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Expression of the wild-type and the Cys-/Trp-less $\alpha_3\beta_3\gamma$ complex of thermophilic F_1-ATPase in *Escherichia coli*

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Abstract

The α, β and γ subunits of F_1-ATPase from thermophilic *Bacillus* PS3 were expressed in *Escherichia coli* cells simultaneously in large amounts. Most of the expressed subunits assembled into a form of $\alpha_3\beta_3\gamma$ complex in *E. coli* cells and this complex was easily purified to homogeneity. The recombinant $\alpha_3\beta_3\gamma$ complex thus obtained showed similar enzymatic properties to the $\alpha_3\beta_3\gamma$ complex obtained by in vitro reconstitution from individual subunits (Yokoyama, K. et al. (1989) J. Biol. Chem. 264, 21837–21841) except that the former had several-fold higher ATPase activity than the latter. Using this expression system, a mutant $\alpha_3\beta_3\gamma$ complex with no Trp and Cys was generated by replacing αCys193 and αTrp463 with Ser and Phe, respectively. This mutant complex was functionally intact, indicating both residues are not essential for catalysis. The Cys-/Trp-less complex is a convenient 'second wild type' enzyme from which one can generate mutants with Trp (as a fluorescent probe) or Cys (as an acceptor of a variety of probes) at desired positions without concern for 'background' Trp and Cys residues.

Keywords: ATPase; ATPase, F_1-; Cys-/Trp-less mutant; F_1; Thermophile; Subunit complex

1. Introduction

F_1-ATPase is a water-soluble catalytic portion of H^+-translocating ATP synthase which synthesizes ATP from ADP and P_i coupled with proton flow across the energy-transducing membranes, such as the plasma membranes of bacteria, the inner membranes of mitochondria, and thylakoid membranes of chloroplasts (reviewed in [1–4]). In general, the F_1-ATPase consists of five different subunits with a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$. The catalytic site is located mainly on the β subunit and noncatalytic site is mainly on the α subunit [5]. The amino acid sequence of the β subunit, as well as the α subunit, is well conserved during evolution. The α and β subunits are also mutually homologous even though the degree of homology is not very high.

We have been studying the structure and function of F_1-ATPase from thermophilic *Bacillus* strain PS3 [6]. This enzyme, TF_1, is highly stable and the function of each subunit has been investigated using the ATPase-active complexes with various subunit compositions reconstituted from individual subunits [7–9]. The reconstitution studies have demonstrated that the $\alpha_3\beta_3$ complex, but not individual α and β subunit alone, exhibits ATPase activity, and thus absolute requirement of both α and β subunits for ATPase activity was proved [10,11]. The properties of the $\alpha_3\beta_3$ complex are, however, rather different from those of TF_1. The $\alpha_3\beta_3$ complex is not heat-stable, it tends to dissociate during ATP hydrolysis, its requirement of divalent metal cations for ATPase activity is much less specific, and its ATPase activity is not sensitive to azide inhibition. In this respect, the $\alpha_3\beta_3\gamma$ complex has similar characteristics to TF_1 [9]. Importance of the γ subunit in 'gating' of proton flow was indicated at first from reconstitution experiment [8]. Recent achievements of molecular

Abbreviations: CW-less $\alpha_3\beta_3\gamma$ complex, a mutant $\alpha_3\beta_3\gamma$ complex with substitutions of αCys193 → Ser and αTrp463 → Phe; F_oF_1, H^+-translocating ATP synthase; F_1-ATPase, catalytic portion of H^+-translocating ATP synthase; TF_1, EF_1, and MF_1, F_1-ATPase from thermophilic *Bacillus* PS3, *Escherichia coli*, and mitochondria, respectively; TF_oF_1, H^+-translocating ATP synthase from *Bacillus* PS3; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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genetic and site-directed fluorescent modification studies on *Escherichia coli* F_1-ATPase (EF_1) [12–15] and X-ray crystallography of bovine heart mitochondrial F_1-ATPase (MF_1) [5] strongly suggest the essential role of the γ subunit in coupling between proton flow and catalysis. Therefore, the $\alpha_3\beta_3\gamma$ complex, rather than $\alpha_3\beta_3$ complex, is thought to be the minimum complex that possesses essential features of F_1-ATPase and has been used to examine the nature of mutant α and β subunits [16–20].

At early stage of our research, the $\alpha_3\beta_3\gamma$ complex was obtained by reconstitution from α, β and γ subunits which were isolated from denatured TF_1 [7] and the procedures were tedious. Later, cloning of TF_1 genes and construction of expression system for each subunit in *E. coli* cells were achieved by Kagawa's group which contributed significantly to improve the reconstitution procedures [17,21]. These methods of reconstitution, however, still have several drawbacks, such as unstable expression, inevitable exposure of the γ subunit to 8 M urea during purification, low water-solubility of the isolated γ subunit, occasional poor reconstitution yield, and possible contamination of the $\alpha_3\beta_3$ complex. We have improved each of problems on the one hand [19,20] and, on the other hand, also have tried to express three subunits as $\alpha_3\beta_3\gamma$ complex in the same *E. coli* cell as a final solution. Although the difficulty had been foreseen because the ATPase-active complex should waste ATP in the expressing *E. coli* cell, here we report the success in construction of the convenient expression system of the active $\alpha_3\beta_3\gamma$ complex of TF_1. Using this system, we have generated a mutant $\alpha_3\beta_3\gamma$ complex of which all Trp and Cys are replaced by other residues. If this mutant is functionally intact, it will be a convenient 'second wild type' enzyme to which the fluorescent (or other) probes can be generated at any defined position by next mutation to introduce Trp or Cys. Cys is to be chemically modified by specific probe reagents.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli strains used here were JM109 [22] for preparation of plasmids, CJ236 [23] for generating uracil-containing single-stranded plasmids for site-directed mutagenesis and BL21(DE3) [24] for gene expression by plasmids carrying T7 promoter. The *unc* deletion mutant JM103 Δ(*uncB-uncD*) [25] was provided from Dr. W.S.A. Brusilow (Wayne State University, Detroit) and used for expression by plasmids derived from pKK223-3. As growth media, 2 × YT was used for preparation of plasmids and for small scale culture to check gene expression, and terrific broth was used for large scale culture to purify gene products [26]. Ampicillin (50–100 μg/ml) was supplemented in the growth media. pUC118α was constructed by transferring the *Sal*I-*Hin*dIII restriction fragment from pTZα [21] to pUC118 [27]. Construction of pUC118β was described previously [16]. pTF1 carrying the entire TF_0F_1 genes was constructed as follows. Genomic DNA isolated from *Bacillus* PS3 was digested with *Pst*I and *Sna*BI and the 6.7 kb fragment was ligated into the *Pst*I-*Sma*I site of pUC119 [27]. pTD · T7 [28] was gifted from Dr. T. Date (Kanazawa Medical School, Uchinada, Japan). Expression plasmid pKK223-3 [29] and helper phage M13 K07 were obtained from Pharmacia Japan, Tokyo.

2.2. Construction of the expression plasmids of the $\alpha_3\beta_3\gamma$ complex

The construction of the expression plasmids of the $\alpha_3\beta_3\gamma$ complex is outlined in Fig. 1. The genes encoding the α, β and γ subunits of TF_1 were derived from pUC118α, pUC118β and pTF1, respectively. The 1.7-kb

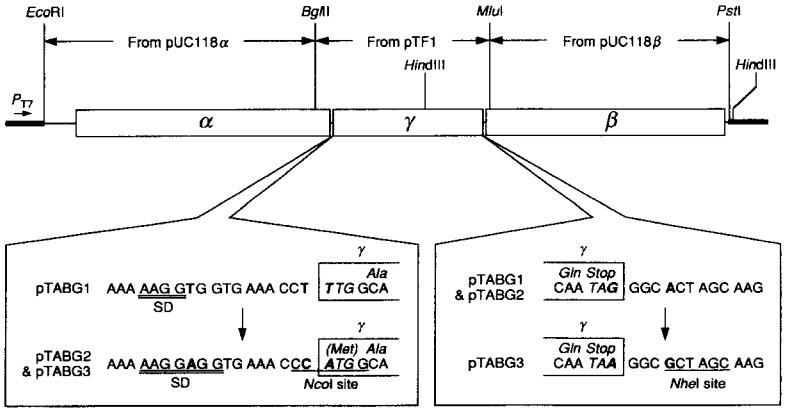

Fig. 1. Construction of plasmids pTABG1, pTABG2, and pTABG3. The genes coding the α, β, and γ subunits of TF_1 were cloned between the *Eco*RI and *Pst*I sites of pTD · T7. pTABG2 and pTABG3 were constructed by introducing mutations into pTABG1. The replaced bases are shown by bold letters. The underlined restriction sites were also introduced. Abbreviations used are: P_{T7}, T7 promoter; SD, Shine-Dalgarno sequence.

*Eco*RI-*Bgl*II fragment from pUC118α, the 1.0-kb *Bgl*II-*Mlu*I fragment from pTF1 and the 1.4-kb *Mlu*I-*Pst*I fragment from pUC118β were ligated into the *Eco*RI-*Pst*I site of pTD · T7 to produce pTABG1. pTABG2 was constructed by site-directed mutagenesis on pTABG1. Using the synthetic oligonucleotide P-γATG, 5′-AACGATGCCA**TG**GGTTTCACC**T**CCTTTTTGCTG-3′ (changed bases are underlined), the mutation was introduced at the initiation region of the γ subunit. pTABG3 was constructed as follows. The termination codon of the γ subunit on pTABG1 was replaced by using the synthetic oligonucleotide P-γTAA, 5′-TAACTTGCTAG**C**GC-C**T**TATTGCAAGGC-3′. After this mutated plasmid was digested with *Hin*dIII, the 1.7-kb fragment was isolated and ligated into the 5.1-kb *Hin*dIII fragment of pTABG2. pKABG1 was constructed by transferring the 4.1-kb *Eco*RI-*Pst*I fragment from pTABG3 to pKK223-3 behind *tac* promoter.

2.3. Construction of the Cys-/Trp-less $\alpha_3\beta_3\gamma$ complex

The expression plasmids for the Cys-/Trp-less (CW-less) mutant of the $\alpha_3\beta_3\gamma$ complex was constructed as follows. The mutation of Cys193 → Ser in the α subunit was introduced into pTABG1 by using the oligonucleotide P-αC193S, 5′-GCGACATAAATA**G**A**T**ATCATGTTT-TGG-3′, and the resulting plasmid with a new *Eco*RV site was designated pTABG1-αC193S. Likewise, the mutation of Trp463 → Phe in the α subunit was carried out by using the oligonucleotide P-αW463F, 5′-GCCGTTTTG-GTC**T**AG**AA**ACAAGTAAAACTC-3′, and the resulting plasmid with a new *Xba*I site was named pTABG1-αW463F. Subsequently, the *Eco*RI-*Bcl*I fragment from pTABG1-αC193S and the *Bcl*I-*Bgl*II fragment from pTABG1-αW463F were ligated into the *Eco*RI-*Bgl*II site of pKABG1 to produce pKABG2.

2.4. Purification of TF_1 and the $\alpha_3\beta_3\gamma$ complex

TF_1 was purified as described previously [6]. Purification of the $\alpha_3\beta_3\gamma$ complex expressed in *E. coli* was carried out as follows. Strain JM103 Δ(*uncB-uncD*)/pKABG1 was grown in 1.5 liter of terrific broth containing 100 μg/ml ampicillin at 37°C. An inducer, isopropyl β-D-thiogalactopyranoside (IPTG), was not added to the culture since the complex was well expressed without induction and the addition of the inducer did not improve the expression. When terrific broth was used as the growth medium, the yield was increased about 3-times more than 2 × YT medium. Cells were harvested in the late logarithmic phase. About 13 g (wet weight) cells were suspended in 50 ml of 50 mM Tris-sulfate (pH 8.0), 1 mM EDTA (buffer A), and they were disrupted by sonication in ice bath. The cell lysate was incubated at 60°C for 30 min and the insoluble denatured proteins were removed by centrifugation for 30 min at 35 000 rpm. After this step, all the procedures were carried out at room temperature. The supernatant was applied to a DEAE-Sephacel column (2.8 × 10 cm) equilibrated with buffer A. The column was washed with 200 ml of buffer A containing 150 mM NaCl and the complex was eluted with a 150–500 mM NaCl linear gradient in buffer A (total 400 ml). The fractions containing the complex were combined and solid ammonium sulfate was added to 0.75 M. After centrifugation for 15 min at 10 000 rpm, the supernatant was applied to a Butyl-Toyopearl column (2.8 × 8 cm) equilibrated with buffer A containing 0.75 M ammonium sulfate. The column was washed with 200 ml of the same buffer and the complex was eluted with a 0.75–0 M ammonium sulfate gradient in buffer A (total 400 ml). The fractions containing the $\alpha_3\beta_3\gamma$ complex were combined and precipitated by the addition of solid ammonium sulfate to a final concentration of 75% saturation. The pellet was collected by centrifugation at 10 000 rpm for 15 min and dissolved in 4 ml of buffer A containing 50 mM NaCl. After insoluble material was removed by centrifugation, the supernatant was subjected to a Sephacryl S-300HR column (2.2 × 46 cm) equilibrated with buffer A containing 50 mM NaCl. The flow rate was 5 ml/min. The fractions containing the complex were pooled and stored in 70% saturated ammonium sulfate suspension at 4°C.

2.5. Assays of ATPase activity

ATPase activities at various temperatures were measured as described below. A reaction mixture (475 ml) containing 50 mM Tris-chloride (pH 8.0), 2 mM $MgCl_2$, and the enzyme (5 pmol) was preincubated at the indicated temperature for 3 min. The reaction was initiated by addition of 25 μl of 0.1 M ATP-Mg (0.1 M ATP + 0.1 M $MgCl_2$) to a final concentration of 5 mM. The reaction was terminated after 5 min by addition of perchloric acid and the amount of released P_i was determined [6]. Under this assay condition, the amount of produced P_i was less than 20% of added ATP. When ATPase activity at various ATP concentrations was measured, a coupled enzyme assay was used [30]. A reaction mixture (1 ml) contained 50 mM Tris-chloride (pH 8.0), 2 mM $MgCl_2$, 10 mM KCl, 2.5 mM phospho*enol*pyruvate, 50 μg pyruvate kinase (rabbit muscle), 50 μg lactate dehydrogenase (pig muscle), 0.2 mM NADH, and the indicated concentrations of ATP. Pyruvate kinase and lactate dehydrogenase were diluted from 50% glycerol stock solutions (Boehringer Mannheim). The reaction was initiated at 25°C by addition of 10 pmol of TF_1 or the complexes. The rate of ATP hydrolysis was monitored as the oxidation rate of NADH by measuring the decrease of the absorbance at 340 nm. The constant slope of the absorbance decrease, which was reached at about 10 min after initiation of the reaction, was taken as a steady-state rate. Usually, ATPase activities measured by the coupled enzyme assay were larger than those obtained from the P_i assay probably due to the presence of initial

lag period after initiation of the assay [31] and the cumulative inhibition of the product ADP in the P_i assay [32]. One unit of activity was defined as the amount of enzyme which hydrolyzes 1 μmol ATP per min.

2.6. General procedures

Recombinant DNA procedures were performed as described in the manual [26]. Site-directed mutagenesis was carried out as described by Kunkel et al. [23]. Protein concentrations were determined by the method of Lowry et al. [33]. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE) was performed on 12.5% acrylamide slab gel according to Laemmli [34]. Gel electrophoresis in non-denaturing condition (Native-PAGE) was performed on 7.5% acrylamide gel. Proteins in the gels were stained with Coomassie brilliant blue R-250. Molecular weight markers from Bio-Rad were used. N-terminal peptide sequencing was carried out as described previously [35]. Analysis of sulfhydryl groups in proteins by using 5,5′-dithiobis-2-nitrobenzoic acid was performed as described previously [20]. Fluorescence was measured with a FP-777 spectrofluorometer (JASCO, Tokyo) at room temperature. Each fluorescence emission spectrum was corrected for background fluorescence by subtracting the corresponding buffer spectrum.

3. Results

3.1. Construction of the expression system of thermophilic $\alpha_3\beta_3\gamma$ complex

To construct an expression plasmid of thermophilic $\alpha_3\beta_3\gamma$ complex, α, β and γ subunit genes isolated from *Bacillus* PS3 were cloned into pTD · T7 after T7 promoter, and the resulting plasmid was designated pTABG1 (Fig. 1). pTD · T7 has only T7 promoter as the expression promoter which is powerful and highly controllable, and it carries the intergenic region of bacteriophage M13 to prepare single-stranded DNA for site-directed mutagenesis [28]. The genes cloned in this plasmid cannot be transcribed in the absence of T7 RNA polymerase, and so, to express the cloned genes, pTABG1 was introduced into strain BL21(DE3) which has T7 RNA polymerase gene following the inducible *lac*UV5 promoter in its genomic DNA. To check whether the cloned genes were expressed

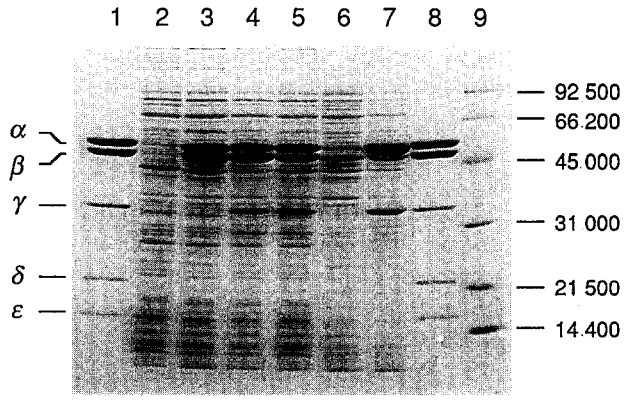

Fig. 2. Expression of the $\alpha_3\beta_3\gamma$ complex from various expression plasmids. *E. coli* cells in which gene expression was induced were broken by sonication. After centrifugation, each supernatant containing 20 μg protein was analyzed by SDS-PAGE (12.5% acrylamide). Lanes 1 and 8, purified TF_1; lane 2, BL21(DE3)/pTD · T7; lane 3, BL21(DE3)/pTABG1; lane 4, BL21(DE3)/pTABG2; lane 5, BL21(DE3)/pTABG3; lane 6, JM103 Δ(*uncB-uncD*)/pKK223-3; lane 7, JM103 Δ(*uncB-uncD*)/pKABG1; lane 9, molecular weight markers. Each molecular size of the standards is shown on right.

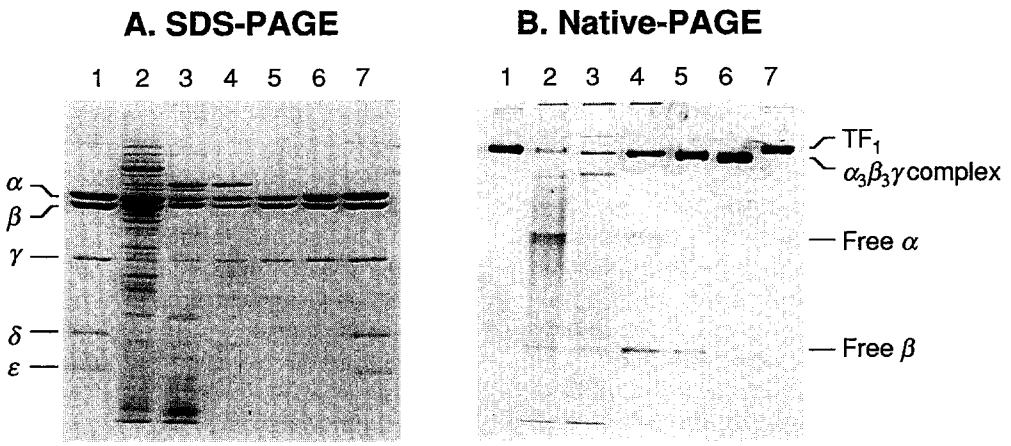

Fig. 3. Purification of the recombinant $\alpha_3\beta_3\gamma$ complex. A sample corresponding to each step in the purification procedure was analyzed by PAGE. (A) SDS-PAGE (12.5% acrylamide). (B) Native-PAGE (7.5% acrylamide). Gels were stained with Coomassie brilliant blue. Lanes 1 and 7, purified TF_1; lane 2, crude cell extract (20 μg); lane 3, supernatant after heat treatment (10 μg); lane 4, DEAE-Sephacel eluate (5 μg); lane 5, Butyl-Toyopearl eluate (5 μg); lane 6, Sephacryl S-300HR eluate (5 μg).

or not, BL21(DE3)/pTABG1 was grown in 2 × YT medium containing ampicillin and gene expression was induced by addition of 1 mM IPTG. The cell lysate was analyzed by PAGE. As shown in Fig. 2, lane 3, very little γ subunit was expressed although the α and β subunits were overexpressed. Most of the α and β subunits expressed in the cell were free and the $\alpha_3\beta_3\gamma$ complex was hardly formed (data not shown). To improve poor expression of the γ subunit, the unusual initiation codon (TTG) of the γ subunit gene of TF_1 [21] was replaced by ATG, and, at the same time, Shine-Dalgarno sequence of the γ subunit gene was also substituted by the consensus sequence by site-directed mutagenesis (Fig. 1). Strain BL21(DE3) harboring the improved plasmid pTABG2 expressed more γ subunit (Fig. 2, lane 4). N-terminal peptide sequence analysis revealed that the expressed γ subunit did not have Met at the N-terminus although the initiation codon was ATG in pTABG2. Native-PAGE showed that the expressed γ subunit was mostly incorporated into the $\alpha_3\beta_3\gamma$ complex (data not shown). Since the strain BL21(DE3) has an intact *unc* (F_oF_1) operon and there is a possibility that the produced $\alpha_3\beta_3\gamma$ complex is not homogeneous but chimeric complex between EF_1 and TF_1, the host strain was changed to strain JM103 Δ(*uncB-uncD*) of which most of F_1-ATPase genes were deleted. Since the strain has *lacI*^{q} gene, inducible expression is possible by using expression plasmids carrying *lac* or *tac* promoter as an expression promoter. Since JM103 Δ(*uncB-uncD*) is also *supE* mutant, the stop codon of the γ subunit gene, TAG, was replaced by TAA (plasmid pTABG3). The BL21(DE3)/pTABG3 also expressed the $\alpha_3\beta_3\gamma$ complex at high yield (Fig. 2, lane 5). The *Eco*RI-*Pst*I fragment containing the α, β and γ gene was transferred from pTABG3 to pKK223-3 which has a powerful *tac* promoter (plasmid pKABG1). In JM103 Δ(*uncB-uncD*)/pKABG1, all of the α, β and γ subunits were expressed, although the expression of the β subunit was less efficient than the other subunits (Fig. 2, lane 7; Fig. 3, lane 1). However, the amount of expressed $\alpha_3\beta_3\gamma$ complex was usually good enough, and we decided to use strain JM103 Δ(*uncB-uncD*)/pKABG1 for large scale preparation of the $\alpha_3\beta_3\gamma$ complex of TF_1.

3.2. Purification of the recombinant $\alpha_3\beta_3\gamma$ complex

The results of a typical purification of the recombinant $\alpha_3\beta_3\gamma$ complex are summarized in Table 1. The purification procedure of the recombinant complex was essentially the same as that of TF_1 except for some modifications. The complex was purified in a four-step procedure including heat treatment, anion-exchange chromatography on DEAE-Sephacel, hydrophobic interaction chromatography on Butyl-Toyopearl, and gel filtration on Sephacryl S-300HR. The first step served to remove a large amount of *E. coli* proteins by heat denaturation (Fig. 3A, lane 2 and 3). In the subsequent two steps of the column chromatographies, most of the residual *E. coli* proteins were removed and the fraction after Butyl-Toyopearl seemed to be almost pure (Fig. 3A, lane 5). However, Native-PAGE showed that it contained a trace amount of the free β subunit (Fig. 3B, lane 5). This free subunit was removed by next Sephacryl S-300HR chromatography (Fig. 3B, lane 6). The purified complex had much higher specific activity ($\sim$ 23 units/mg at 65°C) than the $\alpha_3\beta_3\gamma$ complex prepared from the individual subunits by previous reconstitution method [7,9].

3.3. Characterization of the Cys-/Trp-less $\alpha_3\beta_3\gamma$ complex

The α subunit of TF_1 contains a single Cys (αC193) and Trp (αW463), and other subunits do not have these residues at all [21,36,37]. Consequently, TF_1 has three Cys residues and three Trp residues. The Cys residues corresponding to TF_1-αC193 are conserved in α subunits of F_1-ATPases from all of sources so far known. We replaced this residue by Ser (coincidentally, the residue in the TF_1-β subunit corresponding to TF_1-αC193 is Ser-183). The residues corresponding to the position of TF_1-αW463 are not conserved in MF_1 and EF_1 and they are His and Tyr, respectively. The common property in these residues is the possession of bulky and cyclic side chains. We replaced it by Phe, whose fluorescence is much less than Trp.

The mutant α subunit (αC193S/W463F) assembled normally into $\alpha_3\beta_3\gamma$ complex with the wild-type β and γ

Table 1
Purification table of the recombinant $\alpha_3\beta_3\gamma$-complex

Step	Protein (mg)	ATPase activity at 65°C		Yield (%)
		total (units)	specific (units/mg protein)	
Cell extract	1610	1210	0.752	100
Supernatant after heat treatment	339	1190	3.51	98
DEAE-Sephacel eluate	85.3	938	11.0	78
Butyl-Toyopearl eluate	30.9	523	16.9	43
Sepharecryl S-300HR eluate	14.9	339	22.8	28

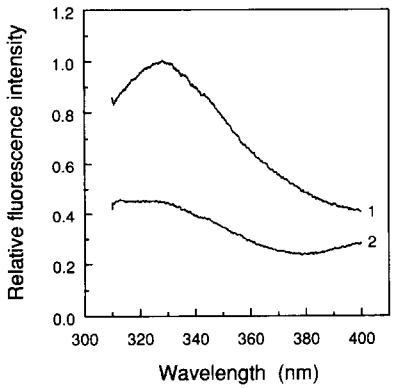

Fig. 4. Fluorescence emission spectra of the wild-type (trace 1) and CW-less $\alpha_3\beta_3\gamma$ complexes (trace 2). The samples contained 50 mM Tris-chloride (pH 8.0), 2 mM $MgCl_2$, and 2 μM wild-type or CW-less $\alpha_3\beta_3\gamma$ complex. Spectra were measured at room temperature. The excitation wavelength was 300 nm, and the excitation and emission bandwidths were set at 1.5 nm and at 10 nm, respectively. Each fluorescence spectrum was corrected for background fluorescence and the Raman scattering peak by subtracting the corresponding buffer spectrum.

subunits in *E. coli* cells. The expressed mutant (CW-less) complex was purified by the same procedures as the wild-type complex and it behaved similarly in all the steps of the purification procedure.

The number of free sulfhydryl groups obtained from titration by 5,5'-dithiobis-2-nitrobenzoic acid in 1% SDS was 0.08 mol/mol for the CW-less mutant complex and 2.94 mol/mol for the wild-type complex, indicating that Cys residue was in fact eliminated in the mutant complex. In the absence of SDS, both CW-less and wild-type complexes did not have detectable sulfhydryl residue (< 0.02 mol/mol).

The fluorescence emission spectra of the wild-type and the CW-less complexes are shown in Fig. 4. Since excitation wavelength was 300 nm, Trp was mainly responsible for the emission. The wild-type complex had an emission maximum at approx. 330 nm (Fig. 4, trace 1), whereas the mutant did not exhibit a peak around the corresponding wavelength (Fig. 4, trace 2), confirming that the residue of αW463 was replaced in the mutant complex.

3.4. Effect of temperature on the ATPase activities of the CW-less $\alpha_3\beta_3\gamma$ complex

The ATPase activities of native TF_1 and the recombinant wild-type and CW-less $\alpha_3\beta_3\gamma$ complexes were measured at various temperatures in the range from 10°C to 80°C (Fig. 5). Similar to TF_1 [7], the wild-type and the CW-less $\alpha_3\beta_3\gamma$ complexes exhibited two peaks of activity in their temperature-activity profiles, but the peak temperatures of both $\alpha_3\beta_3\gamma$ complexes (15°C, 65°C) were about 5°C lower than that of TF_1 (20°C, 70°C) under the condition tested. At whole temperature range except above 70°C where heat inactivation occurred, ATPase activity of the $\alpha_3\beta_3\gamma$ complex, either wild-type or the mutant, was

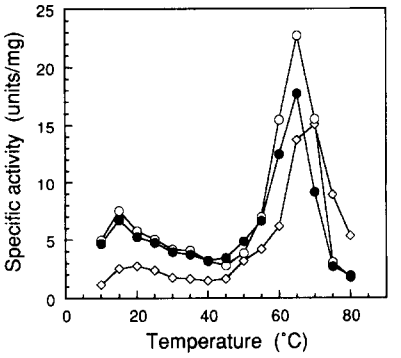

Fig. 5. Temperature dependence of ATPase activities of the complexes. Assays were performed as described in Materials and methods. ◇, TF_1; ○, wild-type $\alpha_3\beta_3\gamma$ complex; ●, CW-less $\alpha_3\beta_3\gamma$ complex.

significantly higher than that of TF_1. The wild-type and mutant complexes had almost the same activity below 60°C. However, above 60°C, activity of the CW-less mutant is lower than that of the wild-type complex, about 80% at 65°C and 60% at 70°C. This difference might be caused by slight decrease of heat stability of the mutant $\alpha_3\beta_3\gamma$ complex.

3.5. Kinetics of the ATPase activities of the CW-less $\alpha_3\beta_3\gamma$ complex

The ATPase activities of TF_1 and the $\alpha_3\beta_3\gamma$ complexes were measured at 25°C at various ATP concentrations in the range from 11 μM to 5.5 mM (Fig. 6). The kinetics of the recombinant wild-type and CW-less $\alpha_3\beta_3\gamma$ complexes were almost indistinguishable. Similar to TF_1 [38], their Eadie-Hofstee plots were concave indicating

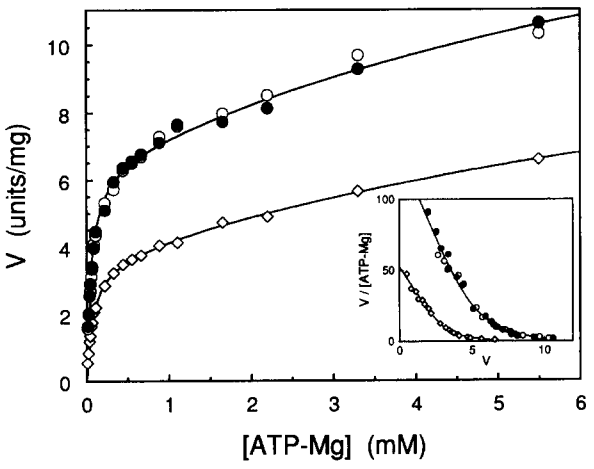

Fig. 6. Kinetics of ATP hydrolysis catalyzed by the complexes. Assays were performed as described in Materials and methods. Inset: Eadie-Hofstee plots. Lines are drawn by a curve fitting program assuming kinetic parameters described in footnote 1. ◇, TF_1; ○, wild-type $\alpha_3\beta_3\gamma$ complex; ●, CW-less $\alpha_3\beta_3\gamma$ complex. V, rate of ATP hydrolysis; [ATP-Mg], concentration of ATP-Mg.

negative cooperativity of catalysis (Fig. 6, inset) [1]. The $\alpha_3\beta_3\gamma$ complex obtained from in vitro reconstitution showed similar kinetics with negative cooperativity but its magnitude of the ATPase activity (2.2 units/mg at 23°C) [9] was much less than that of the recombinant $\alpha_3\beta_3\gamma$ complex. The fact that TF_1 is less active than the $\alpha_3\beta_3\gamma$ complex indicates the inhibitory role of the δ or ϵ subunit or both. The specificity for cation requirement for ATPase activity and sensitivity to azide inhibition of the CW-less $\alpha_3\beta_3\gamma$ complex were similar to those of the wild-type complex and TF_1 (data not shown).

4. Discussion

The $\alpha_3\beta_3\gamma$ complex is the minimum subunit complex which retains many features of TF_1. The complex also provides opportunity to test the roles of δ and ε subunits by adding them to the complex. The previous method of preparing the complex, reconstitution from individual subunits, is significantly improved by the construction of the over-expression system in *E. coli* described here. Firstly, as much as about 20 mg of pure $\alpha_3\beta_3\gamma$ complex can be obtained routinely from a 1 liter culture. Only 1–3 mg of the complex was obtained after laborious work by the previous method. Secondly, a purification procedure for a single protein is necessary to obtain the complex. It includes effective heat treatment to remove host *E. coli* proteins, but the complex is native throughout procedures. This is in contrast to the previous method; three subunits should be individually expressed and purified, purification procedure of the γ subunit which is poorly soluble in water includes exposure to 8 M urea, and further purification of the complex is necessary after reconstitution to remove free subunits [9]. Thirdly, thus purified recombinant $\alpha_3\beta_3\gamma$ complex is very homogeneous and does not contain $\alpha_3\beta_3$ complex. The complex is highly ATPase-active, probably because it is not exposed to denaturation reagent during purification.

The characteristics of ATPase activity of the recombinant $\alpha_3\beta_3\gamma$ complex are essentially very similar to the $\alpha_3\beta_3\gamma$ complex reconstituted from individual subunits except that the former has several-fold higher ATPase activity than the latter. The specificity for cation requirement, azide sensitivity, and negative cooperativity of kinetics of the $\alpha_3\beta_3\gamma$ complex, obtained from either over-expressed *E. coli* or reconstitution, are similar to those of TF_1 [7,9]. The recombinant $\alpha_3\beta_3\gamma$ complex is heat-stable but the degree of stability is slightly lower than TF_1. Heat-stability of the reconstituted $\alpha_3\beta_3\gamma$ complex was the same as the recombinant $\alpha_3\beta_3\gamma$ complex [7]. The ATPase activity of the $\alpha_3\beta_3\gamma$ preparation reported here was higher than those of not only the reconstituted $\alpha_3\beta_3\gamma$ complex but also TF_1. The difference of the activities between the $\alpha_3\beta_3\gamma$ complex and TF_1 should be due to the presence of the δ or ε subunits or both in TF_1. Since the ϵ subunit has been known to play an inhibitory role to the ATPase activity of EF_1-ATPase [39] and F_1-ATPase from chloroplast [40], the higher activity of the recombinant $\alpha_3\beta_3\gamma$ complex over TF_1 may be attributable to the absence of the ε subunit. However, involvement of the δ subunit cannot be excluded, since Xiao et al. demonstrated that the binding of the δ subunit to the $\alpha_3\beta_3\gamma\varepsilon$ complex of EF_1 results in loss of chase promotion in unisite catalysis by addition of excess ATP [41].

The CW-less $\alpha_3\beta_3\gamma$ complex showed almost the same ATPase activity as the wild-type complex. Although the Cys residues corresponding to αC193 of TF_1 are conserved for all F_1-ATPases so far known, Cys at this position does not have direct influence to ATPase activity. This Cys is not accessible from outside [36] and is located in one of β-strands buried inside the protein structure [5]. The residues corresponding to αW463 are not conserved in other F_1-ATPases and it corresponds to αH471 in MF_1, which is located in an α-helix in the C-terminal helical bundle [5]. In the study of intrinsic Trp fluorescence of mitochondrial F_1-ATPase from *Schizosaccharomyces pombe*, an emission maximum was observed at 332 nm in the native enzyme and at 354 nm in the denatured one [42]. This shift of peak wavelength corresponds to the loss of interactions of the Trp residues to other residues inside the native protein structure upon denaturation. Similarly, since the fluorescence emission spectrum of TF_1 [37] and the wild-type $\alpha_3\beta_3\gamma$ complex of TF_1 exhibited an emission maximum at approx. 330 nm as shown in Fig. 4, αW463 is certainly located in the same α-helix as αH471 is in MF_1. αC193 and/or αW463 of the $\alpha_3\beta_3\gamma$ complex may contribute to the stability of protein structure to some extent because the activity of the mutant was smaller than that of the wild-type complex above 60°C.

Fluorescence spectroscopy is a sensitive method to detect rapid conformational changes of proteins and has been applied to the study of F_1-ATPase [43–45]. In order to give clear interpretation, the site of fluorescent probe should be specified. The CW-less $\alpha_3\beta_3\gamma$ complex, which is functionally intact as described in this paper, is a very useful 'second wild type' enzyme in this sense. If Trp is mutationally introduced at a certain site of the CW-less mutant complex, all the Trp fluorescence is attributable to this newly introduced Trp. A mutant EF_1 in which all nine Trp residues were replaced by Tyr, Phe, and Leu was

[1] At least two sets of apparent kinetic parameters are necessary to explain Eadie-Hofstee plots and K_m and corresponding V_{max} values calculated by one of curve fitting programs are; TF_1 (2.8 mM and 4.5 units/mg, 63 μM and 3.2 units/mg), the wild-type $\alpha_3\beta_3\gamma$ complexes (0.97 mM and 5.6 units/mg, 38 μM and 4.9 units/mg), the CW-less $\alpha_3\beta_3\gamma$ complex (1.3 mM and 5.7 units/mg, 39 μM and 5.2 units/mg). However, these values should not be taken as final values since they are vulnerable to the small deviations of the data especially at very high and low concentrations of ATP and easily changed by a simulation program used for curve fitting.

generated by Wilke-Mounts et al. for this purpose [46]. Another way to put fluorescent probe at desired position is modification of Cys by fluorescent probe reagents. Validity of this approach has been demonstrated by Capaldi and his co-workers who modified an introduced Cys residue of EF_1 with fluorescent maleimides and the structural changes in the enzymes were monitored by fluorescent change [14,15,47]. EF_1 contained as much as 15 Cys residues (3 in α, 1 in β, 1 in γ, 2 in δ, and zero in ε subunit) and one has to map the probe binding sites by biochemical methods. In this respect, the CW-less $\alpha_3\beta_3\gamma$ complex of TF_1 is easy; if Cys is mutationally introduced at a certain site, there is no Cys other than the introduced one. We are currently examining the change of protein structure during ATP hydrolysis using secondary mutants of the CW-less $\alpha_3\beta_3\gamma$ complex.

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