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ATPase Activity of a Highly Stable $\alpha_3\beta_3\gamma$ Subcomplex of Thermophilic F_1 Can Be Regulated by the Introduced Regulatory Region of γ Subunit of Chloroplast F_1*

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A mutant F_1-ATPase $\alpha_3\beta_3\gamma$ subcomplex from the thermophilic *Bacillus* PS3 was constructed, in which 111 amino acid residues (Val^{92} to Phe^{202}) from the central region of the γ subunit were replaced by the 148 amino acid residues of the homologous region from spinach chloroplast F_1-ATPase γ subunit, including the regulatory stretch, and were designated as $\alpha_3\beta_3\gamma_{(TCT)}$ (Thermophilic-Chloroplast-Thermophilic). By the insertion of this regulatory region into the γ subunit of thermophilic F_1, we could confer the thiol modulation property to the thermophilic $\alpha_3\beta_3\gamma$ subcomplex. The overexpressed $\alpha_3\beta_3\gamma_{(TCT)}$ 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 dithiothreitol. No loss of thermostability compared with the wild type subcomplex was found, and activation by dithiothreitol was functional at temperatures up to 80 °C. $\alpha_3\beta_3\gamma_{(TCT)}$ was inhibited by the ϵ subunit from chloroplast F_1-ATPase but not by the one from the thermophilic F_1-ATPase, indicating that the introduced amino acid residues from chloroplast F_1-γ subunit are important for functional interaction with the ϵ subunit.

F_0F_1-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 F_1 part with a subunit composition of $\alpha_3\beta_3\gamma\delta\epsilon$ (5) and a hydrophobic F_0 part with a subunit composition of $a_1b_2c_{10-12}$ (6). The subcomplex $\alpha_3\beta_3\gamma$[1] 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 F_1 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 $\alpha_3\beta_3$ 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 $\alpha_3\beta_3$ 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 $\alpha_3\beta_3$ hexamer (14–17) was shown.

The molecular structure of the F_0F_1-ATP synthase of chloroplasts may be basically the same as those of other F_0F_1, but this enzyme has a unique regulation system. The activity of chloroplast F_0F_1 and F_1 (CF_1) is strongly regulated by the reduction and the oxidation of a disulfide bridge in the γ subunit formed between the two cysteines Cys^{199} and Cys^{205} (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 CF_1. *In vitro* reduction can be achieved by dithiothreitol (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 CF_0CF_1 from *Chlamydomonas reinhardtii* (25, 26) resulted in a non-modulated enzyme. However, due to the lack of suitable overexpression systems for the subunits of CF_1 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 F_1 of the thermophilic *Bacillus* PS3 (TF_1) and the recombinant γ subunit from spinach CF_1 (27). The resulting chimeric $\alpha_3\beta_3\gamma$ 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 $\alpha_3\beta_3\gamma$, and large scale preparation was difficult.

In order to understand the molecular mechanism of thiol regulation, we engineered a chimeric $\alpha_3\beta_3\gamma$ subcomplex of TF_1 in which the central half of the γ subunit was replaced by the

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[1] The abbreviations used are: $\alpha_3\beta_3\gamma$ (β-His, γS106C), $\alpha_3\beta_3\gamma$ subcomplex of TF_1 containing histidine tag on the N terminus of the β subunit and the substitution of Ser^{106} of the γ subunit to Cys; CF_1, chloroplast coupling factor 1; DTT, dithiothreitol; Trx-*f*, spinach chloroplast thioredoxin-*f*; TF_1, F_1-ATPase from thermophilic *Bacillus* PS3 plasma membrane; MOPS, 3-[*N*-morpholino]propanesulfonic acid; TNP-ATP (or TNP-ADP), 2′,3′-*O*-(2,4,6-trinitrophenyl) ATP (or ADP); Ni-NTA, nickel nitrilotriacetic acid; $\alpha_{T3}\beta_{T3}\gamma_C$, chimeric $\alpha_3\beta_3\gamma$ complex formed by the recombinant α and β subunits of TF_1 and the recombinant γ subunit of CF_1; HPLC, high pressure liquid chromatography.

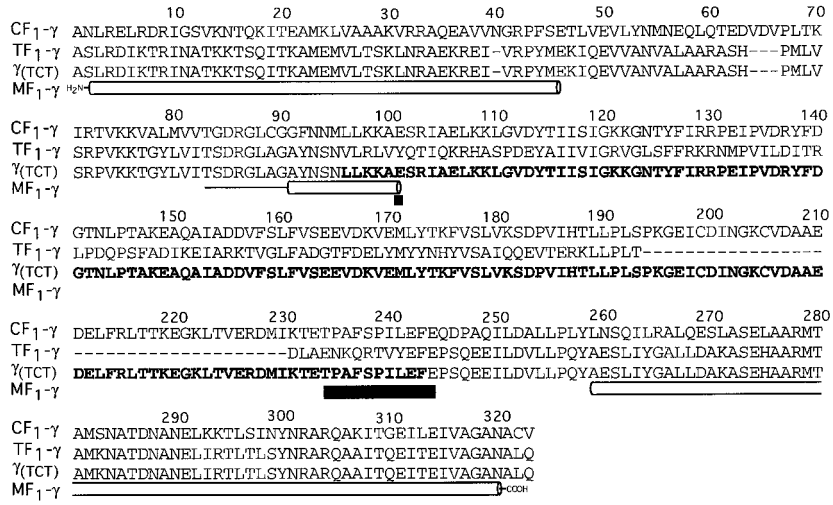

FIG. 1. **Amino acid sequence of $\gamma_{(TCT)}$.** Residues 1–91 (1–95 by CF_1-γ numbering) are derived from the γ subunit of TF_1 (*TF_1-γ*), residues 92–239 (*bold letters*) including the additional regulatory stretch (190–226) from the γ subunit of CF_1 (CF_1-γ), and residues 240–319 again from the γ subunit of TF_1. For comparison the domains resolved in a high resolution x-ray structure of bovine heart mitochondria F_1-ATPase (*MF_1-γ*) are shown (8). The long N-terminal and C-terminal helices form a coiled-coil interacting with the $\alpha_3\beta_3$ hexagon. The *closed boxes* show the position of binding regions of the ϵ subunit reported in Ref. 51.

equivalent part of the γ subunit from CF_1 including the regulatory cysteines (Fig. 1). The remaining parts derived from the TF_1-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 $\alpha_3\beta_3\gamma$ 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 Δ*uncB-D* was used.

Plasmid Construction—A DNA fragment coding for α, γ (S106C), and β (carrying an N-terminal tag of 10 histidine residues) subunits of TF_1 on the expression plasmid, pkkHisCys5 (14), was cloned into the multicloning 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*V restriction sites was determined with the program MOTOJIMAN provided by Fumihiro Motojima, Tokyo Institute of Technology, and introduced by the method as described (31) using the primers 5′-CACGAGGCGCAGCACGTTCGAATTGTACGCGCCAGC and 5′-TTCTTCTTGCGACGGTTCGAATTCGTACACCGTGCG. Then the DNA fragment coding the deduced γ subunit was cut out with the restriction enzymes *Nhe*I and *Bgl*II and cloned into the equivalent position between the genes for the α and β subunits on the pkkHisCys5 vector.

A fragment of spinach chloroplast DNA coding for amino acid residues Leu^{96}-Phe^{243} of the γ subunit of CF_1 was amplified by the polymerase chain reaction method using the phosphorylated primers 5′-CGGGATCCCGTTAGAAGTTGCTGAAGAAGGCTGAGTCTAGG and 5′-CGGGATCCCGTTCGAATTCCAGAATTGGGGAAAATGCTGG with the vector containing the gene for the γ subunit of spinach CF_1 (27) as a template. The obtained DNA fragment was inserted by blunt-end ligation into pBluescript vector, which was previously digested with *Sma*I. Then the vector was digested with *Nsp*V to obtain a cohesive-end DNA fragment. Finally the above described pkkHisCys5 vector with two introduced *Nsp*V sites was digested with *Nsp*V and ligated with the cohesive-end DNA fragment to obtain the expression plasmid for $\alpha_3\beta_3\gamma_{(TCT)}$ (pkkTCT vector). The correct orientation of the DNA fragment inserted into pkkTCT was confirmed by digestion with *Nhe*I and *Afl*II.

Overexpression and Protein Purification of $\alpha_3\beta_3\gamma_{(TCT)}$ and $\alpha_3\beta_3\gamma$ (β-His,γS106C)—pkkTCT was transformed into *E. coli* JM 103 strain, cultivated on LB medium agar plates containing 100 μg/ml ampicillin, and then inoculated into the liquid culture. A 2-liter liquid culture in the same medium was harvested after 9 h cultivation. Cells were collected by centrifugation and disrupted by sonication. The supernatant was 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 $MgCl_2$, 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.

The recombinant $\alpha_3\beta_3\gamma$ subcomplex of TF_1 containing a histidine tag on the N terminus of the β subunit and the substitution of Ser^{106} of the γ subunit to Cys ($\alpha_3\beta_3\gamma$ (β-His, γS106C)) was expressed by using the pkkHisCys5 vector and purified by the method described (11).

Preparation of the ϵ Subunits—ϵ subunit of TF_1 was overexpressed in *E. coli* and isolated as described (32). The recombinant ϵ subunit of CF_1 was expressed as an inclusion body and purified (27). Prior to use, the folded ϵ subunit of CF_1 was prepared as follows: the inclusion body was solved 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 $MgCl_2$ for 6 h.

Preparation of the Recombinant Trx-f—Spinach chloroplast Trx-*f* was overexpressed in *E. coli* and isolated as described (21). The concentration of the purified Trx-*f* was determined by the measurement of the absorbance at 278 nm using the published molar absorption coefficient value of 16,830 $M^{-1}\cdot cm^{-1}$ (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, 100 mM KCl, 5 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 2 mM ATP, 50 μg/ml pyruvate kinase, 50 μg/ml lactate dehydrogenase, and 0.2 mM NADH was used. After a base line was monitored for 1–2 min, the reaction was initiated by the addition of 5–10 μg of $\alpha_3\beta_3\gamma_{(TCT)}$, and the activity was measured by monitoring the decrease of NADH absorption at 340 nm at 25 °C.

In the case where the ATP regenerating system was not applicable, the activity was measured by quantifying the amount of produced phosphate (35). 80 μl of 50 mM MOPS-KOH, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 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 μg) of $\alpha_3\beta_3\gamma_{(TCT)}$. After 2 min, the reaction was quenched by the addition of 100 μl of 2.4% (w/v) trichloroacetic acid. The reaction was carried out at 25 °C unless stated otherwise.

Inhibition by the ϵ Subunit—$\alpha_3\beta_3\gamma_{(TCT)}$ or $\alpha_3\beta_3\gamma$ (β-His, γS106C) were mixed with the ϵ subunit of CF_1 or TF_1 and incubated for 1 h at room temperature. The mixture was then directly applied to the ATP hydrolysis assay using an ATP regenerating system as described above.

ATP Hydrolysis Under Uni-site Conditions—$\alpha_3\beta_3\gamma_{(TCT)}$ was oxidized with 50 μM $CuCl_2$ or reduced with 15 mM DTT for 1 h at 30 °C. DTT in the sample was removed by an HPLC gel filtration column (TSK $3000SW_{XL}$, Tosoh Co., Tokyo, Japan) equilibrated with 50 mM MOPS-KOH, pH 7.0, 100 mM KCl, and 5 mM $MgCl_2$ because DTT directly affected the following quantitative measurement of nucleotides. This step also reduced the amount of intrinsically bound nucleotides from 0.8 mol ADP/mol $\alpha_3\beta_3\gamma_{(TCT)}$ to <0.1 mol ADP/mol $\alpha_3\beta_3\gamma_{(TCT)}$. Each of the complexes was diluted to a protein concentration of 700 nM. A 50-μl aliquot was preincubated in a 25 °C water bath and then mixed with the same volume of 150 nM TNP-ATP. At the indicated period the reaction was terminated by addition of 5 μl of 24% (w/v) trichloroacetic acid. The

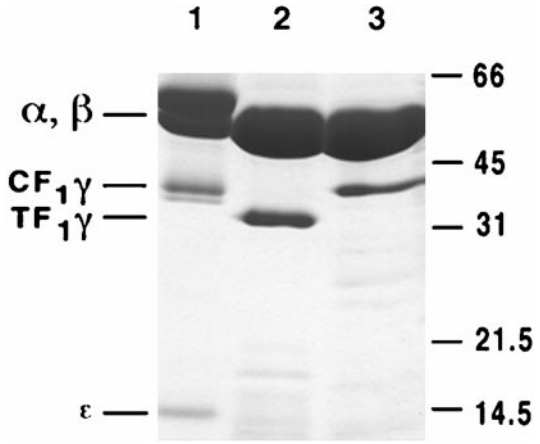

FIG. 2. **Gel electrophoresis of purified $\alpha_3\beta_3\gamma_{(TCT)}$.** Purity and the subunit composition of the complex $\alpha_3\beta_3\gamma_{(TCT)}$ was analyzed by SDS-gel electrophoresis. A 15% (w/v) polyacrylamide gel was used. Each lane contained 15 μg of proteins. *Lane 1,* CF_1; *lane 2,* $\alpha_3\beta_3\gamma$ (β-His, γS106C); *lane 3,* $\alpha_3\beta_3\gamma_{(TCT)}$. *Bars* indicate positions of molecular mass markers (kDa).

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 in the presence of SDS was performed according to Laemmli (36). Protein bands were visualized by the staining with Coomassie Brilliant Blue R-250.

RESULTS

Preparation and Purification of $\alpha_3\beta_3\gamma_{(TCT)}$—$\alpha_3\beta_3\gamma_{(TCT)}$ 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 $\alpha_3\beta_3\gamma_{(TCT)}$ complex was about 80 mg of protein from a 2-liter *E. coli* culture, comparable to the yield of wild type $\alpha_3\beta_3\gamma$ (7) or $\alpha_3\beta_3\gamma$ (β-His, γS106C) (14).

The molecular mass of the γ subunit in the $\alpha_3\beta_3\gamma_{(TCT)}$ complex was about 35 kDa, clearly larger than the γ subunit of $\alpha_3\beta_3\gamma$ (β-His, γS106C) but very similar to the γ subunit of CF_1 (Fig. 2), indicating the successful expression and the formation of the complex with the mutant γ subunit.

Modulation of Activity by Oxidation and Reduction—The activity of ATP hydrolysis catalyzed by the purified $\alpha_3\beta_3\gamma_{(TCT)}$ was measured spectrophotometrically by the pyruvate kinase/lactate dehydrogenase assay that provides continuous regeneration of ATP. Upon addition of oxidized and reduced $\alpha_3\beta_3\gamma_{(TCT)}$ to the reaction mixture, ATP hydrolysis proceeded in a linear manner for at least 10 min without time-dependent inactivation (Fig. 3*A*), but the rates were different as follows: $\alpha_3\beta_3\gamma_{(TCT)}$ 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. 3*B*). Extension of the preincubation time for more than 1 h did not significantly increase activation. 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 $\alpha_3\beta_3\gamma_{(TCT)}$ 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. 3*A*, *inset*). The concentration of Trx-*f* for half-maximal activation was about 200 nM (Fig. 3*B, inset*), considerably lower than that for authentic CF_1, which was more than 1 μM (21). The specific activity of $\alpha_3\beta_3\gamma_{(TCT)}$ 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 $\alpha_3\beta_3\gamma_{(TCT)}$ after reduction with 15 mM DTT and additional 1.7–2.0 mol of SH/mol of $\alpha_3\beta_3\gamma_{(TCT)}$ after reduction with 15 mM DTT plus 500 nM Trx-*f*.

The specific activity of $\alpha_3\beta_3\gamma_{(TCT)}$ in the fully reduced state under the reaction conditions above (pH 7.0) was comparable to that of $\alpha_3\beta_3\gamma$ (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 $\alpha_3\beta_3\gamma$ (7), for TF_1 (37), and for CF_1 (38).

Thermostability of $\alpha_3\beta_3\gamma_{(TCT)}$—The optimum temperature of the ATPase activity of oxidized and reduced $\alpha_3\beta_3\gamma_{(TCT)}$ was at 70 °C, the same as that of $\alpha_3\beta_3\gamma$ (β-His, γS106C) (Fig. 4) and comparable to the values reported for wild type TF_1 and $\alpha_3\beta_3\gamma$ (7). The ratio between the activities of oxidized and reduced $\alpha_3\beta_3\gamma_{(TCT)}$ did not change significantly over the whole temperature range investigated.

Interaction with the ϵ Subunit—The effect of the inhibitory ϵ subunit of CF_1 and that of TF_1 on $\alpha_3\beta_3\gamma_{(TCT)}$ was investigated (Fig. 5*A*). ATPase activity of $\alpha_3\beta_3\gamma_{(TCT)}$ in its oxidized state was inhibited to 50–60% by the ϵ subunit of CF_1. Interestingly, $\alpha_3\beta_3\gamma_{(TCT)}$ in its reduced state was nearly insensitive to the CF_1-ϵ subunit, resulting in about a 4-fold higher activity compared with $\alpha_3\beta_3\gamma_{(TCT)}$ in the oxidized state in the presence of the CF_1-ϵ subunit. As reported previously, neither the CF_1-ϵ subunit nor the TF_1-ϵ subunit had any significant effect on the activity of $\alpha_3\beta_3\gamma$ (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 $\alpha_3\beta_3\gamma_{(TCT)}$ with the TF_1-ϵ subunit (Fig. 5*A*). Again, this effect was less pronounced for $\alpha_3\beta_3\gamma_{(TCT)}$ in the reduced form.

To clarify the cause of the weakly stimulating effect of the ϵ subunit of TF_1 on ATPase activity of $\alpha_3\beta_3\gamma_{(TCT)}$, we examined its binding to the complex. After incubation with ϵ subunit of TF_1, the complex was separated from unbound ϵ subunit by gel filtration HPLC. The $\alpha_3\beta_3\gamma_{(TCT)}$ fraction was then applied to SDS-PAGE (Fig. 5*B*). $\alpha_3\beta_3\gamma_{(TCT)}$ in the oxidized state as well as in the reduced state could bind the ϵ subunit of TF_1 in an approximately stoichiometric ratio, comparable to $\alpha_3\beta_3\gamma$ (β-His, γS106C) under identical conditions.

ATP Hydrolysis Under Uni-site Conditions—When a substoichiometric molar ratio of TNP-ATP was added to the oxidized or reduced $\alpha_3\beta_3\gamma_{(TCT)}$ complexes, the ATP analogue was slowly hydrolyzed. With both complexes it took 20 s to hydrolyze 50% of added TNP-ATP (Fig. 6). An interesting difference was observed upon the addition of chase ATP. Whereas hydrolysis of TNP-ATP by reduced $\alpha_3\beta_3\gamma_{(TCT)}$ was greatly accelerated by ATP, comparable to wild type $\alpha_3\beta_3\gamma$ (32) and $\alpha_3\beta_3\gamma$ (βI389C) (39), chase promotion was obviously lower in the case of the oxidized $\alpha_3\beta_3\gamma_{(TCT)}$.

DISCUSSION

In this study, an expression plasmid for an $\alpha_3\beta_3\gamma$ subcomplex of TF_1 containing a TF_1/CF_1 chimeric γ subunit ($\alpha_3\beta_3\gamma_{(TCT)}$) was constructed, and the desired protein complex was successfully overexpressed and purified with a yield comparable to those of wild type $\alpha_3\beta_3\gamma$ (7) or $\alpha_3\beta_3\gamma$ (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 CF_1 into the central part of the γ subunit of TF_1 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 CF_1-γ subunit and the replaced part of the TF_1-γ sub-

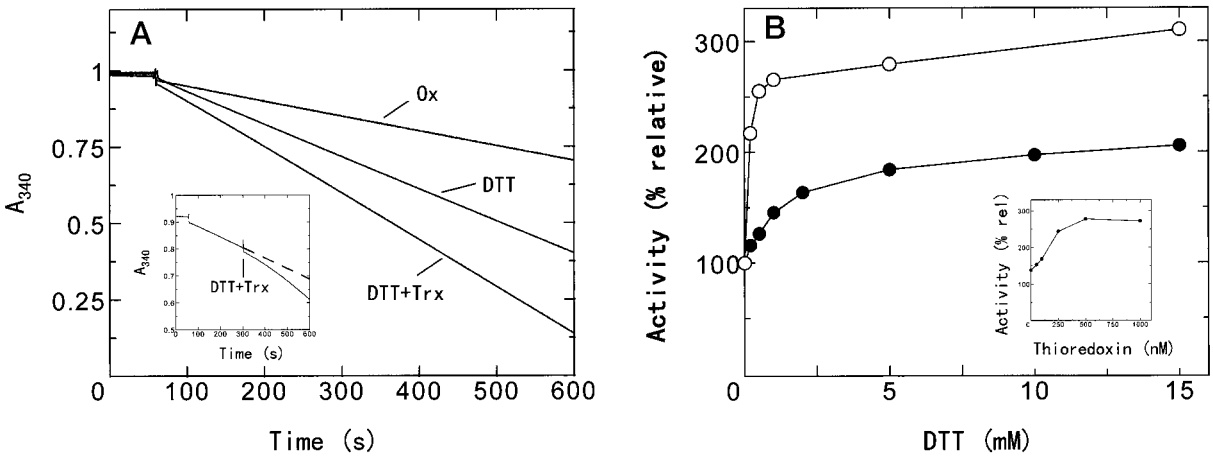

FIG. 3. **Effect of DTT and Trx on ATPase activity of $\alpha_3\beta_3\gamma_{(TCT)}$.** *A*, ATPase activity was measured using an ATP regenerating system monitoring the decrease of NADH absorption at 340 nm. Samples were preincubated for 1 h with 50 μM $CuCl_2$ (*Ox*), 15 mM DTT (*DTT*), or with 15 mM DTT + 500 nM Trx-*f* (*DTT+Trx*) in 50 mM MOPS-KOH, pH 7.0, supplemented with 100 mM KCl and 5 mM $MgCl_2$. The ATPase activity without incubation with DTT or Trx was 1.1 unit/mg and set as 100%. *Inset,* real time activation of oxidized $\alpha_3\beta_3\gamma$(TCT) by addition of 15 mM DTT + 500 nM 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 $\alpha_3\beta_3\gamma_{(TCT)}$ on the DTT concentration. *Closed circles, X* mM DTT; *open circles, X* mM DTT + 500 nM Trx-*f*. *Inset,* dependence of activation on Trx-*f* concentration in the presence of 1 mM DTT. For details see under "Materials and Methods."

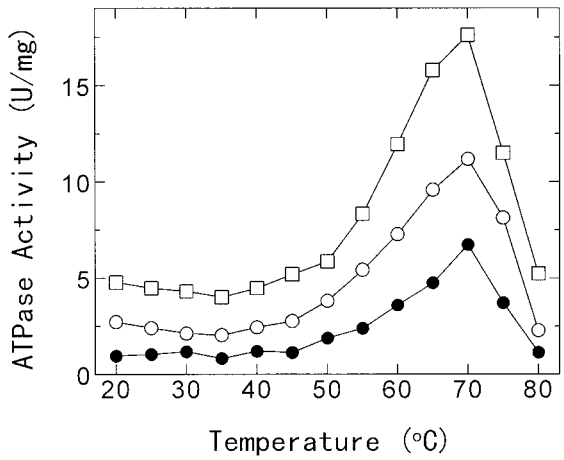

FIG. 4. **Thermostability of $\alpha_3\beta_3\gamma_{(TCT)}$.** ATPase activity was measured in the absence of an ATP regenerating system at the indicated temperatures in 50 mM MOPS-KOH, pH 7.0, supplemented with 100 mM KCl and 5 mM $MgCl_2$. Produced phosphate was quantified by the method described (21). *Closed circles*, $\alpha_3\beta_3\gamma_{(TCT)}$ oxidized with 50 μM $CuCl_2$; *open circles,* $\alpha_3\beta_3\gamma_{(TCT)}$ reduced with 15 mM DTT; *open squares*, $\alpha_3\beta_3\gamma$ (β-His, γS106C).

unit was only about 15% on the amino acid level, proper folding of the γ subunit and insertion into the $\alpha_3\beta_3$ hexagon appears to be unaffected. This is consistent with a recently published intermediate-resolution x-ray structure of *E. coli* F_1, which indicated that four α-helices, thought to be comprised by the central amino acid residues of the γ subunit (roughly *E. coli* F_1 γ100–200, corresponding to residues 100–235 of $\gamma_{(TCT)}$) are located at the tip of the enzyme and not in interaction with the $\alpha_3\beta_3$ hexagon (40).

The ATPase activity of the $\alpha_3\beta_3\gamma_{(TCT)}$ complex was modulated by the reduction or oxidation of the two cysteines that were introduced by the insertion of the respective part of the CF_1-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 CF_1 (3–7-fold) (19–22), most likely the mechanism of thiol activation is the same in the $\alpha_3\beta_3\gamma_{(TCT)}$ complex. Sokolov *et al.* (41) recently proposed that the conformation of the γ subunit of CF_1 may be different from other F_1-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 CF_1 $\alpha_3\beta_3$ 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 F_1 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 TF_1, and the γ subunit of CF_1 ($\alpha_{T3}\beta_{T3}\gamma_C$), was regulated by thiol modulation (27, 28) although the complex showed reduced stability.

Interestingly, a higher degree of activation of the complex $\alpha_3\beta_3\gamma_{(TCT)}$ could be achieved with a mixture of DTT and Trx-*f* than with DTT alone (Fig. 3*B*), although we could not detect a significant difference in the amount of reduced thiol groups. 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 $\alpha_3\beta_3\gamma_{(TCT)}$, similar to $\alpha_3\beta_3\gamma$ (β-His, γS106C), is more stable than a chimeric $\alpha_{T3}\beta_{T3}\gamma_C$, 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 $\alpha_3\beta_3$ 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 vivo* 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 F_1-

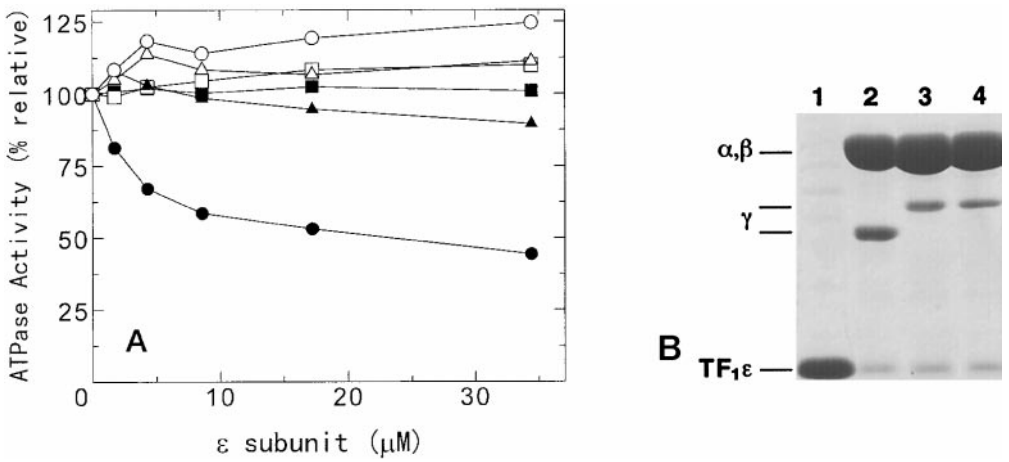

FIG. 5. **Interaction of $\alpha_3\beta_3\gamma_{(TCT)}$ with the ϵ subunit of F_1-ATPase.** *A,* influence of the ϵ subunits on ATP hydrolysis activity. Samples were oxidized with 50 μM $CuCl_2$ (*circles*) or reduced with 15 mM dithiothreitol (*triangles*) and then mixed with the indicated amounts of the ϵ subunit either from CF_1 (*closed symbols*) or from TF_1 (*open symbols*). ATPase activity was measured by an ATP regenerating system; the ATPase activities of $\alpha_3\beta_3\gamma_{(TCT)}$ in the absence of any ϵ subunit were 1.1 and 2.3 units/mg for $\alpha_3\beta_3\gamma_{(TCT)}$ oxidized by $CuCl_2$ and reduced by DTT, respectively, and were set as 100%. $\alpha_3\beta_3\gamma$ (β-His, γS106C) (*squares*) was used as control. *B*, binding of TF_1-ϵ to $\alpha_3\beta_3\gamma_{(TCT)}$ was examined. *B*, isolated $\alpha_3\beta_3\gamma_{(TCT)}$ and $\alpha_3\beta_3\gamma$ (β-His, γS106C) were mixed with TF_1-ϵ, passed through a gel filtration HPLC, and then applied to 12% (w/v) SDS-PAGE. *Lane 1*, TF_1-ϵ; *lane 2*, $\alpha_3\beta_3\gamma$ (β-His, γS106C) + TF_1-ϵ; *lane 3*, $\alpha_3\beta_3\gamma_{(TCT)}$ (oxidized) + TF_1-ϵ; *lane 4,* $\alpha_3\beta_3\gamma_{(TCT)}$ (reduced) + TF_1-ϵ.

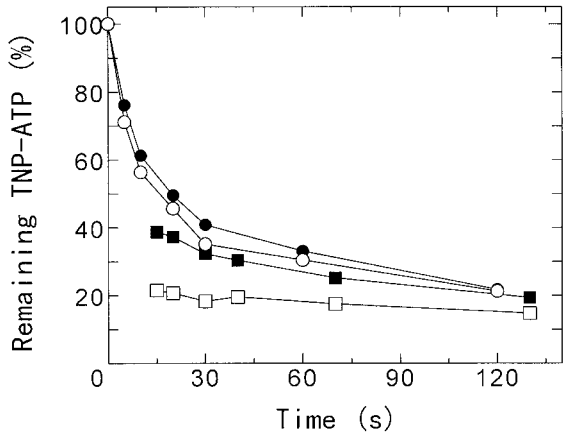

FIG. 6. **Time course of hydrolysis of TNP-ATP by $\alpha_3\beta_3\gamma_{(TCT)}$.** $\alpha_3\beta_3\gamma_{(TCT)}$ was oxidized with 50 μM $CuCl_2$ (*closed symbols*) or reduced with 15 mM DTT plus 500 nM Trx (*open symbols*). 350 nM $\alpha_3\beta_3\gamma_{(TCT)}$ was incubated with 75 nM TNP-ATP at 25 °C for the indicated reaction period. 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 ϵ subunit is mainly responsible for the inhibitory function (44–48). As the ϵ 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 ϵ subunit of CF_1 can efficiently inhibit the ATPase activity of $\alpha_3\beta_3\gamma_{(TCT)}$ but not that of $\alpha_3\beta_3\gamma$ (β-His, γS106C), although the α and β subunits are identical (Fig. 5). This induces the conclusion that an interaction of the ϵ subunit with the γ subunit may be more crucial for inhibition, possibly by providing proper binding and orientation of the ϵ subunit. As the sequence of the amino acid residues 92–239 of the γ subunit of $\alpha_3\beta_3\gamma_{(TCT)}$ is different from $\alpha_3\beta_3\gamma$ (β-His, γS106C), it can be concluded that this region confers the sensitivity to inhibition by the ϵ subunit. Consistent with this finding, *E. coli* F_1 residue γ106 and the region γ202–212 have previously been found to be involved in binding of the ϵ subunit by cross-linking and chemical labeling experiments (51). *E. coli* F_1-γ106 corresponds to $\gamma_{(TCT)}$101, near the N-terminal part of the insertion from CF_1, γ202–212 corresponds to $\gamma_{(TCT)}$ 234–244, located at the C terminus of the insertion (Fig. 1). Taken together, the part of the γ subunit that is of chloroplast origin in $\gamma_{(TCT)}$ appears to be crucial for binding of and inhibition by the ϵ 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 $\alpha_3\beta_3\gamma_{(TCT)}$. 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 $\alpha_3\beta_3\gamma_{(TCT)}$ was comparable to that published for TF_1 (35), wild type $\alpha_3\beta_3\gamma$ (32), and $\alpha_3\beta_3\gamma$ (βI389C) (39). However, significantly lower acceleration was observed for the oxidized $\alpha_3\beta_3\gamma_{(TCT)}$, indicating an impaired interplay between the catalytic sites in the suppressed activity state caused by the formation of the disulfide bridge between Cys^{199} and Cys^{205} of the γ subunit. The regulation of the enzyme activity might be attributed to this difference observed at the chase acceleration level.

The chimeric subcomplex $\alpha_3\beta_3\gamma_{(TCT)}$ combines the property of thiol modulation, a unique feature of CF_1-ATPase, with the stability and relative ease of genetic manipulation of the TF_1-ATPase. It is therefore a very promising tool to investigate the structural basis and functional analyses of thiol modulation more in detail. Further experiments in this direction are under way in our laboratory.

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