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The $\alpha_3\beta_3$ complex, the catalytic core of F_1-ATPase

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ABSTRACT **The $\alpha_3\beta_3$ complex was reconstituted from α and β subunits of the thermophilic bacterium PS3 F_1-ATPase (TF_1) and then isolated. It is less stable at high and low temperatures than TF_1, and the complex dissociates into subunits during native polyacrylamide gel electrophoresis. The $\alpha_3\beta_3$ complex has about 20% of the ATPase activity of TF_1. Its enzymic properties are similar to those of the native TF_1, exhibiting similar cooperative kinetics as a function of ATP concentration, similar substrate specificity for nucleotide triphosphates, and the presence of two peaks in its temperature–activity profile. Differing from TF_1, the ATPase activity of the $\alpha_3\beta_3$ complex is insensitive to N_3^- inhibition, its divalent cation specificity is less stringent, and its optimum pH shifts to the alkaline side. The addition of the γ subunit to the $\alpha_3\beta_3$ complex leads to the formation of the $\alpha_3\beta_3\gamma$ complex, indicating that the $\alpha_3\beta_3$ complex is an intermediate in the process of assembly of the holoenzyme from each subunit. These results definitely show that the essential structure for eliciting the ATPase activity of F_1-ATPase is trimeric $\alpha\beta$ pairs and that the kinetic cooperativity of the F_1-ATPase is an inherent property of this trimeric structure but is not due to the presence of single-copy subunits. In this sense, the $\alpha_3\beta_3$ complex is the catalytic core of F_1-ATPase.**

In energy-transducing membranes of bacteria, mitochondria, and chloroplasts, ATP synthases catalyze ATP synthesis coupled with proton flow (1–3). These enzymes are composed of an integral membrane protein sector, F_0, and a peripheral protein sector, F_1. F_1 has an ATP-hydrolyzing activity, and its molecular weight is about 380,000. The subunit structure of the F_1-ATPase is $\alpha_3\beta_3\gamma\delta\varepsilon$. The catalytic sites reside on each β subunit or at the interfaces of the α and β subunits. This unusual subunit composition raises a number of questions. One concerns the catalytic core of the F_1-ATPase. In reconstitution of the F_1-ATPase from the thermophilic bacterium PS3 (TF_1), it was demonstrated that none of the pure subunits show ATPase activity by itself and that both the $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3\delta$ complexes were ATPase active (4, 5). Similar results, except inability to reconstitute the $\alpha_3\beta_3\delta$ complex, were reported for *Escherichia coli* F_1-ATPase (6, 7). Therefore, it has been reasonably assumed that the catalytic core of F_1-ATPase might be composed of trimeric $\alpha\beta$ pairs. Whereas this assumption is consistent with other observations, the isolation of the ATPase-active $\alpha_3\beta_3$ complex will be the final, conclusive evidence to prove this hypothesis. Another question concerns the symmetry of the F_1-ATPase. As expected from the subunit composition, the electron micrographs of F_1-ATPase (8–11) indicate a gross three-fold symmetry. However, this symmetry is inevitably imperfect because of the presence of single-copy minor subunits: γ, δ, and ε subunits. In other words, three $\alpha\beta$ pairs in the F_1-ATPase cannot be equivalent to each other with respect to the interraction with single-copy subunits. Apparently consistent with the above findings, the binding of adenine nucleotides or some chemical-modification reagents to the F_1-ATPase occurs preferentially to one of three β subunits (12, 13). From these considerations, one might ask if there is an intrinsic functional asymmetry in the F_1-ATPase (14–16).

In this report, we describe the reconstitution and isolation of the $\alpha_3\beta_3$ complex from the α and β subunits of TF_1. This symmetric complex has an ATPase activity with properties closely resembling those of intact TF_1.†

METHODS

Isolation of the $\alpha_3\beta_3$ Complex. The α and β subunits of TF_1 were expressed in the *E. coli* DK-8 strain, a mutant with a deletion of F_0F_1 genes, which was given to us by M. Futai, and were isolated individually as described (17, 18). The isolated subunits were stored in 75% ammonium sulfate suspension at 4°C. Prior to use, the α and β subunits were applied to an HPLC gel permeation column (G3000SWx1, Tosoh, Tokyo) and were eluted at room temperature with 50 mM Tris sulfate buffer (pH 7.0) containing 200 mM Na_2SO_4 at a flow rate 0.5 ml/min. The purified subunits were mixed, incubated at 30°C for 15 min in the same buffer, and the mixture was reapplied to the same gel permeation column. The $\alpha_3\beta_3$ complex was eluted at 14.7 min and was prepared freshly for each experiment.

Assays of ATP Hydrolytic Activity. Steady-state ATP hydrolysis was assayed in two ways. First, assays were performed at 25°C in solutions containing 50 mM Tris sulfate (pH 8.0), 5 mM $MgSO_4$, 10 mM KCl, 5 mM phospho*enol*pyruvate, 10 μg of pyruvate kinase and 10 μg of lactate dehydrogenase per ml, 0.2 mM NADH, and the indicated concentration of ATP. Rate of ATP hydrolysis was measured by the disappearance of NADH, which was monitored by the absorbance at 340 nm. Second, for experiments to determine the substrate specificity, cation specificity, and the sensitivity to chemical reagents, ATP hydrolysis was assayed at 30°C in 50 mM Tris sulfate (pH 8.0), 5 mM $MgSO_4$, 5 mM ATP, and 10 mM KCl with indicated modifications, and the amount of released P_i was measured (4).

Other Methods. Purification of TF_1 from the thermophilic bacterium PS3 was carried out as described (4). The γ subunit of TF_1 was prepared from the extract of *E. coli* DK-8, which harbors the expression plasmid pKKγE (18). Protein concentrations were measured by using Coomassie brilliant blue dye reagent (Bio-Rad). Polyacrylamide gel electrophoresis was performed by methods described previously (4, 18). The concentration of polyacrylamide was 12% for the electrophoresis in the presence of sodium dodecyl sulfate (SDS) and 7.5% in the absence of SDS. Gels were stained by Coomassie brilliant blue R250 or by silver staining.

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Abbreviation: TF_1, F_1-ATPase from the thermophilic bacterium PS3.

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RESULT

Isolation of the $\alpha_3\beta_3$ Complex. Isolated α and β subunits of TF_1 alone have no meaningful ATP hydrolizing activity.‡ However, we found that once these two subunits were mixed, some ATPase activity appeared. This means that α and β subunits assemble to form ATPase-active subunit complexes even in the absence of single-copy subunits. Therefore, we isolated and characterized these complexes. The complex reconstituted from α and β subunits was successfully isolated by gel permeation HPLC (Fig. 1). When pure α or β subunit was applied to the column, each was eluted as a single peak at the elution volume of 9.40 ml (α) or 9.20 ml (β), and aggregates of subunit with higher molecular sizes were not detected (Fig. 1 *A* and *B*). The α subunit was weakly adsorbed to the column and was eluted more slowly than the β subunit. However, in the case of the $\alpha+\beta$ mixture, a high molecular weight complex was eluted as an isolated peak at the elution volume of 7.35 ml (Fig. 1*C*). When the peak fraction at 7.35 ml was rechromatographed in the same HPLC system, it was eluted as a main peak at 7.35 ml, although small peaks appeared at the positions of the respective free subunits (Fig. 1*D*). From the SDS/polyacrylamide gel electrophoresis of the peak fraction at 7.35 ml in Fig. 1*D* (Fig. 1 *Inset*), it is clear that the complex was composed of equal amounts of α and β subunits. The holoenzyme, TF_1, with a molecular weight of 385,000, was eluted at 7.05 ml (Fig. 1*E*). Using ferritin and pyruvate kinase as molecular weight standards, the molecular weight of the complex was estimated to be about 310,000 from its retention time on the HPLC system. Based on molecular weight and the presence of equal amounts of α and β subunits in the complex, we concluded that the complex was composed of three α and β subunits ($\alpha_3\beta_3$). SDS gel electrophoresis of the complex showed that it did not contain the γ, δ, or ε subunit (Fig. 1 *Inset*). Since the α and β subunits were obtained from transformed *E. coli* cells harboring the expression plasmids that carry the α or β subunit gene of TF_1, the possibility that the complex contained a small amount of contaminating γ, δ, or ε subunit or degradation products was excluded. Furthermore, the host *E. coli* strain was DK-8, which has no F_1-ATPase genes in itself. Yohda *et al.* (19) reported that the mutant α subunit (Lys-175 → Ile), α_{K175I}, of TF_1 lost its ability to reconstitute into the $\alpha_3\beta_3\gamma$ complex. Consistently, the mixture of the β and the above mutant α subunits, which was given to us by M. Yohda, did not show ATP hydrolyzing activity. No high molecular weight complexes were detected on examination by HPLC (Fig. 1*F*).

Stability of the $\alpha_3\beta_3$ Complex. Whereas the $\alpha_3\beta_3$ complex was stable during HPLC purification, it was very labile in an electric field. Almost all of the $\alpha_3\beta_3$ complex dissociated into subunits during native polyacrylamide gel electrophoresis, and the band for the $\alpha_3\beta_3$ complex was only seen faintly (data not shown).

In general, the $\alpha_3\beta_3$ complex is less stable than TF_1 at high and low temperatures. For example, when the complex (1 mg of protein per ml) dissolved in 50 mM Tris chloride (pH 8.3 at 25°C) containing 200 mM Na_2SO_4 was incubated at 70°C for 10 min, the complex was denatured, resulting in almost complete loss of activity. The complex is also cold labile; after incubation in the above solution at 0°C for 30 or 60 min, it lost 50% or 90% of its activity, respectively. It was shown from HPLC analysis that exposure to cold induced dissociation of the complex into subunits. Even at room temperature, about 30% of the complex was dissociated after 16 hr. The holoenzyme, TF_1, remained fully active after all of the above treatments. The presence of ammonium sulfate stabilized the complex. Glycerol did not have protective effect. The presence of ATP and/or Mg^{2+} in the solution neither facilitated the complex formation nor protected the complex from dissociation.

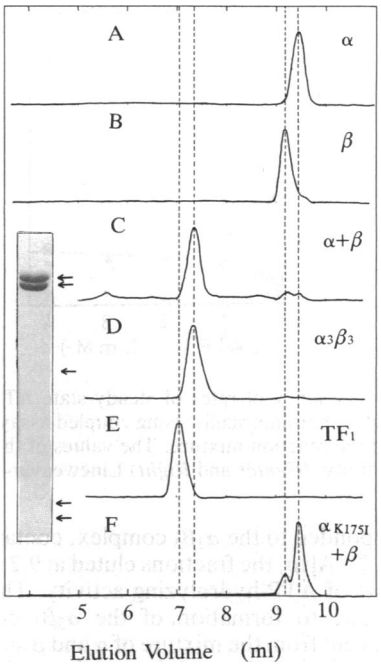

FIG. 1. Elution profiles of the α, β, $\alpha+\beta$, $\alpha_3\beta_3$ complex, TF_1, and $\alpha_{K175I}+\beta$ obtained by gel permeation HPLC at room temperature with a G3000SWx1 column equilibrated and eluted with 50 mM Tris sulfate (pH 7.0) buffer containing 200 mM Na_2SO_4 at a flow rate 0.5 ml/min. The void volume of the column was about 5 ml, and the total volume was about 15 ml. Elutions were monitored with absorbance at 280 nm, and profiles are shown with an arbitrary scale. (*A*) α subunit (1 mg). (*B*) β subunit (1 mg). (*C*) Incubated mixture of α and β subunits (1 mg of each). (*D*) Peak fraction at 7.35 ml of *C* was rechromatographed. (*E*) Holoenzyme TF_1 (1 mg). (*F*) Incubated mixture of the mutant α_{K175I} (Lys-175 → Ile) and β subunits (2 mg and 1 mg, respectively). (*Inset*) SDS/polyacrylamide gel electrophoresis of the peak fraction of *D*. Arrows indicate the electrophoretic positions of the α, β, γ, δ, and ε subunits (from upper to lower). The bands of the γ, δ, and ε subunits are not seen at all.

The $\alpha_3\beta_3$ Complex Is ATPase-Active. Measurement of ATP hydrolyzing activity in each fraction from gel permeation HPLC showed that the protein peak fraction eluted at 7.4 ml,

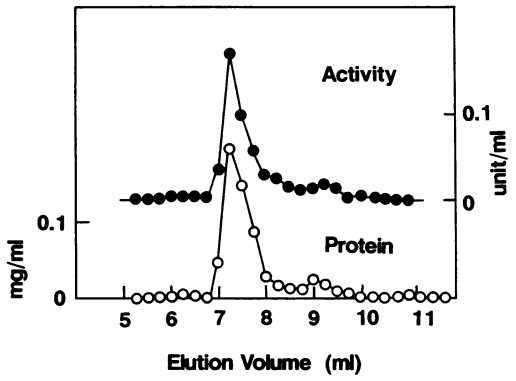

FIG. 2. ATPase activity of the $\alpha_3\beta_3$ complex eluted from a gel permeation HPLC column. The peak fraction corresponding to the $\alpha_3\beta_3$ complex obtained from the $\alpha+\beta$ mixtures was rechromatographed. Each fraction contained 0.25 ml of the eluate. Other conditions were the same as described in the legend of Fig. 1.

‡Very weak ATP hydrolytic activities were detected for pure α and β subunits. The specific activities were 0.001 unit/mg (0.02% of TF_1) for the α subunit and 0.007 unit/mg (0.15% of TF_1) for the β subunit. It is not known if these activities represent or relate to the activity of TF_1.

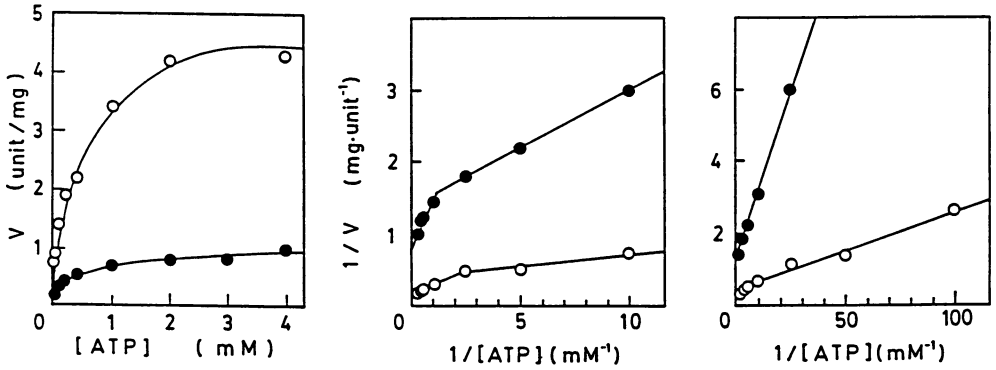

FIG. 3. Dependency of rates of steady-state ATP hydrolysis catalyzed by TF_1 and the $\alpha_3\beta_3$ complex. The rate of ATP hydrolysis was measured spectrophotometrically using coupled assays to NADH oxidation. The reactions were initiated by adding 2 μg of TF_1 (○) or the $\alpha_3\beta_3$ complex (●) to the reaction mixture. The values of the triplicated measurements were averaged. (*Left*) Plots of substrate (ATP) concentration vs. ATPase activity. (*Center* and *Right*) Lineweaver–Burk plot over two concentration ranges. Data for ATP hydrolysis shown in (*Left*) were replotted.

which corresponded to the $\alpha_3\beta_3$ complex, contained ATPase activity (Fig. 2). Also, the fractions eluted at 9.2 ml contained small amounts of ATP-hydrolyzing activity. This activity is most likely due to formation of the $\alpha_3\beta_3$ complex in a postcolumn event from the mixture of α and β subunits in this peak. None of the fractions of the α subunit (Fig. 1*A*) or β subunit (Fig. 1*B*) alone possessed any detectable ATPase activity (data not shown). The kinetics of ATP hydrolysis catalyzed by the $\alpha_3\beta_3$ complex were compared with those of TF_1. The general profile of the curve of activity vs. ATP concentration of the $\alpha_3\beta_3$ complex was very similar to that of TF_1, except that the activity of the $\alpha_3\beta_3$ complex at each ATP concentration was about 20% of that of native TF_1 (Fig. 3 *Left*). The specific activity at 2 mM ATP was 0.80 unit/mg for the $\alpha_3\beta_3$ complex and 4.3 units/mg for TF_1 under the same conditions. These ATP hydrolytic reactions were analyzed by Lineweaver–Burk plots (Fig. 3 *Center* and *Right*). As reported previously (20–22), the Lineweaver–Burk plot of ATP hydrolysis catalyzed by TF_1 appeared to be at least biphasic and concave downward at high ATP concentrations. This was also the case for the $\alpha_3\beta_3$ complex. The apparent K_m and V_{max} values of the $\alpha_3\beta_3$ complex, calculated by a computer from the data shown in Fig. 3 *Center* and *Right* by using the weighted least-squares method, were about 150 μM and 490 μM and 0.80 unit/mg and 0.30 unit/mg, respectively, and those of TF_1 were about 80 μM and 490 μM and 2.6 units/mg and 2.4 units/mg, respectively.§ Whereas the V_{max} values of the $\alpha_3\beta_3$ complex are significantly reduced when compared with TF_1, it should be noted that its K_m values did not differ drastically from those of TF_1.

Other Properties of the Activity of the $\alpha_3\beta_3$ Complex. The substrate specificity of the $\alpha_3\beta_3$ complex is almost the same as that of TF_1. It hydrolyzed ATP, GTP, and ITP but not CTP and UTP at 30°C (data not shown). The $\alpha_3\beta_3$ complex required metal cations for activity since it was inactive in the presence of the cation chelator EDTA. However, the specificity of the $\alpha_3\beta_3$ complex for metal cation differed significantly from that of TF_1 (Table 1). All metal cations tested were effective as cofactors for the ATPase activity of the complex. Thus, the specificity of the metal cation requirement of the complex is much lower than that of TF_1.

The $\alpha_3\beta_3$ complex exhibited less sensitivity to HSO_3^-, an activation anion, and no sensitivity to N_3^-, an inhibitory anion. By including 100 mM Na_2SO_3 in the reaction mixture, the ATPase activity of TF_1 was activated 220%. However, the activity of the $\alpha_3\beta_3$ complex was activated to a much lower extent (130%) under the same conditions. N_3^- is known to be a potent inhibitor of F_1-ATPases. The activity of TF_1 was completely inhibited at 0.2 mM NaN_3. However, the activity of the complex was not inhibited even at 2 mM NaN_3.

Similar to native TF_1, the $\alpha_3\beta_3$ complex exhibited two peaks of activity in its temperature-activity profile, one at about 20°C and the other at about 60°C (data not shown). However, the activity of the $\alpha_3\beta_3$ complex above 60°C decreased more sharply than that of TF_1, reflecting decreased stability of the complex at high temperature. The pH dependency of the ATPase activity for the $\alpha_3\beta_3$ complex was altered. When examined in the reaction mixtures containing 5 mM ATP, 5 mM $MgSO_4$, and 50 mM Tris sulfate buffer adjusted to pH 6.5–9.5, the pH optima of TF_1 and the $\alpha_3\beta_3$ complex at 30°C were pH 7.5–8.0 and pH 8.0–8.5, respectively. Thus, as was observed for the $\alpha_3\beta_3\delta$ complex (5), the pH optimum of the $\alpha_3\beta_3$ complex was shifted to the alkaline pH side.

Reconstitution of the $\alpha_3\beta_3\gamma$ Complex from the $\alpha_3\beta_3$ Complex. We previously reported the reconstitution of the $\alpha_3\beta_3\gamma$

Table 1. The specificity of the metal ion requirement for the ATP-hydrolyzing activity of TF_1 and the $\alpha_3\beta_3$ complex

Metal ion, 1 mM	ATPase activity, unit/mg of protein	
	TF_1	$\alpha_3\beta_3$
Mg^{2+}	4.3	0.80
Mn^{2+}	7.5	0.91
Co^{2+}	1.7	1.4
Zn^{2+}	0.56	1.7
Cd^{2+}	0.52	1.2
Ca^{2+}	0.39	2.1
Fe^{2+}	0.39	1.3
Cu^{2+}	<0.1	1.6
Ni^{2+}	<0.1	1.1
Sr^{2+}	<0.1	1.4
Ba^{2+}	<0.1	1.5
None (+ EDTA)*	<0.1	<0.1

The reaction mixture contained 5 mM ATP, 1 mM metal ions (added as chloride salts), and 50 mM Tris sulfate (pH 8.0). The values were the average of the duplicated measurements.

*Concentration of EDTA was 0.1 mM. To avoid interference in the colorimetric assay for P_i by EDTA, the amount of ATP and ADP in the reaction mixture was measured by using ion-exchange HPLC (21).

§We also replotted the data for an Eadie–Hofstee plot and analyzed by a computer to obtain kinetic parameters to give a best fitting. However, we found these values showed large variation, depending on the calculation programs. Nevertheless, as directly suggested from the fact that ratio of the specific activity of the two enzymes was almost constant through the entire ATP concentration range, the kinetic parameters for them varied in a parallel manner.

complex of TF_1 (6, 7) and suggested that the $\alpha_3\beta_3$ complex appears to be a precursor in the course of assembly of the $\alpha_3\beta_3\gamma$ complex. To examine this possibility directly, an excess of the γ subunit was added to a solution containing freshly isolated $\alpha_3\beta_3$ complex. After a 15-min incubation at 30°C, the mixture was centrifuged and the supernatant was rechromatographed by gel permeation HPLC. A single complex with a molecular weight of about 320,000 was eluted. As opposed to the original $\alpha_3\beta_3$ complex, the complex obtained in this fashion was stable under nondenaturing polyacrylamide gel electrophoresis, and SDS gel electrophoresis of the obtained complex clearly showed that it comprised the α, β, and γ subunits (data not shown). This complex was active as an ATPase even at high temperature, which is characteristic of the $\alpha_3\beta_3\gamma$ complex (6). The specific activity of this complex was 3.5 units/mg at 30°C. These results indicated that the γ subunit was incorporated into the $\alpha_3\beta_3$ complex to form the $\alpha_3\beta_3\gamma$ complex.

DISCUSSION

The presence of interactions between α and β subunits in mixtures of these purified subunits was implicated from studies using infrared absorption (23). However, no complex was detected when the mixture was analyzed by native polyacrylamide gel electrophoresis (4); this now turns out to be due to the lability of the $\alpha_3\beta_3$ complexes in an electric field. In the present study, we successfully isolated the complex by gel permeation HPLC. It has been reasonably assumed that the γ and δ subunits of F_1-ATPases are not involved in catalysis, since both the $\alpha_3\beta_3\gamma$ and the $\alpha_3\beta_3\delta$ complexes are active as ATPases (4, 5). Demonstration of the activity of the $\alpha_3\beta_3$ complex reported in this report confirmed this assumption. The possibility that the γ and/or δ subunits are responsible for the cooperative kinetics was also eliminated since the $\alpha_3\beta_3$ complex shows cooperative kinetics similar to those of the holoenzyme. The possibility exists that the $\alpha_3\beta_3$ complex has an asymmetric structure. However, it seems more likely that the complex has three-fold symmetry. If this is indeed the case, the cooperative kinetics observed for the complex could be interpreted as induction of asymmetry when ATP-Mg^{2+} first binds to the complex, as suggested by Boyer (2). Thus, the trimeric $\alpha\beta$ pairs by themselves are responsible for the cooperative kinetics, and in this sense it is the "catalytic core" of F_1-ATPase. It should be pointed out that this trimeric core structure has been conserved in archaebacterial and eukaryotic endomembrane ATPases (24, 25).

Comparison of the properties of the $\alpha_3\beta_3$ complex with the $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3\delta$ complex reveals some aspects of the roles of the γ and δ subunits. Since only the $\alpha_3\beta_3\gamma$ complex is heat stable and sensitive to N_3^- inhibition, binding of the γ subunit to the $\alpha_3\beta_3$ core induces the stabilization of the $\alpha_3\beta_3$ structure and a conformation that is susceptible to N_3^- inhibition. The γ subunit in the holoenzyme also appears to be responsible for shifting the optimum pH of the activity of the catalytic core to the acidic side since the pH optimum of the $\alpha_3\beta_3\gamma$ complex and that of the holoenzyme are more acidic than those of the $\alpha_3\beta_3$ and $\alpha_3\beta_3\delta$ complexes (5). In general, the properties of the $\alpha_3\beta_3$ complex resemble those of the $\alpha_3\beta_3\delta$ complex.

In conclusion, the $\alpha_3\beta_3$ complex retains some essential features of the ATP-hydrolyzing activity of the holoenzyme, and we have noticed that this complex with a symmetric subunit composition might be ideal because of its subunit composition for some studies on F_1-ATPase such as crystallization.

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