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The $\alpha_1\beta_1$ heterodimer, the unit of ATP synthase

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The $\alpha_3\beta_3$ hexamer (M_r 319582) was reconstituted from the α and β subunits of the TF_1 portion of ATP synthase of thermophilic bacterium PS3 (Kagawa, Y. et al. (1989) FEBS Lett. 249, 67–69). The radius of gyration (R_g) of the $\alpha_3\beta_3$ hexamer determined by small-angle X-ray scattering was 4.64 ± 0.03 nm. However, in the presence of A(D)P-Mg, R_g of the $\alpha\beta$ complex was markedly reduced to 3.47 ± 0.02 nm, indicating the dissociation of the hexamer. The heterodimer was isolated by gel-permeation chromatography of the $\alpha_3\beta_3$ hexamer in the presence of AT(D)P-Mg. Judging from the apparent molecular weight by the gel permeation chromatography, the dissociation product was the $\alpha_1\beta_1$ heterodimer (M_r = 106524). On gel electrophoresis, both the dimer and hexamer gave bands of material with ATPase activity (relative mobilities: $TF_1:\alpha_3\beta_3:\alpha:\alpha_1\beta_1:\beta$ = 1:1.3:2.1:2.9:3.6, in 7.5% polyacrylamide gel at pH 8.8). The dissociation of the hexamer was induced by IT(D)P, but not by unyhydrolyzable ATP analogues – Mg, P_i-Mg and Mg. During gel-permeation column chromatography in the presence of ATP-Mg, the ATPase activity appeared before the peak of the heterodimer (about 100 kDa). This observation strongly suggests the interconversion of the $\alpha_1\beta_1$ dimer to the $\alpha_3\beta_3$ hexamer during catalysis.

Introduction

ATP synthase (F_0F_1) catalyzes oxidative and photosynthetic phosphorylation [1–3] driven by a protonmotive force [4]. F_0F_1 is a multisubunit complex composed of two subcomplexes: a soluble ATPase, F_1, and a proton channel, F_0 [1–3]. The subunit structure of F_1 is $\alpha_3\beta_3\gamma\delta\varepsilon$ [3,5], and the α and β subunits both bind AT(D)P [3,5,6]. The interactions between α and β subunits in mixtures of these purified subunits was implied from their proton–deuteron exchange kinetics [6]. In fact, the presence of an $\alpha_3\beta_3$ hexamer of thermophilic F_1 (TF_1) was demonstrated by the gel-chromatography [7,8], ultracentrifugation [7] and crystallography of the hexagonal molecule [9]. The exact molecular weights of the α (54589.89) and β (51937.58) subunits were determined by sequencing the F_0F_1 operon [10]. The $\alpha_3\beta_3$ hexamer (M_r = 319582) was purified by gel chromatography of a mixture ($\alpha:\beta$ = 1:1) of the α and β subuntis [7,8] that were obtained by overexpressing the genes for these subunits [10].

In these studies [7–9], the molecular weight of the $\alpha_3\beta_3$ hexamer was estimated in the absence of AT(D)P-Mg. However, under the conditions for assay of ATPase (1 mM ATP-Mg), the small-angle X-ray scattering of the hexamer (radius of gyration: R_g = 4.64 ± 0.03 nm) revealed its complete dissociation into a smaller $\alpha\beta$ complex (R_g = 3.47 ± 0.02 nm) [11].

Here we report the isolation of the $\alpha_1\beta_1$ heterodimer in the presence of Mg-nucleotides, and discuss the role of the heterodimer during the catalysis.

Materials and Methods

Materials. All chemicals used were of the highest purity available. The α and β subunits of TF_1 were overexpressed in *Escherichia coli* and were purified as described by Ohta et al. [10]. TF_1 was purified as described by Kagawa and Yoshida [12]. The $\alpha_3\beta_3$ hexamer was reconstituted by mixing stoichiometric amounts of the α and β subunits (1:1) [7] and then purified by high-performance liquid chromatography on a G3000SW column (ToSoh, 7.5 mm × 60 cm) equi-

Abbreviations: AMP-PNP, adenylylimidodiphosphate; AMP-PSP, adenosine-5′-*O*-(3-thiotriphosphate); F_0F_1, ATP synthase; F_1, catalytic portion of F_0F_1; TF_1, thermophilic F_1; F_0, proton channel portion of F_0F_1; HPLC, high performance liquid chromatography; R_g, radius of gyration.

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librated with 0.1 M Na_2SO_4, 50 mM Tris-SO_4 (pH 7.2), 0.1 mM EDTA and 0.1 mM dithiothreitol. The $\alpha_3\beta_3$ hexamer thus obtained was pure enough to be crystallized on addition of poly(ethylene glycol). The protein concentration was determined with Coomassie brilliant blue as described by Bradford [13]. A solution of $\alpha_3\beta_3$ hexamer (1 mg/ml) showed an optical absorbance of 0.505 at 280 nm (absorption max. and min. at 277.5 nm and 250.5 nm, respectively).

[γ-^{32}P]ATP was obtained from Amersham, U.K., AMP-PNP and AMP-PSP were purchased from Boehringer-Mannheim-Yamanouchi, Tokyo. Other reagents were as described in previous reports [6,7,10,12].

Liquid chromatography. A high-performance liquid chromatograph (HPLC) was equipped with a system controller (Waters 600E), detectors (Waters 490, at 280 nm and 214 nm) and operated at a flow rate of 0.5 ml/min, and chart speed of 30 cm/h at 25°C. The G3000SW column (ToSoh, 7.5 mm × 60 cm) was equilibrated with 0.1 M Na_2SO_4, 50 mM Tris-SO_4 (pH 7.2), 0.1 mM $MgSO_4$ and the indicated concentration of nucleotide (when the nucleotide concentration was higher than 0.1 mM, equal concentrations for $MgSO_4$ and nucleotide were used) at 25°C.

Small-angle X-ray scattering. The X-ray source (Rigakukiki, model D9C, 40 kV, 30 mA) was equipped with a Philips fine-focus X-ray tube (focus size = 0.4 × 12 mm), single-mirror Franks optics and proportional counter (resolution = 0.1265 mm). Measurements of samples were performed three times for 3000 s each time. The sample solution contained the purified $\alpha_3\beta_3$ (11.5 mg/ml), 0.1 M Na_2SO_4, 50 mM Tris-SO_4 (pH 7.2), 0.1 mM EDTA and the indicated concentration of nucleotide, $MgSO_4$, P_i or other components, at 25°C. The molecular parameters of TF_1 by this method were reported by Furuno et al. [14]. The details of the experiments will be reported by Harada et al. (unpublished data).

ATPase assay. Activity was assayed spectrophotometrically with an ATP-generating system [7] or radiometrically with [γ-^{32}P]ATP. When samples contained AT(D)P and the regenerating system was used, the full-scale range of the absorbance at 340 nm in Beckman DU70 spectrophotometer was adjusted to 4.0, and if necessary, NADH and phospho*enol*pyruvate were supplied in order to obtain steady-state velocity. The ATPase activity in the presence of high concentrations of ADP was measured as follows. To 200 μl of column eluate containing the $\alpha\beta$ complex, 0.1 M Na_2SO_4, 50 mM Tris-SO_4 (pH 7.2), 0.1 mM $MgSO_4$ and nucleotide (less than 1 mM), 10 μl of 0.2 M [γ-^{32}P]ATP ($6 \cdot 10^5$ cpm per 10 μl), 0.2 M $MgSO_4$ was added and incubation was carried out at 25°C for 30 min. The reaction was stopped by adding 200 μl of 18% perchloric acid, 1 mg/l of I_2, and 200 μl of 8% ammonium molybdate. Then the [^{32}P]P_i liberated was extracted with 2 ml of water-saturated isobutanol/benzene (1 : 1) and the radioactivity (Cherenkov light) in the isobutanol layer was counted in a liquid scintillation counter after washing with 1 ml of water. The ATPase activity of the $\alpha\beta$ complex during gel-permeation HPLC equilibrated with ATP-Mg was measured as follows. The 1 : 1 mixture of the α and β subunits in the solution containing 50 mM Tris SO_4, 0.1 M Na_2SO_4, 0.1 mM DTT and 0.1 mM EDTA was applied onto a HPLC G3000SW column equilibrated with same buffer to isolate the hexamer (11.5 mg/ml). The hexamer solution thus obtained (100 μl) was applied onto the HPLC G3000SW column equilibrated with 50 mM Tris-SO_4. 0.1 M Na_2SO_4, 0.1 mM DTT and 0.1 mM ATP-Mg. The resulting $\alpha_1\beta_1$ dimer was purified by the same column chromatography and the dimer fraction was concentrated to 0.1 mg/ml. The purified fraction (8 μg) was applied to the same column and the eluate was dropped into 0.1 ml of 0.5 M EDTA to stop the ATPase reaction immediately. The amount of ADP in each fraction (0.5 ml) was determined by chromatography as described previously [15].

Polyacrylamide gel electrophoresis. Electrophoresis was carried out according to Kadenbach et al. [16] in slab gels in the absence of sodium dodecylsulfate, using the following solutions with or without 0.1 mM ATP-Mg: the separation gel contained 7.5% acrylamide, 0.375 M Tris-SO_4 (pH 8.8) and 13% glycerol; the stacking gel contained 6% acrylamide and 0.1 M Tris-SO_4 (pH 6.8); the running buffer consisted of 0.025 M Tris and 0.192 M glycine (pH 8.5). Proteins were stained with Coomassie brilliant blue [16] and ATPase activity was detected by soaking the gel in a solution containing 1 mM ATP, 1 mM $MgSO_4$ and 0.05% lead acetate, in running buffer at pH 8.5 and 25°C, for 1 h.

Results and Discussion

Dissociation of the $\alpha_3\beta_3$ hexamer in the presence of nucleotides

Small-angle X-ray scattering of a solution of the $\alpha_3\beta_3$ hexamer showed reduction in the radius of gyration (R_g) of the molecules on addition of AT(D)P and Mg (Table I). This change indicated the dissociation of the $\alpha_3\beta_3$ hexamer (R_g = 4.64–0.03 nm) to $\alpha_1\beta_1$ dimers (R_g = 3.47 ± 0.02 nm) composed of the α (R_g = 3.09 ± 0.05 nm; elliptic cylinder 4.5 × 4.5 × 10.0 nm) and β (R_g = 2.80 ± 0.04 nm; elliptic cylinder 3.5 × 4.0 × 9.0 nm). The $\alpha_1\beta_1$ heterodimer, not the $\alpha\alpha$ and $\beta\beta$ dimers, was the possible molecular species, because X-ray scattering of the solutions of the pure α or β subunits showed only one molecular species. The molecular parameters of TF_1 measured in this study (R_g = 4.92 ± 0.01 nm) were very similar to those reported by Furuno et al. (R_g = 4.97 ± 0.03 nm) [14] and were larger than those of the $\alpha_3\beta_3$ hexamer. In the absence of AT(D)P, the $\alpha_3\beta_3$ hexamer

TABLE I

Small-angle X-ray scattering of the $\alpha_3\beta_3$ hexamer-$\alpha_1\beta_1$ heterodimer solution containing nucleotide and Mg ion

X-ray scattering was measured as described in Materials and Methods.

Addition to $\alpha_3\beta_3$, 11.5 mg/ml	Radius of gyration (R_g in nm)	
	−Mg	+1 mM $MgSO_4$
None	4.64 ± 0.03	4.65 ± 0.03
1 mM ATP	4.48 ± 0.03	3.47 ± 0.02
1 mM ADP	4.47 ± 0.03	3.47 ± 0.02
1 mM ADP + 1 mM P_i	4.29 ± 0.03	3.44 ± 0.02
1 mM AMPPNP	4.66 ± 0.03	4.52 ± 0.02
1 mM P_i	4.67 ± 0.02	4.64 ± 0.03

was not dissociated by dilution to 1.15 mg/ml (R_g = 4.57 ± 0.03 nm), or by the addition of Mg, P_i or P_i + Mg (Table I). A nonhydrolyzable nucleotide, AMPPNP (Table I) did not dissociate the $\alpha_3\beta_3$ hexamer, with or without Mg. In contrast to the results obtained by the HPLC and electrophoresis, where the oligomers are continuously diluted in a moving solvent, those by the X-ray scattering (Table I) enabled us to estimate the dissociation of the $\alpha_3\beta_3$ in a static solution, even at very high protein concentrations.

Isolation of the $\alpha_1\beta_1$ heterodimer

When the $\alpha_3\beta_3$ hexamer was chromatographed on a gel-permeation column (total volume, 26.4 ml; void volume, 8.7 ml) in the absence of nucleotide and Mg^{2+}, it was eluted at 13.2 ml (320 kDa) (Fig. 1A), but in the presence of 0.1 mM ATP-Mg or ADP-Mg, it was eluted at 16.6 ml (107 kDa) (Fig. 1B). When the peak fraction of 107 kDa was rechromatographed in the nucleotide system, it was eluted as a main peak at 16.6 ml (Fig. 1C). However, after removal of the AT(D)P-Mg using a centricolumn equilibrated with the control buffer, it reassembled and gave a peak at 13.2 ml (Fig. 1D), with peaks of the partially dissociated α (19.3 ml) and β (18.2 ml) monomers, due to dilution (Fig. 1D). Thus, the nucleotide-dependent dimer-hexamer interconversion is reversible. Even when hexamer that had not been preincubated with the nucleotide was injected into the HPLC column equilibrated with nucleotide-Mg, its dissociation into the dimer was complete. Therefore, the dissociation is completed within 30 s.

Nucleotide specificity and effective concentration for dissociation of the hexamer

Titration of the dissociation was performed with various nucleotides in the presence of Mg. Diphosphonucleosides and hydrolyzable triphosphonucleosides caused dissociation only in the presence of Mg. As shown in Fig. 2, the concentration of ATP for 50% dissociation of the $\alpha_3\beta_3$ hexamer was 6 μM, and that of

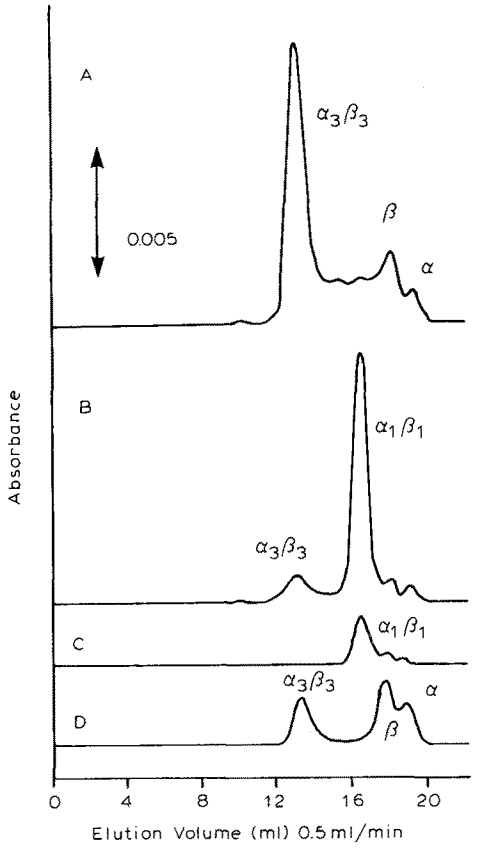

Fig. 1. Elution profiles of the $\alpha_1\beta_1$ heterodimer, $\alpha_3\beta_3$ hexamer, α monomer and β monomer on gel permeation HPLC at 25°C in a G3000SW column at a flow rate of 0.5 ml per min. Curve A: 25 μl of the $\alpha_3\beta_3$ (11.5 mg/ml) was eluted in control buffer consisting of 0.1 M Na_2SO_4, 50 mM Tris-SO_4 (pH 7.2) and 0.1 mM dithiothreitol. Curve B: 25 μl of the $\alpha_3\beta_3$ (11.5 mg/ml) was eluted in the control buffer containing 0.1 mM each of ATP and $MgSO_4$. Curve C: rechromatography of 250 μl of the peak fraction of the $\alpha_1\beta_1$ dimer in control buffer containing 0.3 mM ATP and 0.3 mM $MgSO_4$. Curve D: rechromatography of 250 μl of the peak fraction of the $\alpha_1\beta_1$ dimer in control buffer after removal of nucleotides by centricolumn equilibration with control buffer.

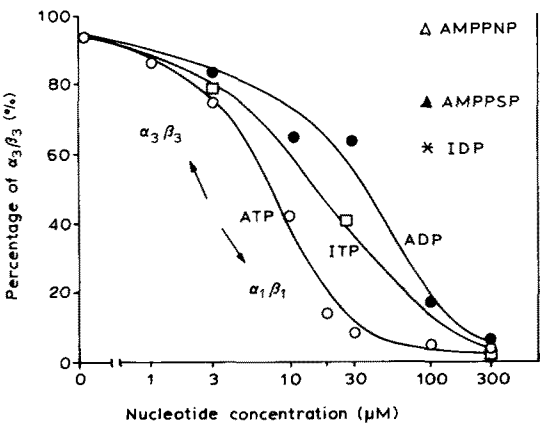

Fig. 2. Effects of various concentrations of nucleotides on the dissociation of the $\alpha_3\beta_3$ hexamer into the $\alpha_1\beta_1$ heterodimer. Gel permeation HPLC was performed as described under 'Materials and Methods' and in the legend for Fig. 1. Open circles, ATP; closed circles, ADP; open squares, ITP; other symbols as indicated.

ADP was 30 μM. The K_d value for ADP-Mg of the β subunit is 25 μM, while that of the α subunit is only 5.2 μM [17]. ITP-Mg and IDP-Mg, which react only with the β subunit [18], also dissociated the hexamer. Therefore, the dissociation was due to interaction between the nucleotide and the β subunit. An additional peak corresponding to $\alpha_2\beta_2$ tetramer (205 kDa) appeared in 30 μM ITP-Mg (data not shown). AMP-PNP seemed to stabilize the hexamer because it reduced the dissociation into the α and β monomer. And when the dimer was preincubated with 20 mM AMP-PNP-Mg in the presence of 0.1 mM ADP-Mg, the peak corresponding to the $\alpha_3\beta_3$ hexamer was increased. Therefore, AMP-PNP not only prevented the dissociation of the hexamer to the dimer but also enhanced the association of the dimer to the hexamer.

Polyacrylamide gel electrophoresis of the $\alpha_1\beta_1$ dimer and $\alpha_3\beta_3$ hexamer

To date, electrophoresis of a mixture of the α and β subunits has given only separate bands corresponding these monomers. However, when the protein concentration was large enough to prevent the dissociation, the $\alpha_1\beta_1$ dimer moved between the α and β monomers in the presence of ATP-Mg (Fig. 3A, B), and the $\alpha_3\beta_3$ hexamer between TF_1 and the α subunit (Fig. 3C, D). The relative electrophoretic mobilities of these bands were $TF_1 : \alpha_3\beta_3 : \alpha : \alpha_1\beta_1 : \beta = 1 : 1.3 : 2.1 : 2.9 : 3.6$, at pH 8.8 in 7.5% polyacrylamide. Both the dimer and hexamer as well as TF_1 showed strong ATPase activity (Fig. 3B, D), but that of the hexamer may represent that of the $\alpha_1\beta_1$ dimer produced in the conditions for ATPase assay. There are other bands with ATPase activity in-

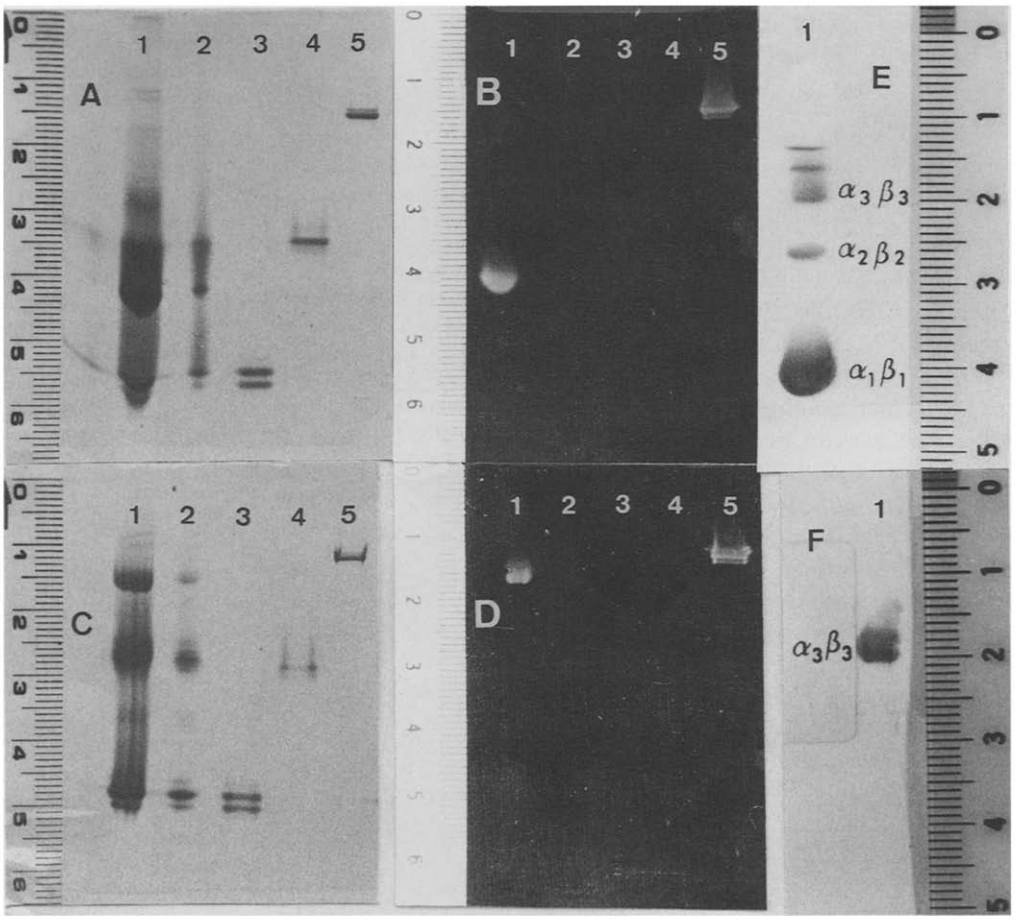

Fig. 3. Polyacrylamide gel electrophoresis of the $\alpha_1\beta_1$ dimer in the presence of ATP-Mg and of the $\alpha_3\beta_3$ hexamer in the absence of nucleotide. Gel electrophoresis was carried out as described under Materials and Methods [18]. A, B and E, with 1 mM ATP Mg; C, D and F, without ATP-Mg. A and C, stained with Coomassie brilliant blue; B and D, stained with 0.05% lead acetate and 0.4 mM ATP-Mg in 50 mM glycine-Tris buffer (pH 8.6). E and F, conversion of lead phosphate in B and D, respectively, to lead sulfite by heating the gel in the presence of 0.005% thioacetoamide at 100 °C for 1 h. Lanes: 1, 10 μl of 11.5 mg/ml $\alpha_3\beta_3$ hexamer; 2, 1 μl of 11.5 mg/ml $\alpha_3\beta_3$ hexamer; lanes 3 to 5, 2 μl of 1 mg/ml solutions of β monomer, α monomer and TF_1, respectively.

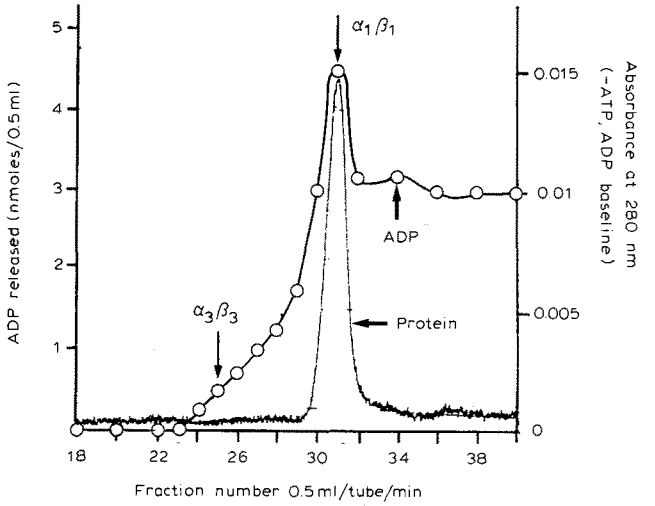

Fig. 4. Hydrolysis of ATP during gel permeation HPLC in the presence of ATP-Mg. The $\alpha_1\beta_1$ dimer was purified by successive HPLC as described in Materials and Methods: once in the absence of nucleotide to obtain the $\alpha_3\beta_3$ hexamer and twice in the presence of 0.1 mM ATP-Mg to isolate the $\alpha_1\beta_1$ dimer. The dimer (80 μl of 0.1 mg/ml) was loaded onto a G3000SW column in HPLC in 0.1 mM ATP, 100 μM $MgSO_4$ 0.1 M Na_2SO_4, 50 mM Tris-SO_4 (pH 7.2), 0.1 mM dithiothreitol at 25 °C. The sample was eluted at the flow rate of 0.5 ml/min, and each fraction (0.5 ml) was dropped into 0.1 ml of 0.5 M EDTA. Open circles indicate the amount of ADP produced by hydrolysis of ATP in each fraction measured by chromatographic analysis [17]. Arrows indicate the position corresponding to the $\alpha_3\beta_3$ hexamer and the $\alpha_1\beta_1$ dimer. The elution profile of the protein was monitored by a detector (Waters Model 490).

cluding that of putative $\alpha_2\beta_2$ tetramer (Fig. 3E). The presence of ATP-Mg during the electrophoresis caused apparent tailing of TF_1 (Fig. 4B). The β subunit showed doublet bands (17) (Fig. 3A, C) and very weak ATPase activity after incubation for 2 days. Both the dimer and hexamer were composed of equal amounts of the α and β subunits, as shown by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Apparent ATPase activity of the $\alpha_1\beta_1$ heterodimer fraction

The isolated 107 kDa peak showed ATPase activity (300–784 nmol/min per mg at 25 °C) in the ATP-regenerating system (4 mM ATP, 0.1–0.4 mg protein/ml). This specific activity was close to that of the $\alpha_3\beta_3$ preparation preincubated for 1 h with or without AT(D)P-Mg (0.1 mM) and then assayed in the same ATP-regenerating system. In the presence of 0.3 mM ADP-Mg, the [γ-^{32}P]ATPase activities of the $\alpha_1\beta_1$ peak and dissociated $\alpha_3\beta_3$ hexamer were both 35.2–54.8 nmol/min per mg at 25 °C. Although the $\alpha_1\beta_1$ dimer may display 'uni-site ATPase activity' in the presence of the stoichiometric amounts of ATP-Mg, the dimer was associated into the hexamer if ATP-Mg concentrations were less than 3 μM (Fig. 2). The ATPase activity of the $\alpha\beta$ complex (less than 100 μg) was gradually lost on partial dissociation of the complex into the α and β monomers, in the absence of 0.1 M Na_2SO_4 as reported previously [7]. However, the activity was kept constant for more than 90 min during dialysis against the ATP-generating system containing 0.1 M Na_2SO_4, even at lower protein concentrations.

Interconversion of the dimer to the hexamer during the catalysis

After the successive purification of the $\alpha_1\beta_1$ dimer in the presence of 0.1 mM ATP-Mg as described under Materials and Methods, the elution profile gave a single peak corresponding the $\alpha_1\beta_1$ dimer. By determining the amount of ADP in each fraction, the constant ADP formation was observed after the peak of the $\alpha_1\beta_1$ dimer (Fig. 4). The ADP formation was proportional to the amount of protein and inverse proportional to the flow rate. The specific ATPase activity during the HPLC was 400 nmol/min per mg, which was essentially equal to that of the mixture as described above. In addition, the distinct ATP hydrolysis was observed in the fractions before the peak (fraction 25 to 29, Fig. 4). Essentially the same results were obtained in five experiments. The possibility of the contamination of any other ATPase was excluded because the activity before the $\alpha_1\beta_1$ dimer fraction was not reduced by the repeated HPLC. Therefore, the activity before the main protein peak is attributed to the $\alpha\beta$ complex. As described above, the dimer–hexamer interconversion was rapid and reversible. The activity before the $\alpha_1\beta_1$ dimer peak may be due to an undetectable amount of the $\alpha_3\beta_3$ hexamer formed during the HPLC. Further information on the kinetic constants in various steps is necessary to analyze this phenomenon precisely. For an approximation, the turnover rate of the catalysis is about 0.5 s (400 nmol/mg per min) during the HPLC. The $\alpha_3\beta_3$ hexamer was eluted about 3 min before the $\alpha_1\beta_1$ dimer. Therefore, judging from the time range, it is possible for a small amount of the $\alpha_3\beta_3$ hexamer to form in each catalytic cycle and to appear in the fraction between the $\alpha_3\beta_3$ hexamer and the $\alpha_1\beta_1$ dimer. When the flow rate was increased to 1 ml/min, in the presence of ATP-Mg, the shape of the elution profile for protein was slightly shifted to the $\alpha_3\beta_3$ hexamer side, while in the presence of ADP-Mg it was not. The idea is also supported by the fact that unhydrolyzable AMPPNP did not cause dissociation of the $\alpha_3\beta_3$ hexamer. As reported earlier [6], the conformational change in the β subunit is induced by its interaction with the α subunit and nucleotides. The relationship between the turnover and dissociation should be discussed on the basis of the detailed kinetic analysis. The combined stopped flow method with the photon factory X-ray scattering of the $\alpha_1\beta_1$ dimer and the $\alpha_3\beta_3$ hexamer in nucleotide-Mg is now in progress.

To the best of our knowledge, the 'active' $\alpha_1\beta_1$ dimer has not previously been isolated by gel permeation

chromatography, or demonstrated by electrophoresis or X-ray scattering. The significance of the $\alpha\beta$ dimer was shown by photoaffinity cross-linking of the two subunits with 3′-arylazido-β-alanyl-2-azido ATP [19]. The cross-linked $\alpha_1\beta_1$ dimer indicates the ATP-binding site at the interface between the α and β subunits. Neutron scattering of the $\alpha_3\beta_3\gamma$ complex also revealed the presence of three $\alpha\beta$ pairs in it [20]. Small-angle X-ray scattering of both the $\alpha_1\beta_1$ dimer and the $\alpha_3\beta_3$ hexamer will be reported in detail elsewhere by Harada et al.

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