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Isolated ϵ Subunit of Thermophilic F_1-ATPase Binds ATP*

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Yasuyuki Kato-Yamada‡ and Masasuke Yoshida§

From the Chemical Resources Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama 226-8503, Japan

F_1-ATPase, a soluble part of the F_0F_1-ATP synthase, has subunit structure $\alpha_3\beta_3\gamma\delta\epsilon$ in which nucleotide-binding sites are located in the α and β subunits and, as believed, in none of the other subunits. However, we report here that the isolated ϵ subunit of F_1-ATPase from thermophilic *Bacillus* strain PS3 can bind ATP. The binding was directly demonstrated by isolating the ϵ subunit-ATP complex with gel filtration chromatography. The binding was not dependent on Mg^{2+} but was highly specific for ATP; however, ADP, GTP, UTP, and CTP failed to bind. The ϵ subunit lacking the C-terminal helical hairpin was unable to bind ATP. Although ATP binding to the isolated ϵ subunits from other organisms has not been detected under the same conditions, a possibility emerges that the ϵ subunit acts as a built in cellular ATP level sensor of F_0F_1-ATP synthase.

F_0F_1-ATPase/synthase (F_0F_1)[1] catalyzes ATP synthesis coupled with the proton flow across the membrane through mechanical rotation of the central shaft subunits relative to the surrounding stator subunits (1, 2). F_1 is the water-soluble portion of F_0F_1 and has ATP hydrolysis activity by itself. It consists of five kinds of subunits with a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ in which the catalytic nucleotide-binding sites are located on the β subunits and the non-catalytic nucleotide-binding sites are on the α subunits. The γ subunit inserts its long coiled-coil helices into the central cavity of the $\alpha_3\beta_3$ cylinder (3), and ATP hydrolysis occurring in the $\alpha_3\beta_3$ drives rotation of the γ subunit along with the ϵ subunit that is associated with the γ subunit (reviewed in Ref. 2).

Responding to the varying energy supply for ATP synthesis in living organisms, the activity of F_0F_1 must be regulated. Eukaryotic organellar F_0F_1 has developed unique regulatory systems; mitochondrial F_0F_1 has a specific ATPase inhibitor protein and its cofactor proteins (4, 5), and chloroplast F_0F_1 is regulated by the reversible formation of a disulfide bond in the γ subunit (reviewed in Ref. 6). More ubiquitous is the inhibition by the ϵ subunit, which was noticed since the early stage of studies of ATP synthase (7–9). The ϵ subunit is a small subunit of 130–140 residues consisting of two distinct domains, an N-terminal β-sandwich domain and a C-terminal helical hairpin domain (10, 11). Accumulating biochemical and structural studies have revealed that the ϵ subunit can adopt at least two different conformational states in F_1 and F_0F_1, "down"-state and "up"-state (12–19). The structures of the isolated ϵ from *Escherichia coli* represent the down-state conformation that does not exhibit the inhibitory effect. The exact conformation of the upstate ϵ subunit in F_1 and F_0F_1 is not known, but it is certain that the C-terminal helical hairpin in the down state is opened in the up state (19) and comes in contact with the α and β subunits (20). The up-state ϵ exerts the inhibitory effect on ATP hydrolysis activity but, interestingly, not on ATP synthesis activity of F_0F_1 (21).

Several factors are known to induce the conformational transitions of the ϵ subunit. Illumination of thylakoid membranes resulted in the change of the arrangement of the ϵ subunit in chloroplast F_0F_1 as probed by the accessibility of the antibody against the ϵ subunit (22, 23). Also addition of ATP to F_1 stabilizes the down-state conformation of the ϵ subunit (17, 24). Since it has been thought that nucleotide-binding sites of F_0F_1 are exclusively located in α and β subunits and the mutant F_1 with incompetent nucleotide-binding sites on α subunits still shows ATP-dependent conformational transition of the ϵ subunit (17), we have concluded that the ATP binding to the catalytic site(s) on the β subunit(s) triggers the conformational transitions of the ϵ subunit. Here, however, we report that the isolated ϵ subunit of F_1 from thermophilic *Bacillus* strain PS3 (TF_1) has a binding site for ATP. This unexpected finding raises a new possibility that the ϵ subunit in F_0F_1 acts not only as a regulator of F_0F_1 but also as a sensor for cellular ATP level.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids for ϵ Subunits—To obtain the wild-type ϵ subunit of TF_1, the expression plasmid pTE2 (14) and pET21c vector carrying TF_1 ϵ subunit gene (20) were used. The expression plasmid for the ϵ subunit of F_1 from *E. coli* (EF_1) was generated as follows. The gene for EF_1-ϵ subunit was amplified by PCR with two primers, one containing *Nde*I site and 5′ region of EF_1-ϵ gene and the other containing complementary sequence to 3′ region of EF_1-ϵ gene and *Hin*dIII site, using a plasmid containing *E. coli* F_0F_1 operon as a template. The resulting DNA fragment was digested with *Nde*I and *Hin*dIII and introduced into the respective sites of the pET21c expression vector (Novagen). The expression plasmid for the ϵ subunit of F_1 from *Bacillus subtilis* (BF_1) was generated by the same procedures by using genomic DNA of *B. subtilis* as a template for PCR. The expression plasmid for a mutant, Lys-109 to Ala (K109A), ϵ subunit of F_1 from *Spinacia oleracea L.* chloroplast (CF_1) was generated by the method of Kunkel *et al.* (25) from an expression plasmid for the wild-type CF_1-ϵ subunit, pMCE1 (26). DNA sequences were confirmed by DNA sequencing.

Purification of Recombinant Proteins—Wild-type and a truncated mutant (Val-90 to Stop) of TF_1-ϵ ($\epsilon^{\Delta C}$) were purified as described (16). The ϵ subunits of EF_1 and BF_1 were expressed in *E. coli* BL21 (DE3)

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‡ Supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists. Present address: Institute of Industrial Science, the University of Tokyo, 4-6-1 Komaba Meguro-ku, Tokyo 153-8505, Japan.

§ To whom correspondence should be addressed: Chemical Resources Laboratory, R-1, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama 226-8503, Japan. Tel.: 81-45-924-5233; Fax: 81-45-924-5277; E-mail: myoshida@res.titech.ac.jp.

[1] The abbreviations used are: F_0F_1, F_0F_1-ATPase/synthase; TF_1, F_1-ATPase from thermophilic *Bacillus* PS3, a soluble portion of F_0F_1; BF_1, F_1-ATPase from *B. subtilis*; CF_1, F_1-ATPase from *S. oleracea L.* chloroplast; EF_1, F_1-ATPase from *E. coli*; $\epsilon^{\Delta C}$, a mutant ϵ subunit of TF_1 truncated after Asp-89; TF_1-ϵ(nd), nucleotide-depleted wild-type TF_1-ϵ subunit; BzATP, 3′(2′)-*O*-(4-benzoyl)benzoyladenosine 5′-triphosphate; CyDTA, 1, 2-cyclohexanediaminetetraacetic acid; HPLC, high performance liquid chromatography.

cells (26). EF_1-ε subunit was purified by the same method as used for the TF_1-ε subunit. BF_1-ε subunit was also purified by the same method except that flow-through fractions of the butyl-Toyopearl column chromatography were used because BF_1-ε subunit did not bind to the column. The isolation of the mutant (K109A) CF_1-ε subunit was carried out at 4 °C as follows. The cells were disrupted by sonication (S-250 Sonifier, Branson Ultrasonics, Japan), and the inclusion bodies were collected by a centrifugation at 8400 × *g* for 20 min. The precipitate was suspended in 50 mM Tris-HCl (pH 8), 1 mM EDTA, and 4% (v/v) Triton X-100. The mixture was mildly shaken for 30 min at room temperature and centrifuged at 8400 × *g* for 20 min. The washing was repeated 3 times. Then the precipitate was suspended in distilled water and centrifuged at 19,000 × *g* for 20 min. The precipitate was dissolved in 50 mM Tris-HCl (pH 8), 1 mM EDTA, and 6 M urea and was subjected to a DEAE-toyopearl column equilibrated with the same buffer. The flow-through was collected, and urea was removed by dialysis against 50 mM Tris-HCl (pH 8), and 1 mM EDTA. The solution was subjected to a butyl-Toyopearl column and eluted with the same conditions as used for TF_1-ε subunit purification. The mutant (K109A) CF_1-ε subunit, whose inhibitory effect on ATPase activity of CF_1 was unchanged, was used in the experiments. For simplicity, we call this mutant (K109A) CF_1-ε subunit as just CF_1-ε in this paper. Molecular masses of purified ε subunits examined by mass spectrography agreed with the values predicted from amino acid sequences (data not shown).

Preparation of Nucleotide-depleted TF_1-ε—The TF_1-ε subunits purified as above contained 0.3 to 1.1 mol/mol of bound ATP. To remove this bound ATP, TF_1-ε subunit was mixed with hexokinase (Roche Diagnostics) (15 units/ml) in 50 mM Tris-HCl buffer (pH 8), 100 mM KCl, 4 mM $MgCl_2$, and 200 mM glucose. The mixture was dialyzed against a 200-fold volume of the same buffer without hexokinase overnight at room temperature. Then the mixture was concentrated by a centrifugal ultrafiltration device (MWCO 5000K, Vivaspin, Vivascience) and subjected to a gel filtration HPLC column (Superdex 200HR 10/30, inner diameter 10 × 300 mm, Amersham Biosciences) equilibrated with 50 mM Tris-HCl (pH 8) and 100 mM KCl to remove hexokinase and glucose. The collected peak contained 0.06 mol of bound ATP/mol of ε (termed as ε(nd) for nucleotide-depleted ε subunit) and used in the experiments. As the ε subunits from other sources did not contain bound nucleotides, they were used for the gel filtration assay without such treatment.

Detection of ATP Binding to ε Subunit—TF_1-ε(nd) or ε subunits from other F_1-ATPase (25 μM) were preincubated with the indicated concentrations of nucleotides in 50 mM Tris-HCl buffer (pH 8), 100 mM KCl, and 4 mM $MgCl_2$ for 10 min at room temperature. Then the mixtures were subjected to a Sephadex G-25F (Amersham Biosciences) HPLC column (inner diameter 10 × 300 mm) at room temperature and eluted with 50 mM Tris-HCl buffer (pH 8), and 100 mM KCl at flow rate 1.5 ml/min. The elution of proteins was monitored by absorbance at 290 nm, and the elution of the nucleotides was monitored by absorbance at 260 nm. In the case when Mg^{2+} requirement was examined, $MgCl_2$ was omitted from the incubation buffer and 10 mM EDTA or 1,2-cyclohexanediaminetetraacetic acid (CyDTA) was included instead. For ADP, hexokinase (15 units/ml) and 200 mM glucose were included in the mixture during preincubation to remove the contaminated ATP in the commercial ADP.

Photoaffinity Labeling of ε Subunit by BzATP—TF_1-ε(nd) (5 μM) was incubated with 20 μM 3′(2′)-*O*-(4-benzoyl)benzoyladenosine 5′-triphosphate (BzATP, Sigma) (27) for 10 min in 50 mM Tris-HCl (pH 8), 100 mM KCl, and 4 mM $MgCl_2$. UV irradiation was carried out with a UV transilluminator (CSF-10CF, Cosmo bio, Japan) with 5-cm spacing for 10 min. DTT (10 mM) was added after UV irradiation, and 30 μl of the reaction mixture was subjected to analysis with 15% SDS-PAGE. The proteins were stained with Coomassie Blue R-250.

Other Procedures—UV absorption spectra of TF_1-ε subunit were measured with a UV absorbance meter (V-550, Jasco, Japan) with a slit width at 5 nm. Protein concentrations were determined by the method of Bradford (28) using bovine serum albumin as a standard. For TF_1-ε subunit, the values of Bradford assays were corrected according to the quantitative amino acids analyses.

RESULTS

Unusual Absorption Spectrum of Purified TF_1-ε—We noticed that the UV absorption spectra of the TF_1-ε subunit isolated from expressing *E. coli* were unusual because the peak was ~265 nm and magnitude was varied from preparation to preparation (Fig. 1). As the difference spectra among the preparations showed the peak around 262 nm, it was suspected that the TF_1-ε preparations might contain bound nucleotides. We

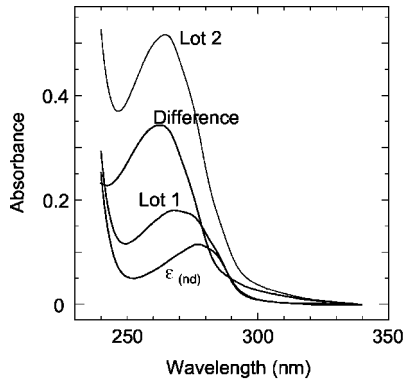

FIG. 1. **UV absorption spectra of TF_1-ε subunit.** UV absorption spectra of different lots of TF_1-ε preparations (25 μM) were measured. The difference spectra (*lot 2* to *lot 1*) and that of 25 μM TF_1-ε(nd) are also shown.

denatured TF_1-ε by acid treatment, removed the denatured proteins, analyzed the supernatant fraction with HPLC as described (29), and found that the preparations indeed contained 0.3–1.1 mol of ATP per mol of ε subunit (data not shown). When the isolated TF_1-ε was incubated with hexokinase and glucose (see "Experimental Procedures"), the amount of the bound ATP decreased to 0.06 mol/mol of TF_1-ε, and the UV absorption spectrum of the thus treated TF_1-ε(nd) (Fig. 1) agreed roughly to the spectrum expected from the content of aromatic amino acid residues in TF_1-ε (two Tyrs and no Trp).

TF_1-ε(nd) Binds ATP—To know if TF_1-ε(nd) was able to bind ATP again, TF_1-ε(nd) was mixed with ATP, preincubated, and subjected to the gel filtration column chromatography. The TF_1-ε was eluted at 6.2 min, and the small peak of TF_1-ε(nd) without ATP preincubation seen in the elution profile monitored at 260 nm was attributed to the absorption of TF_1-ε(nd) itself (Fig. 2*A*). Elution of the TF_1-ε(nd):ATP (1:0.4) mixture showed the 6.2-min peak with increased height and no peak corresponding to the free ATP, indicating that all of the added ATP was associated to TF_1-ε(nd). The 6.2-min peak height was further increased for the elution of the TF_1-ε(nd):ATP (1:1) mixture but saturated for the 1:2 mixture elution in which the peak of free ATP appeared. These results suggest that TF_1-ε(nd) binds 1 mol of ATP and that the TF_1-ε-ATP complex is stable as far as giving rise to an isolated peak in the gel filtration column chromatography. A notice should be added, however, that some fraction of the complex dissociated during chromatography because there was small tailing after the 6.2-min peak in the elution of the TF_1-ε(nd):ATP (1:1) mixture. Mg^{2+} was not required for ATP to bind to TF_1-ε(nd), because even when we omitted Mg^{2+} from the mixture and supplemented EDTA or CyDTA instead, ATP was still co-eluted with TF_1-ε(nd) at 6.2 min (Fig. 2*B*).

Photoaffinity Label of TF_1-ε by BzATP—ATP binding to TF_1-ε(nd) was also confirmed by a different means. A photoaffinity ATP analog, BzATP, was added to the solution of TF_1-ε(nd) and irradiated by UV. As shown in Fig. 3, the irradiated TF_1-ε(nd) generated a new band in SDS-PAGE analysis (Fig. 3, *arrowhead*). This represents the TF_1-ε(nd) labeled by BzATP. The new band disappeared by addition of ATP, ensuring that BzATP competes for the same site with ATP.

Specificity of Nucleotides That Bind to TF_1-ε—The bindings of ADP, GTP, UTP, and CTP were also tested by gel filtration chromatography. As shown in Fig. 2*C*, none of them changed the height of the 6.2-min peak of TF_1-ε(nd) but rather they were eluted as free nucleotides. Thus TF_1-ε(nd) only binds ATP but not other nucleotides under the examined conditions. This is in sharp contrast to the nucleotide binding specificity to the

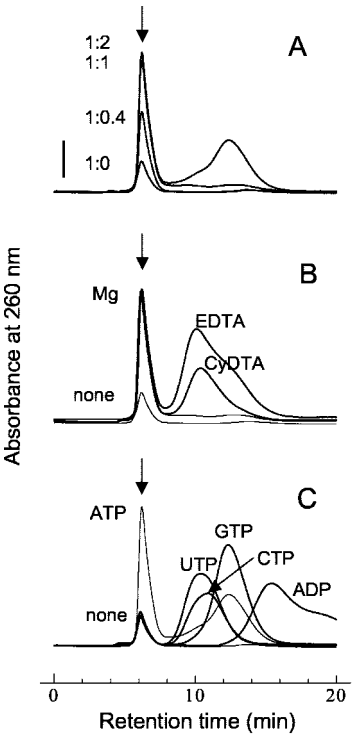

FIG. 2. **Gel filtration analyses of the nucleotides binding to TF_1-ϵ subunit.** The mixtures of ϵ subunits (25 μM) and nucleotides at indicated molar ratios containing 4 mM Mg^{2+} (except for *B*) were incubated at room temperature, and binding of nucleotide to ϵ subunit was analyzed with a Sephadex G-25F HPLC column. Elutions monitored by absorbance at 260 nm are shown. ϵ subunit was eluted at 6.2 min (*arrows*). *A*, binding analysis of ATP to TF_1-ϵ(nd). The mixtures with 1:0, 1:0.4, 1:1, and 1:2 TF_1-ϵ(nd):ATP molar ratios were analyzed. *B*, binding analysis of Mg^{2+}-free ATP to TF_1-ϵ(nd). The TF_1-ϵ(nd):ATP (1:1) mixtures in Mg^{2+}-free buffer supplemented with 10 mM EDTA or CyDTA were analyzed. Elutions of TF_1-ϵ(nd) only and the TF_1-ϵ(nd):ATP (1:1) mixture with Mg^{2+} are also shown as *thin lines* for reference. *C*, binding analysis of the ADP, GTP, CTP, and UTP to TF_1-ϵ(nd). The TF_1-ϵ(nd):nucleotide (1:2) mixtures were analyzed. Elutions of TF_1-ϵ(nd) only and the TF_1-ϵ(nd):ATP (1:2) mixture are also shown as *thin lines* for reference. The *vertical scale bar* in *A* represents absorbance unit of 0.005 at 260 nm. Other experimental conditions are described under "Experimental Procedures."

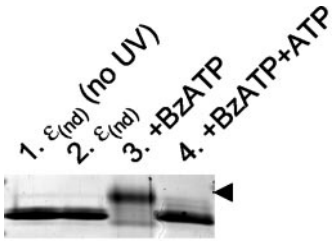

FIG. 3. **Photoaffinity labeling of TF_1-ϵ subunit by BzATP.** The TF_1-ϵ(nd) was incubated with BzATP and irradiated with UV light. The reaction mixtures were subjected to SDS-PAGE analysis. *Lane 1*, TF_1-ϵ(nd) without UV irradiation; *lane 2*, TF_1-ϵ(nd) irradiated without nucleotides; *lane 3*, TF_1-ϵ(nd) irradiated in the presence of 20 μM of BzATP; *lane 4*, TF_1-ϵ(nd) irradiated in the presence of 20 μM BzATP and 200 μM ATP. A newly appeared band by irradiation in the presence of BzATP is marked with an *arrowhead.* Only the region around ϵ subunit bands is shown.

α and β subunits of F_1; ADP and GTP can bind to the α and β subunits (30, 31). The strict discrimination of ATP from ADP by TF_1-ϵ is indicative of a possible role of the ϵ subunit as a sensor for cellular ATP concentration.

TF_1-$ϵ^{ΔC}$ Did Not Bind ATP—It has been demonstrated that the C-terminally truncated TF_1-$ϵ^{ΔC}$ (Met^1–Val^{90}) is capable of mediating the binding of F_1 to F_0 to form F_0F_1 that catalyzes

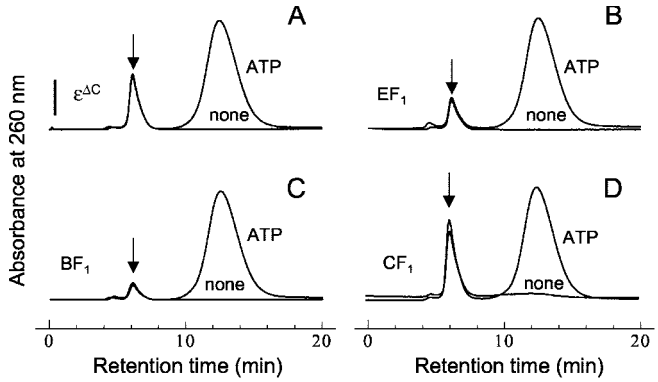

FIG. 4. **Gel filtration analyses of the nucleotides binding to TF_1-$ϵ^{ΔC}$ and ϵ subunits from other sources.** *A*, binding analysis of ATP to TF_1-$ϵ^{ΔC}$. Elution of TF_1-$ϵ^{ΔC}$ only is shown as a reference. *B–D,* binding analysis of ATP to EF_1-ϵ, BF_1-ϵ and CF_1-ϵ. The ϵ:ATP (1:2) mixtures were analyzed. Other conditions of analyses were the same as described in the legend of Fig. 2.

ATP-driven H^+ pumping although it does not exert an inhibitory effect on ATPase activity (16). We examined the binding of ATP to the isolated TF_1-$ϵ^{ΔC}$, but the binding was not detected (Fig. 4*A*). Therefore, the helical hairpin of the C-terminal domain is indispensable to generate the ATP-binding site on the ϵ subunit.

F_1-ϵ Subunits from Other Sources Did Not Bind ATP—Expecting that the ATP binding nature of F_1-ϵ would be common to wide range of organisms, we tested ATP binding to the isolated F_1-ϵ subunits from three sources as follows: a Gram-negative bacterium, *E. coli*; a Gram-positive bacterium, *B. subtilis*; and chloroplasts of the plant *Spinacia oleracea* L. However, none of them bound ATP under the conditions where TF_1-ϵ bound ATP (Fig. 4, *B–D*).

DISCUSSION

Since its isolation as the smallest subunit of F_1 3 decade ago (32), the idea has never come to attention that the ϵ subunit has the ability to bind nucleotide. No typical nucleotide-binding motif has been found in the amino acid sequence of the ϵ subunit, and no typical nucleotide binding domain has been noticed in the three-dimensional structures of the ϵ subunit either as an isolated one or in the F_1 (11–19). However, now we know that the ϵ subunit, at least the one from thermophilic *Bacillus*, is an ATP-binding protein. It is surprising that such a small protein (14 kDa) can generate a very specific ATP-binding site in its structure, and probably TF_1-ϵ is one of the smallest proteins that bind nucleotide.

The finding raises an attractive possibility that the ϵ subunit of F_0F_1 senses the varying cellular ATP concentration, *i.e.* when ATP concentration is elevated, ATP binding to the ϵ subunit in the down state facilitates the conformational transition of the ϵ subunit in F_0F_1 from the up state, which is an ATP synthesis mode, to the down state, which is presumably the mode less favorable for ATP synthesis. In this hypothetical scenario, the ϵ subunit plays dual roles, sensing the ATP level in the cell and regulating the activity of F_0F_1 by conformational transition. However, apparently inconsistent with this hypothesis, the ϵ subunits from other sources do not bind ATP. Also, ATP binding to the ϵ subunit assembled in F_0F_1 (or F_1) has not been demonstrated yet. The affinity of ATP to the ϵ subunit in F_0F_1 is probably around the cellular ATP concentration, *i.e.* the order of mM, for effective sensing of cellular energy conditions. If so, detection of such weak binding might be difficult by usual binding measurements. The isolated TF_1-ϵ shows strong affinity to ATP probably only at temperatures much lower than the living temperature of the organism. Actually, ATP was disso-

ciated from TF_1-ε rapidly at 50 °C.[2] We expect that the finding reported here will turn out to be the beginning of exploring a new regulation system of F_0F_1.

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