Mutations in the β-Subunit Thr$^{159}$ and Glu$^{184}$ of the *Rhodospirillum rubrum* F$_0$F$_1$ ATP Synthase Reveal Differences in Ligands for the Coupled Mg$^{2+}$- and Decoupled Ca$^{2+}$-dependent F$_0$F$_1$ Activities

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Lubov Nathanson$^3$ and Zippora Gromet-Elhanan$^\S$

From the Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

In the crystal structure of the mitochondrial F$_1$-ATPase, the β-Thr$^{163}$ residue was identified as a ligand to Mg$^{2+}$ and the β-Glu$^{188}$ as directly involved in catalysis. We replaced the equivalent β-Thr$^{159}$ of the chromatophore F$_0$F$_1$ ATP synthase of *Rhodospirillum rubrum* with Ser, Ala, or Val and the Glu$^{184}$ with Gln or Lys. The mutant β subunits were isolated and tested for their capacity to assemble into a β-less chromatophore F$_0$F$_1$ and restore its lost activities. All of them were found to bind into the β-less enzyme with the same efficiency as the wild type β subunit, but only the β-Thr$^{159}$ → Ser mutant restored the activity of the assembled enzyme. These results indicate that both Thr$^{159}$ and Glu$^{184}$ are not required for assembly and that Glu$^{184}$ is indeed essential for all the membrane-bound chromatophore F$_0$F$_1$ activities. A detailed comparison between the wild type and the β-Thr$^{159}$ → Ser mutant revealed a rather surprising difference. Although this mutant restored the wild type levels and all specific properties of this F$_0$F$_1$ proton-coupled ATP synthase as well as Mg$^{2+}$- and Mn$^{2+}$-dependent ATP hydrolysis, it did not restore at all the proton-decoupled CaATPase activity. This clear difference between the ligands for Mg$^{2+}$ and Mn$^{2+}$, where threonine can be replaced by serine, and Ca$^{2+}$, where only threonine is active, suggests that the β-subunit catalytic site has different conformational states when occupied by Ca$^{2+}$ as compared with Mg$^{2+}$. These different states might result in different interactions between the β and γ subunits, which are involved in linking F$_1$ catalysis with F$_0$ proton-translocation and can thus explain the complete absence of Ca-dependent proton-coupled F$_0$F$_1$ catalytic activity.

The F$_0$F$_1$ ATP synthase-ATPase complexes, found in the inner membranes of mitochondria and chloroplasts and in bacterial plasma membranes, couple ATP synthesis and hydrolysis to electrochemical proton gradients. These complexes are composed of a membrane-intrinsic F$_0$ sector, which mediates proton translocation, and an extrinsic F$_1$ sector, which carries the catalytic sites. All isolated F$_1$ complexes are composed of five subunits with a stoichiometry of α$_3$β$_2$γδε (1–6).

The crystal structure of bovine mitochondrial MF$_1$ (7) presents the large α and β subunits arranged alternately in a closed hexamer around the N- and C-terminal helices of the γ subunit. The three catalytic β subunits show a clear difference in bound nucleotides resulting in different conformational states. The asymmetric structure imposed on this hexamer by the interaction of the γ-subunit with these different β-subunits, supports the binding change mechanism (8), which proposed that ATP synthesis and hydrolysis involve transitions between different but interacting catalytic sites via rotation of the γ subunit relative to an αβ$_2$ subassembly. Several models suggested that proton-translocation through F$_0$ results in a coupled rotation of the F$_0$-γ and F$_1$-γ subunits (8–11). However full elucidation of the detailed mechanism of action of the F$_0$F$_1$ ATP synthase will depend on identification of the specific residues and/or whole domains that participate in proton-coupled ATP synthesis and hydrolysis as well as in the regulation of these reversible activities.

Tight regulation of ATP hydrolysis is especially important in photosynthetic organisms where it prevents the depletion of essential cellular ATP pools in the dark (3–5). One stringent regulatory pathway operating in plant chloroplasts (12) as well as in bacterial chromatophores (13, 14) is their high sensitivity to inhibition by excess free Mg$^{2+}$ ions, which results in optimal MgATPase activity at Mg$^{2+}$/ATP ratios around 0.5 and its drastic decrease at higher ratios. F$_1$-α, β, and γ subunits from photosynthetic sources are therefore very interesting targets for mutational analysis, based on the available MF$_1$ crystal structure, of amino acid residues participating in coupled catalysis and its regulation.

The F$_1$-α and β subunits of the photosynthetic bacterium *Rhodospirillum rubrum* are most suitable for such studies. RrF$_1$β$^1$ was isolated in large amounts from the chromatophore membrane-bound RrF$_0$F$_1$ by a specific LiCl treatment (15, 16) and recently also cloned and expressed in soluble form (17) in *Escherichia coli* cells lacking the whole unc operon. The recombinant WT RrF$_1$β was found to be as active as the native β-subunit (18) in a large number of earlier developed *in vitro* assays, including the binding of nucleotides (19) and rebinding to the less β-less RrF$_0$F$_1$ resulting in restoration of both ATP synthase and hydrolysis activities (15, 16). The rebinding of the highly purified native and WT RrF$_1$β subunits was, however, found to require the presence of small amounts of RrF$_1$α (18), which were released with the bulk of the β subunit from the LiCl-treated chromatophores (17, 20, 21). This finding provided a direct assay also for the isolated α-subunit. It has been used

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$^\S$ To whom correspondence should be addressed. Tel.: 972-8-9342729; Fax: 972-8-9344118; E-mail: z.gromet-elhananweizmann.ac.il.

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$^1$ The abbreviations used are: RrF$_1$β, RrF$_1$α, CF$_1$β, CF$_1$α, EcF$_1$β, MF$_1$β, MF$_1$α, and TF$_1$β, α and β subunits of the F$_1$-ATPase of *R. rubrum*, chloroplasts, *E. coli*, mitochondria, and thermophilic *Bacillus* PSS1, respectively; BChl, bacteriochlorophyll; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; WT, wild type; PAGE, polyacrylamide gel electrophoresis.
to follow the refolding of the recombinant RrFα, expressed only in insoluble inclusion bodies (22), into a functional monomer that assembles with the WT RrFβ monomer into active αβ dimers (23). If the released α/β ratio can be controlled, the α-depleted β-less chromatophores together with the recombinant RrFα and β monomers could provide suitable systems for studying the effect of mutagenized α as well as β on the in vitro assembly and activity of both the membrane-bound F₀F₁ and soluble F₁ complexes.

In this investigation we have defined the conditions for LiCl treatment of *R. rubrum* chromatophores that release the bulk of their RrFβ together with specific amounts of RrFα. β-less chromatophores, which lost at least a third of this α subunit were used for preparing hybrid RrFα/F₁-CF₁ complexes containing either only CF₁β or CF₁βα and at least one copy of CF₁α (50). β-less chromatophores containing 90% of their α subunit were used here for testing the effect of mutations in RrFβ-βThr159 and Glu184, which are equivalent to MF₁β-βThr166 and Glu186 in the catalytic nucleotide binding site (7). These fully conserved F₁β residues have been mutated only in respiratory F₁-ATPases (6), except for the equivalent CF₁β-βThr159 of *Chlamydomonas reinhardtii*, which has recently been mutated to serine and shown to increase dramatically the MgATPase activities of the soluble CF₁α and αβγ complexes (24). Our studies revealed that in the membrane-bound F₀F₁, the RrFβ-T159S, but not the T159A or T159V, could restore the proton-coupled Mg- and Mn-dependent ATP synthesis and hydrolysis activities to the extent restored by WT RrFβ. Moreover, even the active β-T159S did not restore the proton-decoupled CaATPase activity. These results indicate that the conserved WT β-Thr159 is an absolutely essential ligand for Ca²⁺, which could not be replaced by serine. The RrFβ-E184Q and E184K mutants did bind to the β-less chromatophores, but the assembled mutant RrF₀F₁βS were completely inactive.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—** *R. rubrum* cells were grown as described previously (15). The *E. coli* LM3115 strain (25) lacking the tetC gene was used as host for the recombinant plasmids (17) carrying the WT and mutated RrFβ genes. This strain was found to express large amounts of RrFβ as a soluble protein when grown in LB medium, supplemented as described by Nathanson and Gromet-Elhanan (18), at 22 °C to about 85 mg of BChl/ml.

**Site-directed Mutagenesis—** The RrFβ-βThr159 mutants were obtained by a modification of the PCR-based mutagenesis method of Ho et al. (17). For each mutation the start and end fragments of the cloned WT RrFβ gene (17) were amplified separately in two independent PCR reactions, using the start-forward primer with the earlier introduced EcoRI site (17), and the reverse primer with a newly introduced BamHI site. Each PCR reaction contained a complementary mutagenic primer (26). The following mutagenic primers for T159V, T159A, and T159S-directed mutagenesis were used: 5′-AAC-GAG-GTC-AGC-CTG-GCC-GAC-GCC-G and 5′-CTT-GCC-GAC-GCC-GAC-GCC-G. Each primer contained a new, underlined site for ScaI restriction analysis and full DNA sequencing. All three mutants had only the above stated nucleotide changes.

For mutagenesis of RrFβ-βGlu184, the pBSKS′-WTβ plasmid (17) was transformed into *E. coli* C3296 and single-stranded uracil-containing DNA was prepared after infection with helper phage VCS M13 (27).

Site-directed mutagenesis was performed as described by Kunkel et al. (28), using the following oligonucleotides to introduce the β-E184Q and E184K mutations, respectively: 5′-GCC-CTC-AGC-GTC-AGC-CTG-GCC-GAC-GCC-G and 5′-GCC-CTC-AGC-GTC-AGC-CTG-GCC-GAC-GCC-G. They also contained a new, underlined site for *MluI*, introduced by a single base change, indicated by a bold letter as the bases changed to give the Glu or Lys codons. The mutagenized DNA was transformed into *E. coli* HB101 cells, and the mutations were confirmed by *MluI* restriction analysis and full DNA sequencing. The β-E184K gene had only the stated mutation. However, β-E184Q had one additional change in the nucleotide 249, altering the codon from GCC to GGT, both coding for glycine.

All the expressed WT and mutant RrFβ subunits were isolated from the cytoplasmic fraction of the *E. coli* LM3115 cells and purified as described previously (18).

**Preparation of LiCl-treated *R. rubrum* Chromatophores and Determination of the Amounts of Released RrFα and β Subunits—** LiCl treatment of the chromatophores was carried out as outlined by Gromet-Elhanan and Khananshvili (16) with the following modifications. 1 mM protease inhibitor PMSF was present in the final 1.9 M LiCl buffer, and the concentration of the treated chromatophores was varied as stated in the text. The LiCl supernatant was separated from the treated chromatophores, and the dissolved 60% ammonium sulfate precipitate (16) was subjected to SDS-PAGE (29, 30), transferred to nitrocellulose (31), and probed with antibodies raised against RrFα and β subunits.

**Other Procedures—** Chromatophores treated with 1.9 M LiCl at 1.2 mg of BChl/ml were washed to remove all traces of LiCl (16), reconstituted with WT or mutated RrFβ in presence of RrFα at a ratio of α/β of 0.2, and assayed for restored ATP synthesis and hydrolysis as described previously (18). Published methods were used for measurements of protein concentration (32, 33) and the BChl content of chromatophores (34).

**Materials—** *E. coli* LM3115 was a gift of Dr. P. R. Jensen (The Netherlands Cancer Institute, Amsterdam). Oligonucleotides were synthesized by Dr. Ora Goldberg (Biological Services, Weizmann Institute of Science, Rehovot, Israel). Restriction enzymes, T4 DNA polymerase, and ligase were from New England Biolabs. Plasmid pBSKS′ and helper phage VCS M13 were from Stratagene.

[32P]Pi was obtained from the Nuclear Research Center, Negev, Israel. All other reagents were of the highest purity available.

**RESULTS**

**Preparation of β-less Chromatophores Containing about 90% of the RrF₀F₁α Subunit—** Earlier preparations of β-less chromatophores were obtained by treatment with 2 mM LiCl and no added protease inhibitors (16). Similar treatments of spinach (35) as well as lettuce and tobacco (36) chloroplasts were also found to release some CF₁α together with all the CF₁β. Furthermore Western immunoblots probed with antibodies raised against spinach CF₁α and β subunits revealed that some of the released CF₁α was nicked by proteases and ran in SDS-PAGE together with the β subunit. This proteolysis was fully blocked only in the presence of a mixture of three protease inhibitors (37).

Since antibodies against CF₁α show no cross-reaction with RrFβ (50), we have raised antibodies against RrFα and found proteolyzed RrFα in LiCl extracts of earlier treated chromatophores. Addition of 1 mM PMSF blocked completely this proteolysis, thus enabling a clear determination of the relative amounts of the RrFα and β subunits released from chromatophores treated by 1.9 M LiCl at 0.4, 0.8, or 1.2 mg of BChl/ml (Fig. 1, lanes 2–4). About 0.65, 0.52, and 0.48 % of the RrFα were, respectively, released as compared with practically all their β subunit (Fig. 1, lane 5 and Fig. 2, lane 5). Chloroplasts treated with LiCl at 1.2 mg of BChl/ml were used for evaluating the effect of the β-T159 and β-E184 mutations when reconstituted with the various β subunits in presence of RrFα at a ratio of α/β of 0.2.

**Activities of RrFβ Mutants at Position 159—** RrFβ-βThr159 is the last residue in the glycine rich p-loop sequence found in α
and \( \beta \) subunits of all \( F_{i} \)-ATPases and many other nucleotide-binding proteins (38). The parallel MF\( _{\beta} \) \( \beta \)-O\( \gamma \)-T163 was identified in the crystal structure as a ligand to Mg\( ^{2+} \) (7). In the GTP-binding Ras protein, a serine, which replaces the threonine in this sequence, was also found to be a ligand to Mg\( ^{2+} \) (39). We have mutated the RrF\( _{\beta} \)-Thr\( ^{159} \) into serine (RrF\( _{\beta} \)-Thr\( ^{159} \)) as well as alanine (RrF\( _{\beta} \)-Thr\( ^{159} \)) and valine (RrF\( _{\beta} \)-Thr\( ^{159} \)). All of them were found to assemble into the \( \beta \)-less \( R. \ rubrum \) chromatophores as efficiently as WT RrF\( _{\beta} \) (Fig. 2, lanes 1–4). However, the RrF\( _{\beta} \)-Thr\( ^{159} \) and RrF\( _{\beta} \)-Thr\( ^{159} \) reconstituted with WT, T159A, T159V, and T159S RrF\( \beta \), respectively; lane 5, unconstituted \( \beta \)-less chromatophores.

The ATP synthesis restored by the \( \beta \)-Thr\( ^{159} \) was similar or slightly higher than the WT rate and showed a similar MgCl\( _{2} \) requirement (Fig. 3). Values of \( K_{m} \) of 175 and 238 \( \mu \)M MgCl\( _{2} \) and \( V_{\text{max}} \) of 352 and 429 pmol of ATP formed/h per mg of BChl were calculated for chromatophores reconstituted with the WT and the \( \beta \)-Thr\( ^{159} \) mutant, respectively. The maximal rate of this restored ATP synthesis, as that observed in control \( \beta \)-less chromatophores (14), was obtained at MgCl\( _{2} \) concentrations below 10 \( \mu \)M and Mg\( ^{2+} \)/ADP ratios below 5. At 40 \( \mu \)M MgCl\( _{2} \), the rates restored by the WT and T159S mutant were inhibited by 50% and 30%, respectively (Fig. 3). The lower sensitivity of the mutant to inhibition by increasing MgCl\( _{2} \) concentrations could explain its capacity to restore somewhat higher maximal rates of ATP synthesis.

A very similar pattern was observed also for restoration of MgATPase activities by the WT and T159S mutant (Fig. 4A). Mg- and Mn-dependent ATP hydrolysis in control \( R. \ rubrum \) chromatophores (14), as in \( \beta \)-less chromatophores reconstituted with WT RrF\( \beta \) (18), were reported to be much more sensitive to inhibition by excess free Mg\( ^{2+} \) or Mn\( ^{2+} \) ions than their respective ATP synthesis activities. This difference in sensitivity provides the basis for the tight regulation of ATP hydrolysis in photosynthetic organisms, which enables them to limit the depletion of essential cellular ATP pools in the dark and light.

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DISCUSSION

RrF1β-Thr159 and Glu184 are fully conserved in all sequenced F1 complexes. The equivalent MFβ-Thr163 was identified in the catalytic nucleotide binding site of the bovine MF1 crystal structure (7) as a ligand to Mg2+, and the MFβ-Glu188 was suggested to be directly involved in catalysis. Our analysis of the equivalent RrFβ-E181Q and E184K mutants yielded assembled but fully inactive enzymes (Table I). Similar results were reported for the equivalent EcFβ-E181 mutations (41, 42), where even the β-E181D mutant had only 1.4% of the control MgATPase activity. Only the equivalent TFβ-E190D mutant (43) had 7% of the WT MgATPase activity. These results confirm that this residue is essential for catalysis in respiratory as well as photosynthetic F1 ATP synthases, but do not really clarify its function (see Ref. 6). Residues equivalent to RrFβ-Thr159 were mutated to serine in several respiratory systems (44–46) and recently also in C. reinhardtii (24), and checked extensively on the MgATPase activity of isolated F1, or αβγ complexes. In all tested cases this mutation resulted in a 3–10-fold increase in the MgATPase activity. It also eliminated the stimulation of the MgATPase by oxyanions or alcohols, and reduced the sensitivity of the MgATPase activity to inhibition by azide.

The three mutants RrFβ-T159S, T159A, and T159V have, however, been tested for their in vitro assembly into the β-less membrane-bound RrFβF1, as well as for their capacity to restore all the divalent cation-dependent ATP synthesis and hydrolysis activities of the assembled RrF0F1 complex. All three mutants did bind into the β-less chromatophores (Fig. 2), but only the assembled RrF0F1 containing the β-T159S was active. It did restore the WT rates and specific properties of ATP synthesis as well as Mg2- and Mn-dependent ATP hydrolysis, including the tight regulation of these ATPase activities, and their effective stimulation by sulfite. However, even this active mutant could not restore any CaATPase activity.

Two unexpected results emerged from the present study with the RrFβ-T159S. One is the large difference between the membrane-bound activities and properties obtained here with the mutant, which were very similar to those obtained with WT RrFβ, and the much higher activities and different properties than the WT ones that were earlier observed in soluble F1 or αβγ complexes containing the equivalent threonine to serine mutants of CF1 (24) MF1 (44), and TF (46).

The second unexpected result is the inability of the RrFβ-T159S mutant to restore the proton-decoupled CaATPase, while effectively restoring the proton-coupled Mg- and Mn-ATPases. The capacity to restore CaATPase has not been tested with any other equivalent mutant, but an opposite effect of restoration of Ca- but not MgATPase activity was earlier observed with the RrFβ-E195G mutant (18). These results indicate that the ligands for Ca2+ and Mg2+ must be different, since only in the case of Ca2+ the threonine cannot be replaced.

and E195K mutants (18). These results demonstrate that the RrFβ-Glu184, although not required for assembly, is absolutely essential for all the above tested membrane-bound RrF0F1-activities.

TABLE I

| Assayed activities | Activities restored by the following RrFβ subunits | MgATPase (μmol/h per mg Bchl) |
|--------------------|---------------------------------------------------|-------------------------------|
|                    | WT | T159S | E184K or E184Q |<0.1 |
| ATP synthesis      | 340 | 390   |<0.1            |
| MgATPase           | 110 | 124   |<0.1            |
| MgATPase + sulfite | 320 | 205   |<0.1            |
| MnATPase           | 115 | 85    |<0.1            |
| MnATPase + sulfite | 350 | 220   |<0.1            |
| CaATPase           | 57  |<1     |<0.1            |

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by serine (Fig. 4C). This difference in ligands might lead to a
different conformational state of the catalytic site occupied by
Ca$^{2+}$ as compared with Mg$^{2+}$ or Mn$^{2+}$. It can explain the
inability of the Ca$^{2+}$-occupied catalytic sites to carry out ATP
synthesis and proton-coupled ATP hydrolysis, by Ca-induced
changes in the interactions between the $\beta$ and $\gamma$ subunits that
may be involved in linking catalysis to proton translocation.

Omot et al. (47) have recently reported that they observed
similar rotation and torque generation in engineered WT EcF$_1$-
$\alpha\beta_2\gamma$ and an uncoupled mutant EcF$_1$-$\alpha\beta_3\gamma$M23K. This
mutant is unable to translocate protons through F$_0$ but has about
60% of the WT EcF$_1$-MgATPase activity. These properties are
very similar to those recorded with the proton-decoupled mem-
brane-bound and the soluble RfF$_1$-CaATPase activity. It would
therefore be most interesting to test whether rotation can occur
also during CaATPase as well as MgATPase activity. Although
CaATPase activity has been tested very thoroughly in various
photosynthetic F$_1$-ATPases (2–5), however, it has not been
studied in detail in respiratory bacterial F$_1$-ATPases, whereas
a direct full rotation of $\gamma$ has not been demonstrated as yet with
any engineered photosynthetic F$_1$-$\alpha\beta_3\gamma$. Incubation of the
recombinant WT RfF$_1$-$\alpha$ and $\beta$ monomers (23) with a recombinant
spinach CF$_1$, $\gamma$ subunit (48, 49) has recently been found to result
in assembly of a very active hybrid RfF$_1$$\alpha\beta_3$$\gamma$c, which has both
Ca- and MgATPase activities. This hybrid can be engineered
to be used in direct rotation assays, and could thus enable the
examination of rotation in presence of CaATP as well as MgATP.

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