase activities of the $RrF_1$ and both hybrid WT and mutant complexes were very different from their MgATPase activities (compare Figs. 5 and 6). They were dependent on the presence of CaATP, reaching saturation at a Ca/ATP ratio of 1.0, but showed no inhibition even at a ratio of 5.0. They were thus rather similar to the MgATPase activities of the dimers and hexamers (see Fig. 5, inset).

A detailed comparison of the Ca$^{2+}$- and Mg$^{2+}$-dependent ATPase activities of $RrF_1$ and all assembled complexes (Table I) demonstrated a number of additional most interesting differences as follows:

1) Between the dimers and hexamers. The dimers have practically no CaATPase activity, although their MgATPase is similar in its rates and properties to that of the hexamers (Fig. 5 and Table I). These results illuminate clear differences in the structure of catalytic nucleotide-binding sites occupied by Ca$^{2+}$ versus Mg$^{2+}$, since CaATP can bind to these sites and enable the appearance of CaATPase activity only in the closed hexamer. On the other hand, the identical and very low dimer and hexamer MgATPase activities, which in the dimers cannot have any catalytic cooperativity, suggest its absence also in the hexamers. Indeed, both dimer and hexamer MgATPases do not respond to any MgATPase effectors of $CF_1$ and $RrF_1$. They are not stimulated by sulfite or inhibited by azide (Table I).

2) Between both dimers and hexamers and the $RrF_1\pm$AlF$_3$ as well as the hybrid WT complex. The $\gamma$-containing complexes show much higher Ca- and MgATPase activities as well as clear differences between the functional properties of these two ATPase activities. Their MgATPases are tightly regulated by excess free Mg$^{2+}$ ions (Fig. 5), highly stimulated by sulfite and methanol (not shown) and inhibited by azide (Table I). But their 3–10-fold higher CaATPase activities are not regulated (Fig. 6) and, as both dimer and hexamer MgATPase activities, do not respond to any tested MgATPase effectors (Table I).

3) Between the $RrF_1$, its assembled $\alpha_\beta_\gamma$ dimers, and hybrid WT $\alpha_\beta_\gamma\beta$-T159S complexes. The MgATPase activities of $RrF_1$, and the dimers (\(\gamma\)), hybrid WT (\(\gamma\)), and mutant (\(\gamma\)) complexes were measured as described under “Experimental Procedures” using 4 mM ATP and the indicated concentrations of MgCl$_2$. For comparing the activities of $RrF_1$, the dimers (\(\gamma\)) and the hexamers (\(\gamma\)), the dimers were first assembled into the hexamers by incubation in the presence of 10 mM NaF, 0.5 mM AlCl$_3$, and 1 mM MgADP as described under “Experimental Procedures,” and $RrF_1$ underwent the same treatment. Both complexes were diluted into the assay mixtures described above, except that all of them contained also 10 mM NaF and 0.5 mM AlCl$_3$. The highly active hybrid WT (\(\gamma\)) and mutant (\(\gamma\)) complexes are presented in the main figure. Inset presents the much lower activities of $RrF_1$, (\(\gamma\)), the dimers (\(\gamma\)), and hexamers (\(\gamma\)).

The MgATPase activities of $RrF_1$, its assembled $\alpha_\beta_\gamma$ dimers, and hybrid WT $\alpha_\beta_\gamma\beta$-T159S complexes were dependent on the presence of CaCl$_2$, reaching saturation (Fig. 6) and, as both dimer and hexamer MgATPase activities, do not respond to any tested MgATPase effectors (Table I).

4) Between the hybrid WT and mutant complexes. Both $RrF_1$ and the hybrid WT showed a 3–10-fold higher CaATPase than MgATPase activities. On the other hand, the hybrid mutant showed a much higher MgATPase activity than either $RrF_1$ or the hybrid WT complex but a lower CaATPase activity (Figs. 5 and 6). Its CaATPase, as well as all CaATPase activities, was not regulated and did not respond to any tested MgATPase effectors. But the very high MgATPase activity of this mutant was also much less responsive to these effectors (Table I).

DISCUSSION

In this study large amounts of highly active hybrid WT and mutant photosynthetic $F_1\alpha_\beta_\gamma$ complexes were assembled by refolding the recombinant $R.\ rubrum\ RrF_1\alpha$ and WT $\beta$ (26) or mutant $\beta$-T159S subunits (27) together with the spinach $CF_1\gamma$ (11). All ATPase activities of both isolated hybrid complexes showed, unlike those of $RrF_1$, the specific thiol modulation (24) of their unique $\gamma$ disulfide bond. Also all ATPase activities of the hybrid WT $\alpha_\beta_\gamma\beta$-T159S, which were between 9- and 30-fold higher than those of the $\epsilon$-containing $RrF_1$ (Table I), retained the catalytic properties of both $RrF_1$ and $CF_1$-ATPases. This includes the specific regulation of the photosyn-
Specific Properties of Assembled RrF₁αββ₁/CF₁γ Hybrids

Table I

| Tested complexes | MgATPase None | MgATPase +Sulfite | CaATPase None | CaATPase +Sulfite | CaATPase +Na₃ |
|------------------|---------------|------------------|---------------|------------------|---------------|
| α₁β₁β₂γ R⁺ | 0.10          | 0.12             | 0.11          | 0.02             | 0.02          |
| α₁β₁β₂γ R⁺ +AlF₅⁻ | 0.10       | 0.13             | 0.12          | 0.10             | 0.14          |
| RrF₁β₁γ | 0.49          | 7.81             | 0.06          | 4.41             | 6.43          |
| RrF₁β₁γ +AlF₅⁻ | 0.42       | 3.81             | 0.09          | 1.10             | 1.96          |
| α₁β₁β₂γ γ R⁺ | 16.1         | 35.7             | 0.1           | 41.8             | 38.3          |
| α₁β₁β₂γ γ R⁺ | 31.5         | 36.3             | 11.9          | 10.7             | 8.8           |

Assembly and/or isolation of all complexes is described under “Experimental Procedures.” The α₁β₁β₂γ R⁺ hexamers were assembled and assayed in the presence AlCl₃ and NaF, which form AlF₅⁻, and the RrF₁β₁γ R⁺ was incubated and assayed under identical conditions. The ATPase activities were measured as described under “Experimental Procedures” with 4 mM ATP and 2 mM MgCl₂ or 4 mM CaCl₂, either with no additions (None) or with 50 mM sulfite or 2 mM azide.

The hybrid WT α₁β₁β₂γ R⁺ complex provides a most suitable candidate for studies aimed at elucidating the molecular mechanism involved in the γ subunit interaction with the α₂β₃ catalytic subcomplex. This hybrid complex provides a most suitable candidate for studies aimed at elucidating the molecular mechanism involved in the γ subunit interaction with the α₂β₃ catalytic subcomplex. This hybrid complex provides a most suitable candidate for studies aimed at elucidating the molecular mechanism involved in the γ subunit interaction with the α₂β₃ catalytic subcomplex. This hybrid complex provides a most suitable candidate for studies aimed at elucidating the molecular mechanism involved in the γ subunit interaction with the α₂β₃ catalytic subcomplex.
the geometry of either the WT or mutant βc catalytic sites when occupied by Ca2+ as compared with Mg2+. One is operating in the soluble state and a different one in the membrane-bound state, whose βc-T159S-containing RrF0F1 shows the maximal difference between the fully operative proton-coupled Mg2+-occupied sites and the complete absence of any active Ca2+-occupied catalytic sites. The similar catalytic properties of the highly active hybrid mutant MgATPase and the hybrid WT CaATPase make them very promising tools for obtaining information on the as yet unknown factors that couple ATP binding and hydrolysis to rotational motion of the γ subunit of the catalytic F1-ATPase. Since azide does not inhibit the CaATPase activity, another inhibitor will be required for such comparative studies. A very suitable candidate is the specific CF1 effector tentoxin, which at low concentrations inhibits but at high concentrations stimulates both CF1 Ca- and MgATPase activities (30). RrF1 is, however, completely resistant to tentoxin. We have therefore assembled another set of hybrids composed of WT and mutated αg together with βc and γc. They provide the possibility of assaying both γc thiol modulation and rotational catalysis in the presence of inhibitory as well as stimulating tentoxin concentrations.

REFERENCES

1. Gromet-Elhanan, Z. (1985) in Anoxygenic Phototrophic Bacteria (Blankenship, R. E., Madigan, M. T., and Bauer, C. E., eds) pp. 807–830, Kluwer Academic Publishers Group, Dordrecht, The Netherlands
2. Weber, J., and Senior, A. E. (1997) Biochim. Biophys. Acta 1359, 19–58
3. Boyer, P. D. (1981) Annu. Rev. Biochem. 50, 717–749
4. Richter, M. L., Hein, R., and Huchzermeyer, B. (2000) Biochim. Biophys. Acta 1458, 326–342
5. McCarty, R. E., Evron, Y., and Johnson, E. A. (2000) Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 83–109
6. Yoshida, M., Sone, N., Hirata, H., and Kagawa, Y. (1977) J. Biol. Chem. 252, 3480–3485
7. Futai, M. (1977) Biochem. Biophys. Res. Commun. 70, 1231–1237
8. Noami, T., Azuma, M., Shimomura, S., Maeda, M., and Futai, M. (1987) J. Biol. Chem. 262, 14978–14982
9. Yohda, M., Ohba, T., Hisabori, T., and Kagawa, Y. (1988) Biochim. Biophys. Acta 933, 156–164
10. Gao, F., Lipscomb, B., Wu, 1., and Richter, M. (1995) J. Biol. Chem. 270, 9763–9769
11. Sokolov, M. L., Tucker, G., Gao, F., Gengerheimer, P. A., and Richter, M. L. (1999) J. Biol. Chem. 274, 13824–13829
12. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621–628
13. Boyer, P. D. (1991) Biochim. Biophys. Acta 1140, 215–250
14. Boyer, P. D., and Kohlrenner, W. E. (1981) in Energy Coupling in Photosyn...