Reconstitution of F_1-ATPase activity from *Escherichia coli* subunits α, β and subunit γ tagged with six histidine residues at the C-terminus

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Abstract An engineered γ subunit of *Escherichia coli* F_1-ATPase with extra 14 and 20 amino acid residues at the N- and C-termini (His-tag γ), respectively, was overproduced in *E. coli* and purified. Six histidines are included in the C-terminal extension. The reconstituted F_1 containing α, β, and His-tagged γ exhibited sixty percent of the wild-type ATPase activity. The reconstituted αβHis-tag γ complex was subjected to affinity chromatography with nickel-nitrilotriacetic acid (Ni-NTA) agarose resin. ATPase activity was eluted specifically with imidazole. These results implied that the tag sequence protruded to the surface of the complex and did not seriously impair the activity. The reconstituted αβHis-tag γ complex, even after its binding to the resin, exhibited ATPase activity suggesting that the γ subunit, when fixed to a solid phase, may rotate the αβ complex. This system may provide a new approach for analysis of the rotation mechanisms in F_1-ATPase.

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Key words: F_1-ATPase; Reconstitution; Fusion subunit; Rotation mechanism

1. Introduction

The proton-translocating ATPase (F_1F_0) has a key role in energy transduction of living cells [1–7]. This enzyme has a similar structure among various organisms and exists on energy-transducing membranes such as mitochondrial, bacterial, and chloroplast membranes. The enzyme from *E. coli* is composed of a membrane integral portion (F_0) with three different subunits (a, b and c), and a membrane peripheral portion (F_1) with five different subunits (α, β, γ, δ and ε). The F_0 portion forms a proton channel, while the F_1 portion has the ATPase activity. The proton transfer through F_0 and ATP synthesis or hydrolysis are coupled, although the mechanisms have not been clarified.

The ATPase activity was reconstituted from purified *E. coli* α, β and γ subunits [8,9]. The β subunit has a catalytic site with a sequence that is highly homologous among various organisms. Based on the atomic structure of the bovine α and β and a portion of γ, the atomic structure of a portion of the bovine γ subunit shows that this subunit contains 45- and 63-residue long α helical structures at the N- and C-terminal regions, respectively [10]. These α helical segments run through the central cavity surrounded by the three pairs of α

and β. Recently, in the ATPase of thermophilic bacteria PS3, the γ subunit has been shown to rotate like a shaft [11]. In this experiment, the β subunits were fixed to a solid phase and the rotation of γ was monitored with fluorescence-labeled actin filaments attached to γ. With this system, it is difficult to answer the question which subunits other than α, β and γ are involved in the rotation. However, if the N- or C-terminus of the γ subunit could be fixed to a solid phase and fluorescent actin filaments could be attached to the α and/or β, it will be possible to determine whether the ε, δ and b subunits are involved in the rotation. As a first step in preparing this system, we report the preparation of a functionally active γ that has six histidines at the C-terminus, which makes it possible to bind γ to a solid phase.

2. Materials and methods

2.1. E. coli strains and culture medium

E. coli DH5 and JM109 [12] were used for recombinant plasmid propagation and cultured in L-broth medium [13]. For overproduction of F_1 subunits, *E. coli* BL21 cells harboring the expression plasmid with α, β, γ, or histidine-tagged γ gene were cultured in M9ZB medium at 37°C with vigorous shaking. For selection of expression plasmids, appropriate antibiotics were added to an agar plate or the liquid culture medium.

2.2. Construction of plasmid for His-tag γ

The coding sequence of the F_1 γ was amplified as described previously [14] by the polymerase chain reaction (PCR) [15] with primers EKU05 (5′-GATTACCTGTACGAACCCGATCCGAAG-3′) and EKU06 (5′-AAAGAGCTGCAGTTGGTATACAACAAA-3′) corresponding to the amino- and carboxy-termini, respectively, and with the wild-type *E. coli* DNA from KM230 [13] as the template and *Pfu* DNA polymerase [14]. EKU05 and EKU06 contain sequences for restriction sites of *Bam*HI and *Eco*RI, respectively. The amplified DNA was digested by these enzymes and then the coding sequence was inserted into these sites on expression plasmid pET21b(+) (Novagen) [16] which has an epitope sequence for T7 polymerase and six histidines (His-tag), at the N- and C-termini, respectively. The nucleotide sequence of γ amplified by PCR was verified by sequencing.

2.3. Overproduction of F1 subunits and purification

pET21b carrying γ with a His-tag was introduced into *E. coli* BL21. When growth of this transformant cultured in M9ZB medium reached the mid-logarithmic phase of growth ($OD_{600} = 0.6$), 0.4 mM isopropylthio-β-galactoside (IPTG) was added to induce endogenous T7 polymerase and the cells were cultured for another 2 h. Cells were harvested, suspended in buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 10% glycerol and 1 mM benzamidine) and disrupted by sonication. After undisrupted cells were removed by centrifugation at $4000 \times g$ for 10 min, insoluble γ was collected by centrifugation at $10\,000 \times g$ for 20 min. From the precipitate, the His-tag γ was purified by SDS polyacrylamide gel-electrophoresis and subsequently eluted from the gel as described previously [17]. The purified His-tag γ was concentrated with cold acetone and denatured by guanidine HCl as described previously [17]. The active γ was prepared by diluting gua-

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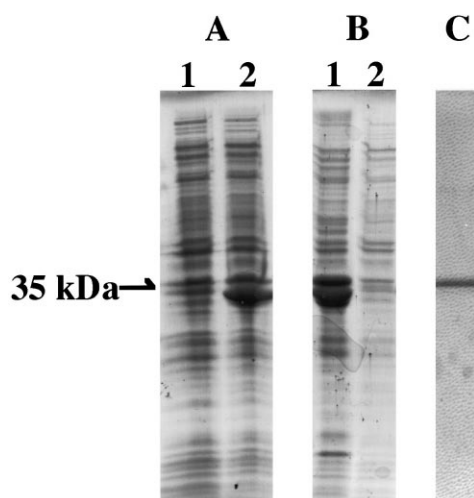E-mail: kanazawa@biotech.okayama-u.ac.jp

ATGGCTAGCATGACTGGTGGACAGCAAATGGGT CGGGATCCC

Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly | Arg Asp Pro

γ subunit   287 a.a

T7 Tag

AAGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCCAG CACCACCACCACCACCAC TGA

Lys Asn Ser Ser Ser Val Asp Lys Leu Ala Ala Ala Leu Glu | His His His His His His | END

His Tag

Fig. 1. Tagged sequence in His-tagged γ subunit. Extra amino acid sequences attached to the N- and C-termini of the γ subunit together with their nucleotide sequences are framed. Spacer sequences created between the T7-epitope, or His-tag and the reading frame of the γ subunit are also shown.

nidine HCl with dilution buffer (50 mM Succinate-Tris pH 7.6, 1 mM DTT, 0.1 mM EDTA, 150 mM NaCl, 20% glycerol) as described previously [17].

2.4. Reconstitution of the ATPase and affinity chromatography

The α, β and γ subunits were purified from *E. coli* cells that over-produced these subunits as described previously [9,17]. 180 μg α and 165 μg β, and 100 μg γ or γ (His-tag) were mixed together and dialyzed against the reconstitution buffer (50 mM Succinate-Tris pH 6.0, 5 mM MgCl$_2$, 0.5 mM EDTA, 1 mM DTT, 5 mM ATP, 10% glycerol) for 8 h at 25°C. ATPase activity and proteins were measured as described previously [18,19].

The reconstituted materials were applied to nickel-nitrilotriacetic acid (Ni-NTA) Sepharose (QIAGEN, 0.5 ml) column [20] which was equilibrated with the reconstitution buffer. After washing the unbound materials, the bound materials were eluted with 250 mM imidazole. Aliquots of the eluent were applied to non-denaturing polyacrylamide gel-electrophoresis or SDS polyacrylamide gel-electrophoresis [14]. For immunological detection of the subunits, the subunits were blotted onto a GHVP membrane and visualized with antibodies against the α, β, γ or T7 polymerase and with an ABC vectastain kit as described previously [21].

2.5. Immobilization of the reconstituted ATPase and ATPase assay

The reconstituted αβγ (His-tag) or αβγ complex (2.3 units) was incubated with 100 μl Ni-NTA agarose resin equilibrated with the reconstitution buffer for 2 h at 25°C. After the resin was washed three times (100 μl each), 250 mM imidazole, or the reconstitution buffer as the control, was added to release the bound materials. After centrifugation for 5 min at 15 000×*g*, the supernatant and precipitate fractions were subjected to the ATPase assay.

2.6. DNA manipulation and sequencing

Preparation of plasmid, digestion and ligation of the DNA fragments and other techniques related to handling of DNA were performed according to the published procedures [12]. The nucleotide sequence of the amplified γ subunit gene was determined by using isolated plasmids as the template for the dideoxynucleotide chain termination reaction [22] with [α-^{35}S]dCTP (37 TBq/mmol).

2.7. Reagents and enzymes

Restriction endonucleases, T4 DNA ligase, *Tth* and *Pfu* DNA polymerase, and T7 DNA polymerase were purchased from Bethesda Research Labs, Toyobo Co., New England Biolabs, and Takara Co. Oligonucleotides were synthesized by DNAgency (Malvern, PA). Other materials were of the highest grade commercially available.

3. Results

3.1. Overproduction and purification of His-tagged γ

To overproduce the γ subunit tagged with six histidine residues at the C-terminus, we constructed an expression plasmid derived from pET21b into which the wild-type γ sequence was inserted. The pET21b had an 11-residue sequence corresponding to an epitope of T7 polymerase recognized by a monoclonal, and three spacer residues at the N-terminus of the γ subunit (Fig. 1). It also had 14 extra spacer residues and six histidines at the C-terminus. Based on the procedure of Chou and Fasman [23], the extra residues at the C- and N-termini were estimated to contain a single α helix and a random coil, respectively. If the 20 residues at the C-terminus formed an α helix as predicted, the segment containing six histidines was expected to be extruded to the surface of the αβ complex on the basis of the three-dimensional structure revealed for bovine α and β and a portion of the γ subunit [10].

His-tagged γ subunit, γ (His-tag), was overproduced in *E. coli* cells harboring the pET expression vector upon induction of T7 polymerase (Fig. 2A) and was found exclusively in the insoluble fraction (Fig. 2B), which we previously reported for the wild-type γ [17]. The insoluble γ could be purified by SDS gel-electrophoresis and subsequent elution from the gel matrix as an active protein. This was then used with purified α and β for in vitro reconstitution of ATPase. γ (His-tag) was also purified by following this procedure for the wild-type (Fig. 2C).

3.2. Reconstitution of the ATPase activity from the purified α, β and γ (His-tag)

The ATPase activity was reconstituted from combinations of the purified wild-type α and β, together with γ or γ (His-tag). The reconstituted materials were applied to an affinity column using Ni-NTA agarose. With the addition of excess imidazole, ATPase activity was eluted from the column when it contained F$_1$ reconstituted with γ (His-tag) (Fig. 3, closed squares) but not when the column contained F$_1$ reconstituted

Table 1
Reconstitution of the ATPase activity from purified α, β and tagged γ

Reconstituted complex	ATPase activity (units/mg)	Relative activity (%)
α+β+γ	35.6	100
α+β+γ (His-tag)	21.5	60.4
α+β	1.0	2.8

The purified α (10 μg), β (10 μg) and γ or His-tag γ (6 μg) were mixed and dialyzed against the reconstitution buffer for 8 h at 25°C. An aliquot of this reconstituted αβγ complex and the peak fraction of reconstituted αβγ (His-tag) that eluted after addition of imidazole was used for the ATPase assay and protein measurement.

Fig. 2. Overproduction and purified γ (His-tag). _E. coli_ BL21 harboring the expression plasmid of γ (His-tag) derived from pET21b was cultured and expression of the tagged γ was induced with addition of IPTG. Whole cell extracts (A1, IPTG⁻; A2, IPTG⁺) and fractions after centrifugation at $15\,000 \times g$ for 10 min of cell extract derived from sonicated cells (B1, supernatant: B2, precipitate) were subjected to SDS polyacrylamide gel-electrophoresis and stained with Coomassie Brilliant Blue. The 35-kDa band corresponding to the overproduced γ subunit in a gel similar to that shown in B1 was cut out from the gel matrix and eluted and an aliquot was electrophoresed again (C).

Fig. 3. Affinity chromatography of the reconstituted ATPase using a Ni-NTA agarose column. The ATPase activity was reconstituted as described in Section 2. The reconstituted αβγ or αβγ (His-tag) was applied to Ni-NTA agarose resin (0.5 ml). Unbound materials were washed out with reconstitution buffer (5 ml) and then bound materials were eluted with imidazole (250 mM, 5 m) added at the position shown by an arrow. An aliquot (500 ul) of each fraction eluted from the column was used to measure ATPase activity. Open circles, αβγ; closed squares, αβγ (His-tag).

with the wild-type γ (open circles). The eluted peak fraction exhibited a high specific activity of ATPase, but the combination of α and β without γ or γ (His-tag) did not show such a high activity (Table 1). The materials eluted with imidazole were subjected to native polyacrylamide gel-electrophoresis. The peak fraction showed a single band at the same position for the αβγ complex (Fig. 4A), confirming that the αβγ (His-tag) formed an active complex. The eluted materials were also analyzed by Western blotting with anti-serum or antibody against the subunits. The peak fraction contained α (55 kDa, Fig. 4D) and β (50 kDa, Fig. 4D). The peak fraction also contained γ (His-tag), as shown by its molecular size, which was larger than the wild-type (Fig. 4C) and by its reactivity to T7 polymerase antibody (Fig. 4B). These results indicated that γ (His-tag) could reconstitute the active ATPase even with the extra sequence including the His-tag.

3.3. The ATPase activity of αβγ (His-tag) bound to Ni agarose resin

Since the reconstituted αβγ (His-tag) was found to be able to bind Ni-NTA agarose resin, we determined whether this complex retains the normal ATPase activity even after its

binding to the resin. The reconstituted αβγ (His-tag) or αβγ complex was bound to Ni-NTA agarose and further washed four times with the reconstitution buffer. After these washes, about 30 percent of the added ATPase activity (48×10^{-2} units) was recovered in the resin fraction. When imidazole was added in the fourth wash (imidazole + in Table 2), 30 percent of the added ATPase activity (42×10^{-2} units) was released from the resin and recovered in the wash. The control αβγ without His-tag exhibited low ATPase activity in the resin fraction before and also after addition of imidazole. These results indicated that the αβγ (His-tag) complex keeps its activity even after binding to the Ni-NTA agarose resin and suggested that the αβ core rotated around the bound γ.

4. Discussion

The atomic structure of α, and β, and a portion of γ from bovine F₁-ATPase revealed the presence of α helical domains at the N- and C-termini of γ. These helical domains exist in the central cavity formed by the three pairs of αβ [10]. The cavity distal to F₀ is open, suggesting that artificial extension of the helical structure at the C-terminus of γ may protrude to the outside of the αβ core complex. We tested this possibility in the present study. The results indicated that an extra 20 amino acid residues at the C-terminus and an extra 14 amino

Table 2
The ATPase activity of reconstituted αβγ (His-tag) bound to Ni-NTA agarose resin

Fraction	ATPase activity (units)			
	αβγ (His)		αβγ	
	Imidazole +	Imidazole −	Imidazole +	Imidazole −
Resin-bound	0.12	0.48	0.09	0.08
Wash	0.42	0.16	0.11	0.08

2.3 units of reconstituted ATPase of the αβγ (His-tag) or αβγ was mixed with 100 μl Ni-NTA agarose and incubated at 25°C for 2 h. After the resin was washed four times with the reconstituted buffer, the wash and resin-bound fractions were separated by centrifugation at $15\,000 \times g$ for 5 min. 250 mM imidazole was added to the resin to release the bound materials at the fourth wash (lane, imidazole +). An aliquot of each fraction was used for the ATPase assay.
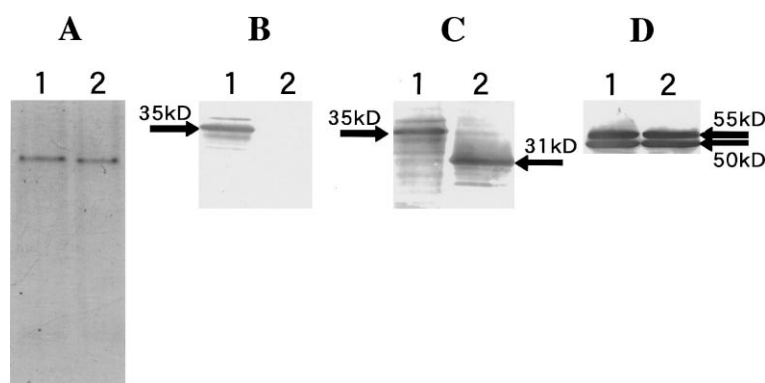
Fig. 4. Polyacrylamide gel-electrophoresis of the fractions eluted with affinity chromatography. Fractions eluted from the affinity column described in Fig. 3 were subjected to native polyacrylamide gel-electrophoresis (A) or SDS polyacrylamide gel-electrophoresis and subsequent Western blotting (B, C, D). A: An aliquot of eluted material (5 μg) in the washed fraction for the αβγ complex (lane 2) and in the eluted fraction with addition of imidazole for the αβγ (His-tag) (lane 1) was subjected to native polyacrylamide gel-electrophoresis (12.5% acrylamide) and subsequent staining with Coomassie Brilliant Blue. B: An aliquot of the fraction for αβγ (His-tag) was subjected to SDS polyacrylamide gel-electrophoresis and the resulting bands were blotted to a membrane filter. The immuno-reactive materials against anti-T7 polymerase (B), anti γ (C), or anti α and β antibodies (D) were detected by an ABC vectastain kit as described previously [14].

acid residues at the N-terminus basically did not impair the function of the wild-type γ and that the extra histidine sequence at the C-terminus is located outside of the αβ complex. The present results are thus consistent with the structure shown by the X-ray crystallographic data for bovine F_1. The secondary structures of the C- and N-terminal extra sequences were predicted to be an α helix and a random coil, respectively. The prediction for the C-terminal sequence is consistent with the present observations. In this connection, it might be interesting to test whether the predicted non-helical sequence added to the C-terminus might affect the function of the γ subunit. Jeanteur-De Beukelaer et al. [23] reported that reconstituted ATPase using a γ subunit with an extension at the C-terminus did not have activity. This is because the extra 14 residues at the N-terminus might impair the function of the γ subunit. Since this subunit has been shown to be located within the αβ complex [11], the extra sequence at the N-termini might interfere with its rotation within the central cavity, leading to the decrease of ATPase activity shown by αβγ (His-tag).

It has been reported that a cross-link between the γ and β subunits blocked the ATPase activity for *E. coli* [24,25]. Rotation of γ was visualized in the ATPase of the thermophilic *bacillus* PS3 [11] when β was fixed to a solid phase [23]. These previous observations suggested that the αβ portion may rotate when the γ subunit is fixed to a solid phase. Here we observed that the αβγ (His-tag) bound to Ni agarose resin had a high ATPase activity, suggesting that the αβ may rotate around γ (His-tag) efficiently. This visualization may be important as demonstrated by the following experiments. If the rotation of the αβ complex is real, the αβγ (His-tag) system will be useful for determining whether the δ, ε and a cytoplasmic portion of subunit b are involved in the rotation. Recently, we have shown that δ and the cytoplasmic region of subunit b interact and contribute to the F_1-F_0 interaction independently of the γ-c and ε-c interactions [26]. It was also reported that a cross-link between α and δ did not affect the ATPase activity [27]. These observations suggested that the b-δ interaction forms an immobile stator, while the γ-c and ε-c interactions might be involved in the rotation. When the rotation of the αβ complex is visualized with the present system, these possibilities will be clarified.

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