Isolated $\alpha$-Subunit of Thermophilic F$_{1}$-ATPase Binds ATP

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Running title: F$_{1}$-ATPase $\alpha$-subunit binds ATP
SUMMARY

F₁-ATPase, a soluble part of F₄F₁-ATP synthase, has subunit structure [1,2,3] in which nucleotide binding sites are located in the [1] and [2] subunits, and, as believed, in none of other subunits. However, we report here that the isolated [1] subunit of F₁-ATPase from thermophilic Bacillus strain PS3 can bind ATP. The binding was directly demonstrated by isolating the [1] subunit-ATP complex with gel-filtration chromatography. The binding was not dependent on Mg²⁺ but highly specific for ATP; ADP, GTP, UTP and CTP failed to bind. The [1] subunit lacking C-terminal helical hairpin was unable to bind ATP. Although ATP binding to the isolated [1] subunits from other organisms has not been detected under the same conditions, a possibility emerges that the [1] subunit acts as a built-in cellular ATP level sensor of F₄F₁-ATP synthase.
F$_o$F$_1$-ATPase/synthase (F$_o$F$_1$) catalyzes ATP synthesis coupled with the proton flow across 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$_o$F$_1$ and has ATP hydrolysis activity by itself. It consists of five kinds of subunits with a stoichiometry of $\square$,$\square$,$\square$, in which the catalytic nucleotide binding sites are located on the $\square$ subunits and the non-catalytic nucleotide binding sites are on the $\square$ subunits. The $\square$ subunit inserts its long coiled-coil helices into the central cavity of the $\square$, cylinder (3) and ATP hydrolysis occurring in the $\square$, drives rotation of the $\square$ subunit along with the $\square$ subunit that is associated to the $\square$ subunit (reviewed in 2).

Responding to the varying energy supply for ATP synthesis in living organisms, the activity of F$_o$F$_1$ must be regulated. Eukaryotic organellar F$_o$F$_1$ has developed unique regulatory systems; mitochondrial F$_o$F$_1$ has a specific ATPase inhibitor protein and its cofactor proteins (4, 5) and chloroplast F$_o$F$_1$ is regulated by the reversible formation of a disulfide bond in the $\square$ subunit (reviewed in 6). More ubiquitous is the inhibition by the $\square$ subunit, which was noticed since the early stage of studies of ATP synthase (7-9). The $\square$ subunit is a small subunit of 130-140 residues consisting of two distinct domains, an N-terminal $\square$ sandwich domain and a C-terminal helical hairpin domain (10, 11). Accumulating biochemical and structural studies have revealed that the $\square$ subunit can adopt at least two different conformational states in F$_1$ and F$_o$F$_1$, “down”-state and “up”-state (12-19). The structures of the isolated $\square$ from Escherichia coli represent the down-state conformation that does not exhibit the inhibitory effect. The exact conformation of the up-state $\square$ subunit in F$_1$ and F$_o$F$_1$ 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 become in contact with the $\square$ and $\square$ subunits (20). The up-state $\square$ exerts the inhibitory effect on ATP hydrolysis activity but, interestingly, not on ATP synthesis activity of F$_o$F$_1$ (21).

Several factors are known to induce the conformational transitions of the $\square$ subunit. Illumination of thylakoid membranes resulted in the change of the arrangement of the $\square$ subunit in chloroplast F$_o$F$_1$ as probed by the accessibility of the antibody against $\square$ subunit (22, 23). Also addition of ATP to F$_1$ stabilizes the down-state conformation of the $\square$ subunit (17, 24). Since it has been thought that nucleotide binding sites of F$_o$F$_1$ are exclusively located in $\square$ and $\square$ subunits and the mutant F$_1$ with incompetent nucleotide
binding sites on ⫸ subunits still shows ATP-dependent conformational transition of the ⫸ subunit (17), we have concluded that the ATP binding to the catalytic site(s) on the ⫸ subunit(s) triggers the conformational transitions of the ⫸ subunit. Here, however, we report that the isolated ⫸ subunit of F₁ from thermophilic Bacillus strain PS3 (TF₁) has a binding site for ATP. This unexpected finding raises a new possibility that the ⫸ subunit in F₂F₁ acts not only as a regulator of F₂F₁ but also as a sensor for cellular ATP level.

EXPERIMENTAL PROCEDURES

Construction of expression plasmids for ⫸ subunits ----- To obtain the wild-type ⫸ subunit of TF₁, the expression plasmid pTE2 (14) and pET21c vector carrying TF₁ ⫸ subunit gene (20) were used. The expression plasmid for the ⫸ subunit of F₁ from Escherichia coli (EF₁) was generated as follows. The gene for EF₁-[?] subunit was amplified by polymerase chain reaction with two primers, one containing NdeI site and 5’ region of EF₁-[?] gene and the other containing complementary sequence to 3’ region of EF₁-[?] gene and HindIII site, using a plasmid containing E. coli F₁,F₁ operon as a template. The resulting DNA fragment was digested with NdeI and HindIII and introduced into the respective sites of the pET21c expression vector (Novagen). The expression plasmid for the ⫸ subunit of F₁ from Bacillus subtilis (BF₁) was generated by the same procedures by using genomic DNA of B. subtilis as a template for polymerase chain reaction. The expression plasmid for a mutant, Lys 109 to Ala (K109A), ⫸ subunit of F₁ from Spinacia oleracea L. chloroplast (CF₁) was generated by the method of Kunkel et al. (25) from an expression plasmid for the wild type CF₁-[?] 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 TF₁-[?](R5) were purified as described (16). The ⫸ subunits of EF₁ and BF₁ were expressed in E. coli BL21 (DE3) cells (26). EF₁-[?] subunit was purified by the same method as used for the TF₁-[?] subunit. BF₁-[?] subunit was also purified by the same method except that flow-through fractions of the Butyl-Toyopearl column chromatography were used because BF₁-[?] subunit did not bind to the column. The isolation of the mutant (K109A) CF₁-[?] subunit was carried out at 4 °C as follows. The
cells were disrupted by sonicication (S-250 Sonifier, Branson Ultrasonics, Japan) and the inclusion bodies were collected by a centrifugation at 8400 xg for 20 min. The precipitate was suspended in 50 mM Tris-HCl (pH 8), 1 mM EDTA and 4 % (v/v) Triton X100. The mixture was mildly shaken for 30 min at room temperature and centrifuged at 8400 xg for 20 min. The washing was repeated 3 times. Then the precipitate was suspended in distilled water and centrifuged at 19000 xg 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, subunit purification. The mutant (K109A) CF, subunit, whose inhibitory effect on ATPase activity of CF, was unchanged, was used in the experiments because it was more resistant to protease digestion during purification procedures than the wild-type CF, (Y. Kikuchi and T. Hisabori, personal communication). For simplicity, we call this mutant (K109A) CF, subunit as just CF, in this paper. Molecular masses of purified subunits examined by mass spectroscopy agreed with the values predicted from amino acid sequences (data not shown).

Preparation of nucleotide-depleted TF, subunit ----- The TF, subunits purified as above contained 0.3 to 1.1 mol/mol of bound ATP. To remove this bound ATP, TF, subunit was mixed with hexokinase (Roche diagnostics) (15 U/ml) in 50 mM Tris-HCl buffer (pH 8), 100 mM KCl, 4 mM MgCl₂ and 200 mM glucose. The mixture was dialyzed against 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, Viva science) and subjected to a gel filtration HPLC column (Superdex 200HR 10/30, ID 10 mm x 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 subunit and termed as subunit (nucleotide-depleted subunit) and used in the experiments. As the 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 subunit ----- TF, subunits from other F₁’s
(25 μM) was preincubated with indicated concentrations of nucleotides in 50 mM Tris-HCl buffer (pH 8), 100 mM KCl and 4 mM MgCl₂ for 10 min at room temperature. Then the mixtures were subjected to a Sephadex G25F (Amersham Biosciences) HPLC column (ID 10 mm x 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 Mg²⁺ requirement was examined, MgCl₂ was omitted from the incubation buffer and 10 mM ethylenediaminetetraacetic acid (EDTA) or 1, 2-cyclohexanediaminetetraacetic acid (CyDTA) was included instead. For ADP, hexokinase (15 U/ml) and 200 mM glucose were included in the mixture during preincubation to remove the contaminated ATP in the commercial ADP.

*Photo-affinity labeling of subunit by BzATP* ---- TF₁-[nd] (5 μM) was incubated with 20 μM of 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 MgCl₂. 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 TF₁-[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 TF₁-[subunit, the values of Bradford assays were corrected according to the quantitative amino acids analyses.

**RESULTS**

*Unusual absorption spectrum of purified TF₁-[* ---- We noticed that the UV absorption spectra of the TF₁-[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 TF₁-[subunit preparations might contain bound nucleotides. We denatured TF₁-[subunit 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 to 1.1 mol of ATP per mol of □ subunit (data not shown). When the isolated TF₁-□ was incubated with hexokinase and glucose (see EXPERIMENTAL PROCEDURES), amount of the bound ATP decreased to 0.06 mol / mol of TF₁-□ and the UV absorption spectrum of thus treated TF₁-□ (Fig. 1) agreed roughly to the spectrum expected from the content of aromatic amino acid residues in TF₁-□ (two Tyr’s, no Trp).

*TF₁-□(nd)* binds ATP ---- To know if TF₁-□(nd) was able to bind ATP again, TF₁-□(nd) was mixed with ATP, preincubated, and subjected to the gel-filtration column chromatography. The TF₁-□ was eluted at 6.2 min and the small peak of TF₁-□(nd) without ATP preincubation seen in the elution profile monitored at 260 nm was attributed to the absorption of TF₁-□(nd) itself (Fig. 2A). Elution of the 1:0.4 TF₁-□(nd):ATP 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 TF₁-□(nd). The 6.2-min peak height was further increased for the elution of the 1:1 TF₁-□(nd):ATP mixture but saturated for the 1:2 mixture elution in which the peak of free ATP appeared. These results suggest that TF₁-□(nd) binds one mol of ATP and that the TF₁-□ ATP complex is stable as far as giving rise an isolated peak in the gel-filtration column chromatography. A notice should be added, however, that some fraction of the complex dissociated during chromatography since there was small tailing after the 6.2-min peak in the elution of the 1:1 TF₁-□(nd):ATP mixture. Mg²⁺ was not required for ATP to bind to TF₁-□(nd), because even when we omitted Mg²⁺ from the mixture and supplemented EDTA or CyDTA instead, ATP was still co-eluted with TF₁-□(nd) at 6.2 min (Fig. 2B).

Photo-affinity label of TF₁-□ by BzATP ---- ATP binding to TF₁-□(nd) was also confirmed by a different means. A photo-affinity ATP analog, BzATP was added to the solution of TF₁-□(nd) and irradiated by UV. As shown in Fig. 3, the irradiated TF₁-□(nd) generated a new band in SDS-PAGE analysis (Fig. 3, an arrowhead). This represents the TF₁-□(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 TF₁-□ ---- The bindings of ADP, GTP, UTP and CTP were also tested by gel-filtration chromatography. As shown in Fig. 2C, none of them did not change the height of the 6.2-min peak of TF₁-□(nd) but rather they were eluted as free nucleotides. Thus TF₁-□(nd) only binds ATP but not other nucleotides
under the examined conditions. This is in sharp contrast to the nucleotide binding specificity to the \( \square \) and \( \square \) subunits of \( F_1 \); ADP and GTP can bind to the \( \square \) and \( \square \) subunits (30, 31). The strict discrimination of ATP from ADP by \( TF_1 - \square \) is indicative of a possible role of the \( \square \) subunit as a sensor for cellular ATP concentration.

\( TF_1 - \square^{TC} \) did not bind ATP ---- It has been demonstrated that the C terminally truncated \( TF_1 - \square^{TC} \) (Met 1-Val 90) is capable of mediating the binding of \( F_1 \) to \( F_o \) to form \( F_oF_1 \) that catalyzes ATP driven H\(^+\) pumping although it does not exert inhibitory effect on ATPase activity (16). We examined the binding of ATP to the isolated \( TF_1 - \square^{TC} \) but the binding was not detected (Fig. 4A). Therefore, the helical hairpin of the C-terminal domain is indispensable to generate the ATP binding site on the \( \square \) subunit.

\( F_1 - \square \) s from other sources did not bind ATP ---- Having expected that the ATP binding nature of \( F_1 - \square \) would be common to wide range of organisms, we tested ATP binding to the isolated \( F_1 - \square \) s from three sources; a Gram negative bacterium, \( E. coli \), a Gram positive bacterium, \( Bacillus subtilis \), and chloroplasts of a plant, \( Spinacia oleracea \) \( L \). However, none of them bound ATP under the conditions where \( TF_1 - \square \) bound ATP (Fig. 4B-4D).

**DISCUSSION**

Since its isolation as the smallest subunit of \( F_1 \) at three decade ago (32), the idea has never come to our thought that the \( \square \) subunit has ability to bind nucleotide. No typical nucleotide binding motif has been found in the amino acid sequence of the \( \square \) subunit and no typical nucleotide binding domain has been noticed in the three dimensional structures of the \( \square \) subunit either as an isolated one or in the \( F_1 \) (11-19). However, now we know that the \( \square \) 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 \( TF_1 - \square \) is one of the smallest proteins that bind nucleotide.

The finding raises an attractive possibility that the \( \square \) subunit of \( F_oF_1 \) senses the varying cellular ATP concentration, that is, when ATP concentration is elevated, ATP binding to the \( \square \) subunit in the down-state facilitates the conformational transition of the \( \square \) subunit in \( F_oF_1 \) 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 subunit plays dual roles, sensing the ATP level in the cell and regulating the activity of $F_oF_1$ by conformational transition. However, apparently inconsistent to this hypothesis, the subunits from other sources do not bind ATP. Also, ATP binding to the subunit assembled in $F_oF_1$ (or $F_i$) has not been demonstrated yet. Probably, the affinity of ATP to the subunit in $F_oF_1$ is around the cellular ATP concentration, that is, the order of mM, for effective sensing of cellular energy conditions. If so, detection of such weak binding might be difficult by usual binding measurement. The isolated $TF_1$ shows strong affinity to ATP probably only at temperatures much lower than the living temperature of the organism. Actually, ATP was dissociated from $TF_1$ rapidly at 50 °C (unpublished result). We expect that the finding reported here will turn out to be a start to explore a new regulation system of $F_oF_1$.

Acknowledgment----We thank K. Yamane at Tsukuba Univ. for providing us Bacillus subtilis strain and M. Odaka at RIKEN institute for quantitative amino acid analyses. We also thank Y.-H. Watanabe, S.P. Tsunoda, H. Taguchi, E. Azami, T. Amano, T. Hisabori, E. Muneyuki and other members in our laboratory for their help and critical discussions. Y. Kato-Yamada is supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.
FOOTNOTES

1 Abbreviations used are: TF₁, F₁-ATPase from thermophilic Bacillus PS3, a soluble portion of F₉F₁; BF₁, F₁-ATPase from Bacillus subtilis; CF₁, F₁-ATPase from Spinacia oleracea L. chloroplast; EF₁, F₁-ATPase from Escherichia coli; [□]^C, a mutant □ subunit of TF₁ truncated after Asp89; TF₁-[□]ₙₐ, nucleotide-depleted wild-type TF₁-[□] subunit; BzATP, 3′(2′)-O-(4-benzoyl)benzoyladenosine 5′-triphosphate; CyDTA, 1, 2-cyclohexanediaminetetraacetic acid; HPLC, high performance liquid chromatography.

2 The nucleotide sequence of TF₁-[□] which we have analyzed, is deposited to DDBJ with an accession No. AB044942. The amino acid sequence deduced from the DNA sequence differs in several amino acids from the previously reported one (accession No. X07804).
REFERENCES


FIGURE LEGENDS

Fig. 1  **UV absorption spectra of TF₁-[] subunit.** UV absorption spectra of different lots of TF₁-[] preparations (25 []M) were measured. The difference spectrum (lot 2 - lot 1) and that of 25 []M TF₁-[]nd) are also shown.

Fig. 2  **Gel-filtration analyses of the nucleotides binding to TF₁-[] subunit.** The mixtures of [] subunits (25 []M) and nucleotides at indicated molar ratios containing 4 mM Mg²⁺ (except for B) were incubated at room temperature and binding of nucleotide to [] subunit was analyzed with a SephadexG25F HPLC column. Elutions monitored by absorbance at 260 nm are shown. [] subunit was eluted at 6.2 min (arrows).  A, Binding analysis of ATP to TF₁-[]nd). The mixtures with 1:0, 1:0.4, 1:1, and 1:2 TF₁-[]nd):ATP molar ratios were analyzed.  B, Binding analysis of Mg²⁺-free ATP to TF₁-[]nd). The 1:1 TF₁-[]nd):ATP mixtures in Mg²⁺-free buffer supplemented with 10 mM EDTA or CyDTA were analyzed. Elutions of TF₁-[]nd) only and the 1:1 TF₁-[]nd):ATP mixture with Mg²⁺ are also shown in thin lines as references.  C, Binding analysis of the ADP, GTP, CTP, and UTP to TF₁-[]nd). The 1:2 TF₁-[]nd):nucleotide mixtures were analyzed. Elutions of TF₁-[]nd) only and the 1:2 TF₁-[]nd):ATP mixture are also shown in thin lines as references. The vertical scale bar in panel A represents absorbance unit of 0.005 at 260 nm. Other experimental conditions are described in “EXPERIMENTAL PROCEDURES.”

Fig. 3  **Photo-affinity labeling of TF₁-[] subunit by BzATP.** The TF₁-[]nd) was incubated with BzATP and irradiated with UV light. The reaction mixtures were subjected to SDS-PAGE analysis.  *Lane 1*, TF₁-[]nd) without UV irradiation;  *lane 2*, TF₁-[]nd) irradiated without nucleotides;  *lane 3*, TF₁-[]nd) irradiated in the presence of 20 []M of BzATP;  *lane 4*, TF₁-[]nd) irradiated in the presence of 20 []M BzATP and 200 []M ATP. A newly appeared band by irradiation in the presence of BzATP is marked with an arrowhead. Only the region around [] subunit bands is shown.

Fig. 4  **Gel-filtration analyses of the nucleotides binding to TF₁-[]³ and [] subunits from other sources.**  A, Binding analysis of ATP to TF₁-[]³. Elution of TF₁-
only is shown as a reference. B, C and D, Binding analysis of ATP to EF1, BF1, and CF1. The 1:2 ATP mixtures were analyzed. Other conditions of analyses were the same as described in legend of Fig. 2.
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J. Biol. Chem. published online July 1, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M306140200

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