ATPase Activity of a Highly Stable αβγ Subcomplex of Thermophilic F₁ Can Be Regulated by the Introduced Regulatory Region of γ Subunit of Chloroplast F₁*

(Received for publication, December 6, 1999, and in revised form, January 24, 2000)

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A mutant F₁-ATPase αβγγ subcomplex from the thermophilic Bacillus PS3 was constructed, in which 111 amino acid residues (Val⁹² to Phe⁴⁰⁹) from the central region of the γ subunit were replaced by the 148 amino acid residues of the homologous region from spinach chloroplast F₁-ATPase γ subunit, including the regulatory stretch, and were designated as αβγγ(Trx) (Thermophilic-Chloroplast-Thermophilic). By the insertion of this regulatory region into the γ subunit of thermophilic F₁, we could confer the thiol modulation property to the thermophilic αβγ subcomplex. The overexpressed αβγγ(TCT) was easily purified in large scale, and the ATP hydrolyzing activity of the obtained complex was shown to increase up to 3-fold upon treatment with chloroplast thioredoxin-f and dithiorthiol. No loss of thermostability compared with the wild type subcomplex was found, and activation by dithiorthiol was functional at temperatures up to 80 °C. αβγγ(TCT) was inhibited by the ε subunit from chloroplast F₁-ATPase but not by one from the thermophilic F₁-ATPase, indicating that the introduced amino acid residues from chloroplast F₁γ subunit are important for functional interaction with the ε subunit.

F₁F₁-ATP synthases ubiquitously occur in eucaryotic and procaryotic cells and synthesize ATP from ADP and inorganic phosphate at the expense of a trans-membrane electrochemical proton gradient (1–4). The bacterial enzyme consists of a hydrophilic F₁ part with a subunit composition of α₁ββε (5) and a hydropholic F₀ part with a subunit composition of α₁ββε₁γδε (6). The subcomplex αβγγε is the minimum complex that is capable of hydrolyzing ATP (7). A high resolution x-ray structure of a major part of the bovine heart mitochondrial F₁ revealed an alternating hexagonal arrangement of the three α and the three β subunits with two α-helices of the γ subunit forming a coiled-coil in the central cavity (8). The crystal structure of an αβγ complex from the thermophilic Bacillus PS3 is completely symmetric (9), but the incorporation of the γ subunit into this complex induces a functional asymmetry among the three catalytic sites (10) residing on the β subunits at the interface to the α subunits. Rotation of the γ subunit relative to αβγ, had been suggested from kinetic analyses (11), biochemical experiments (12), and biophysical measurements (13). Finally, by a single molecular observation technique, rotation of the γ subunit during the ATP hydrolysis reaction in the αβγ hexamer (14–17) was shown.

The molecular structure of the F₁F₁-ATP synthase of chloroplasts may be basically the same as those of other F₁F₁, but this enzyme has a unique regulation system. The activity of chloroplast FₒF₁ and F₁ (CF₁) is strongly regulated by the reduction and the oxidation of a disulfide bridge in the γ subunit following the two cysteine Cys¹⁹⁹ and Cys²⁰⁵ (spinach chloroplast) (18); this regulation system is called thiol modulation (19). Reduction of the disulfide bond elicits the latent ATP hydrolyzing activity of the isolated CF₁. In vitro reduction can be achieved by dithiorthiol (DTT) or other dithiols, but the natural reductant is reduced thioredoxin-f (Trx-f) (20–22). Introduction of nine amino acids comprising the regulatory sequence into the cyanobacterial γ subunit induced thiol modulation in Synechocystis (23, 24). Conversely, replacement of the two cysteines by serines in γ subunit of CFₒCF₁ from Chlamydomonas reinhardtii (25, 26) resulted in a non-modulated enzyme. However, due to the lack of suitable overexpression systems for the subunits of CF₁ and because of its insufficient ability for the reconstitution of the complete enzyme complex, little information is available about the details of this regulation mechanism. Recently we have succeeded in the reconstitution of a chimeric complex from recombinant α and β subunits of F₁ of the thermophilic Bacillus PS3 (TF₁) and the recombinant γ subunit from spinach CF₁ (27). The resulting chimeric αβγ complex, which had substantial ATPase activity, was clearly regulated by the disulfide/dithiol state of the two regulatory cysteine residues. We could demonstrate the importance of the region around the disulfide bridge of γ subunit for the regulatory interaction with ε subunit which is known to inhibit activity (28). However, stability of this chimeric complex was still lower than that of wild type thermophilic αβγ, and large scale preparation was difficult.

In order to understand the molecular mechanism of thiol regulation, we engineered a chimeric αβγγ subcomplex of TF₁ in which the central half of the γ subunit was replaced by the

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equivalent part of the γ subunit from CF₁ including the regulatory cysteines (Fig. 1). The remaining parts derived from the TF₁-γ subunit mainly consists of the N-terminal and C-terminal α-helices forming the coiled-coil and a small additional α-helix. The chimeric α₁β₁γ subcomplex was successfully overexpressed in *Escherichia coli* and purified as an active ATPase. Then we investigated the enzymatic property of this complex under reduced and oxidized conditions.

**MATERIALS AND METHODS**

**Chemicals**—Restriction enzymes were purchased from Toyobo Co., Tokyo, Japan. DTT and MOPS were from Nacalai Tesque, Kyoto, Japan. TNP-ATP was synthesized and purified by the method as described (29, 30). All other chemicals were of the highest grade commercially available.

**Bacterial Strains**—Plasmid amplification was carried out with *E. coli* strain JM 109, and single-stranded uracil-containing DNA was obtained from *E. coli* strain CJ 236, and for protein overexpression *E. coli* strain JM 103 ΔuncB-D was used.

**Plasmid Construction**—A DNA fragment coding for α₁γ (*yCTC*), and β (carrying an N-terminal tag of 10 histidine residues) subunits on the expression plasmid, pkkHisCys5 (14), was cloned into the multi-cloning site of the M13mp18 vector, and single-stranded uracil-containing DNA was obtained by the method of Ref. 31. The location for two silent mutations to construct *Nsp*/*Nhe* restriction sites was determined with the program MOTOJIMAN provided by Fumihito Motojima, Tokyo Institute of Technology, and introduced by the method of Ref. 31. Two introduced 5′-CCCGGACCCGCACGCTGTTTCAAGTTGGAGCCG with the vector containing the gene for the γ subunit of spinach chloroplast DNA (Fig. 2) as a template. The obtained DNA fragment was inserted by blunt-end ligation into pBlueScript vector, which was previously digested with NsiI. Then the vector was digested with NsiI and ligated with the cohesive-end DNA fragment to obtain the expression plasmid for α₁β₁γ. The correct orientation of the DNA fragment inserted into pKKCTCT was confirmed by digestion with Nhel and AflIII.

**Overexpression and Protein Purification of α₁β₁γ** —Overexpression and protein purification of α₁β₁γ expressed in *E. coli* were performed as described. The recombinant α₁β₁γ subcomplex of TF₁ containing a histidine tag on the N-terminus of the β subunit and the substitution of Ser₁⁰⁶ of the γ subunit to Cys (α₁β₁γ-β-His, yCTC) was expressed by using the pkkHisCys5 vector and purified by the method described (11). The concentration of the purified α₁β₁γ was determined by measuring the absorbance at 278 nm using the published molar absorption coefficient value of 16,830 M⁻¹cm⁻¹ (33).

**ATP Hydrolysis Activity**—ATP hydrolysis activity was measured in the presence of an ATP regenerating system (34) with a spectrophotometer model U-3100 (Hitachi, Tokyo, Japan) equipped with a stirrer. The reaction mixture containing 50 mM MOPS-KOH, pH 7.0, 100 mM KCl, 5 mM MgCl₂, and 10 mM imidazole was incubated with 8 M urea, 50 mM Tris-Cl, pH 8.0, 1 mM EDTA, and 0.5 mM DTT and dialyzed against 50 mM MOPS-KOH, pH 7.0, 100 mM KCl, and 5 mM MgCl₂ for 6 h.

**Preparation of the Recombinant TrxF—** Spinach chloroplast TrxF was purified by chromatographs (35). 50 mM MOPS-KOH, pH 7.0, 100 mM KCl, 5 mM MgCl₂, and 2 mM ATP were incubated in a water bath for 2 min, and then the reaction was started by the addition of 20 µL (2 µM) of α₁β₁γ. After 2 min, the reaction was quenched by the addition of 100 mM of 2,4-dinitrophenol (4/νl) to stop the reaction. The mixture was then directly applied to a nickel-NTA (nitrilotriacetic acid) superflow column (Qiagen, Hilden, Germany) equilibrated with 50 mM MOPS-KOH, pH 7.0, 100 mM KCl, 5 mM MgCl₂, and 10 mM imidazole. The column was washed with the same buffer containing 25 mM imidazole and then the enzyme was eluted with the same buffer containing 250 mM imidazole.

**TF₁-α₁β₁γ Containing the Regulatory Region of CF₁-γ**

![Image](https://www.jbc.org/content/281/1/12758/F1)

**Fig. 1. Amino acid sequence of yCTC.** Residues 1–91 (1–95 by CF₁-γ numbering) are derived from the γ subunit of TF₁ (TF₁-γ), residues 92–239 (bold letters) including the additional regulatory stretch (190–226) from the γ subunit of CF₁ (CF₁-γ), and residues 240–319 again from the γ subunit of TF₁. For comparison the domains resolved in a high resolution x-ray structure of bovine heart mitochondria F₁-ATPase (MF₁-γ) are shown (8). The long N-terminal and C-terminal helices form a coiled-coil interacting with the α₁β₁ hexagon. The closed boxes show the position of binding regions of the ε subunit reported in Ref. 51.
amounts of TNP-ATP and TNP-ADP were determined by reversed-phase HPLC (35). In case of chase acceleration experiments, 10 μl of 100 μM ATP was added instead of trichloroacetic acid and incubated for further 10 s. Then the reaction was stopped by the addition of trichloroacetic acid as stated above.

**Gel Electrophoresis**—Polyacrylamide gel electrophoresis was performed according to Laemmli (36). Protein bands were visualized by the staining with Coomassie Brilliant Blue R-250.

**RESULTS**

**Preparation and Purification of αβ3γ(TCT)—αβ3γ(TCT)** was overexpressed in *E. coli* JM 103 and purified by Ni-NTA affinity chromatography. The purity of the protein and the occurrence of the individual subunits in the complex were confirmed by SDS-gel electrophoresis (Fig. 2). The yield of αβ3γ(TCT) complex was about 80 mg of protein from a 2-liter *E. coli* culture, comparable to the yield of wild type αβ3γ (β-His, γS106C) (14).

The molecular mass of the γ subunit in the αβ3γ(TCT) complex was about 35 kDa, clearly larger than the γ subunit of αβ3γ (β-His, γS106C) but very similar to the γ subunit of CF1 (Fig. 2), indicating the successful expression and the formation of the complex with the mutant subunit.

**Modulation of Activity by Oxidation and Reduction**—The activity of ATP hydrolysis catalyzed by the purified αβ3γ(TCT) was measured spectrophotometrically by the pyruvate kinase/lactate dehydrogenase assay that provides continuous regeneration of ATP. Upon addition of oxidized and reduced αβ3γ(TCT) to the reaction mixture, ATP hydrolysis proceeded in a linear manner for at least 10 min without time-dependent inactivation (Fig. 3A), but the rates were different as follows: αβ3γ(TCT) was clearly activated by preincubation with DTT. When DTT alone was used for reduction, up to 2-fold activation was obtained (Fig. 3, A and B). Activation was saturated at 15 mM DTT (Fig. 3B). Extension of the preincubation time for more than 1 h did not significantly increase activity. Interestingly, the activation was higher if reduction was carried out with a combination of DTT and Trx-f (Fig. 3, A and B). When ATP hydrolysis reaction was initiated by addition of oxidized αβ3γ(TCT), and 15 mM DTT plus 500 nM Trx-f (final concentration) was added to the reaction mixture after 5 min, remarkable activation could be observed within several minutes (Fig. 3A, inset). The concentration of Trx-f for half-maximal activation was about 200 nM (Fig. 3B, inset), considerably lower than that for authentic CF1, which was more than 1 μM (21). The specific activity of αβ3γ(TCT), in the oxidized state was 1.0–1.2 units/mg, and upon reduction with 15 mM DTT it increased to 2.2–2.6 units/mg, and with 15 mM DTT plus 500 nM Trx-f 3.0–3.3 units/mg were measured. Quantification of thiol groups indicated additional 1.6–1.9 mol of SH/mol of αβ3γ(TCT) after reduction with 15 mM DTT and additional 1.7–2.0 mol of SH/mol of αβ3γ(TCT), after reduction with 15 mM DTT plus 500 nM Trx-f.

The specific activity of αβ3γ(TCT), in the fully reduced state under the reaction conditions above (pH 7.0) was comparable to that of αβ3γ (β-His, γS106C) under the same conditions (4.5 units/mg). The pH optimum was between 8.5 and 9.0, similar to the values reported for wild type αβ3γ (7), for TF1 (37), and for CF1 (38).

**Thermostability of αβ3γ(TCT)**—The optimum temperature of the ATPase activity of oxidized and reduced αβ3γ(TCT), was at 70 °C, the same as that of αβ3γ (β-His, γS106C) (Fig. 4) and comparable to the values reported for wild type TF1, and αβ3γ (7). The ratio between the activities of oxidized and reduced αβ3γ(TCT) did not change significantly over the whole temperature range investigated.

**Interaction with the ε Subunit**—The effect of the inhibitory ε subunit of CF1 and that of TF1 on αβ3γ(TCT), was investigated (Fig. 5A). ATPase activity of αβ3γ(TCT), in its oxidized state was inhibited to 50–60% by the ε subunit of CF1. Interestingly, αβ3γ(TCT), in its reduced state was nearly insensitive to the CF1-ε subunit, resulting in about a 4-fold higher activity compared with αβ3γ(TCT), in the oxidized state in the presence of the CF1-ε subunit. As reported previously, neither the CF1-ε subunit nor the TF1-ε subunit had any significant effect on the activity of αβ3γ (β-His, γS106C) when the ATP concentration was relatively high (32). However, a slight but significant increase of activity was observed upon incubation of oxidized αβ3γ(TCT) with the TF1-ε subunit (Fig. 5A). Again, this effect was less pronounced for αβ3γ(TCT), in the reduced form.

To clarify the cause of the weakly stimulating effect of the ε subunit of TF1, on ATPase activity of αβ3γ(TCT), we examined its binding to the complex. After incubation with ε subunit of TF1, the complex was separated from unbound ε subunit by gel filtration HPLC. The αβ3γ(TCT), fraction was then applied to SDSPAGE (Fig. 5B). αβ3γ(TCT), in the oxidized state as well as in the reduced state could bind the ε subunit of TF1, in an approximately stoichiometric ratio, comparable to αβ3γ (β-His, γS106C) under identical conditions.

**ATP Hydrolysis Under Uni-site Conditions**—When a subsite occupancy of TF1-ε subunit was constructed, and the desired protein complex was successfully overexpressed and purified with a yield comparable to those of wild type αβ3γ (7) or αβ3γ (β-His, γS106C) (14). This proves that combining two halves of γ subunits from different organisms and the insertion of additional amino acid residues from the γ subunit of CF1, into the central part of the γ subunit of TF1, are no major obstacles for biosynthesis and in vivo stability of this protein complex in the employed expression system. Thus, although the homology between the transferred part of CF1-γ subunit and the replaced part of the TF1-γ sub-
Fig. 3. Effect of DTT and Trx on ATPase activity of αβγ(TCT). A, ATPase activity was measured using an ATP regenerating system monitoring the decrease of NADH absorption at 340 nm. Samples were preincubated for 1 h with 50 μM CuCl2 (Ox), 15 mM DTT (DTT), or with 15 mM DTT + 500 mM Trx-f (DTT+Trx) in 50 mM MOPS-KOH, pH 7.0, supplemented with 100 mM KCl and 5 mM MgCl2. The ATPase activity without incubation with DTT or Trx was 1.1 unit/mg and set as 100%. Inset, real time activation of oxidized αβγ(TCT) by addition of 15 mM DTT + 500 mM Trx-f at the indicated time. The dashed line shows the time course without addition of DTT and Trx-f. B, dependence of ATPase activity of αβγ(TCT) on the DTT concentration. Closed circles, X mM DTT; open circles, X mM DTT + 500 mM Trx-f. Inset, dependence of activation on Trx-f concentration in the presence of 1 mM DTT. For details see under "Materials and Methods."

TF₁-αβγ Containing the Regulatory Region of CF₁-γ

Thermostability of αβγ(TCT). ATPase activity was measured in the absence of an ATP regenerating system at the indicated temperatures in 50 mM MOPS-KOH, pH 7.0, supplemented with 100 mM KCl and 5 mM MgCl2. Produced phosphate was quantified by the method described (21). Closed circles, αβγ(TCT), oxidized with 50 μM CuCl2; open circles, αβγ(TCT), reduced with 15 mM DTT; open squares, αβγ(β-His, γS106C).

The ATPase activity of the αβγ(TCT) complex was modulated by the reduction or oxidation of the two cysteines that were introduced by the insertion of the respective part of the CF₁-γ subunit. An up to 3-fold activation could be observed by the complete reduction of these cysteines (Fig. 3). As this activation ratio is in the same range as that reported for CF₁ (3–7-fold) (19–22), most likely the mechanism of thiol activation is the same in the αβγ(TCT) complex. Sokolov et al. (41) recently proposed that the conformation of the γ subunit of CF₁ may be different from other F₁-γ subunits. Their conclusion is based on former results of energy transfer measurements between γ subunit and the catalytic sites on β subunits (42, 43) and their investigation of the ATPase activity of a complex formed from the isolated CF₁ αβγ subcomplex and recombinant γ subunit of which the C terminus was partially deleted. In their model, the tip of the C terminus of the γ subunit does not act as a spindle for rotation. However, our results indicate that a bacterial γ subunit can be transformed into a functional chloroplast-like γ subunit. Thus the essential structural features and basic functions of both types of γ subunits should be the same. This is also consistent with previous reports that upon introduction of the chloroplast γ-like regulatory stretch into the γ subunit of cyanobacterial F₁, conferred thiol modulation on this enzyme (23, 24). In this mutant both ATP hydrolysis and ATP synthesis are controlled by thiol modulation. Furthermore, ATP hydrolysis activity of a reconstituted chimeric complex containing the α and β subunits of TF₁, and the γ subunit of CF₁ (αβγ(TCT)), was regulated by thiol modulation (27, 28) although the complex showed reduced stability.

Interestingly, a higher degree of activation of the complex αβγ(TCT) could be achieved with a mixture of DTT and Trx-f than with DTT alone (Fig. 3B), although we could not detect a significant difference in the amount of reduced thiols. This is consistent with our recently proposed model that a conformational change, which affects the enzyme activity, is induced by the interaction with Trx-f (21).

The measurement of the temperature optimum of ATPase activity shows that αβγ(TCT), similar to αβγ(β-His, γS106C), is more stable than a chimeric αβγ(β-His, γS106C), which displayed the highest activity at 55–60 °C (Fig. 4, compare with Fig. 2 of Ref. 27). This result implies that the introduction of the 148 amino acid residues from a mesophilic enzyme does not affect thermostability of the complex significantly. The most probable explanation is that the interaction between the coiled-coil structure of γ subunit and the inner surface of the cavity of αβ hexagon is especially important for the stability of the complex. Although the homology of this coiled-coil region of the γ subunit is relatively high (27), some varying amino acid residues in the coiled-coil region might be very important for the stability of the complex. Activation by DTT was not affected by the temperature, so although thiol modulation in vitro usually occurs at temperatures <30 °C in plants, it is also functional at temperatures as high as 80 °C.

The ε subunit is known to be an inhibitory subunit of F₁-
function (44–48). As the sequence of the e subunit could be cross-linked to the subunit of F1-ATPase. It is of chloroplast origin in the γ(TCT) appears to be crucial for binding of and inhibition by the e subunit.

As supposed from the suggested mechanism for the uni-site catalysis (29, 30), no significant differences could be detected in the rate of uni-site hydrolysis by oxidized or by reduced αβγ(TCT). However, we found a difference in chase acceleration of hydrolysis of TNP-ATP by the addition of excess amounts of ATP (Fig. 6). The range of the chase acceleration for the reduced αβγ(TCT) was comparable to that published for TF1 (35), wild type αβγ(TCT), and αβγ (β-His, γS106C) was examined. B, isolated αβγ(TCT) and αβγ (β-His, γS106C) were mixed with TF1-e, passed through a gel filtration HPLC, and then applied to 12% (w/v) SDS-PAGE. Lane 1, TF1-e; lane 2, αβγ(TCT) (oxidized) + TF1-e; lane 3, αβγ(TCT), (oxidized) + TF1-e; lane 4, αβγ(TCT), (reduced) + TF1-e. The results of acid quench (circles) and chase acceleration (squares) were obtained from the measurement of remaining TNP-ATP and produced TNP-ADP by reversed-phase HPLC as described (35).

ATPases. It has been proposed that the C-terminal helical domain of the e subunit is mainly responsible for the inhibitory function (44–48). As the e subunit could be cross-linked to the β subunit (49, 50), the inhibition may be achieved by binding to the DELSEED region of the β subunit. We found that the e subunit of CF1 can efficiently inhibit the ATPase activity of αβγ(TCT), but not that of αβγ (β-His, γS106C), although the α and β subunits are identical (Fig. 5). This induces the conclusion that an interaction of the e subunit with the γ subunit may be more crucial for inhibition, possibly by providing proper binding and orientation of the e subunit. As the sequence of the amino acid residues 92–239 of the γ subunit of αβγ(TCT) is different from αβγ (β-His, γS106C), it can be concluded that this region confers the sensitivity to inhibition by the e subunit. Consistent with this finding, E. coli F1, residue γ106 and the region γ202–212 have previously been found to be involved in binding of the e subunit by cross-linking and chemical labeling experiments (51). E. coli F1-γ106 corresponds to γ(TCT)101, near the N-terminal part of the insertion from CF1, γ202–212 corresponds to γ(TCT) 234–244, located at the C terminus of the insertion (Fig. 1). Taken together, the part of the γ subunit that
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*J. Biol. Chem.* 2000, 275:12757-12762.
doi: 10.1074/jbc.275.17.12757

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