Interaction of the δ and b Subunits Contributes to F1 and F0 Interaction in the Escherichia coli F1F0-ATPase*

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Interactions of the F1F0-ATPase subunits between the cytoplasmic domain of the b subunit (residues 26–156, bcyt) and other membrane peripheral subunits including α, β, γ, δ, ε, and putative cytoplasmic domains of the α subunit were analyzed with the yeast two-hybrid system and in vitro reconstitution of ATPase from the purified subunits as well. Only the combination of bcyt fused to the activation domain of the yeast GAL-4, and δ subunit fused to the DNA binding domain resulted in the strong expression of the β-galactosidase reporter gene, suggesting a specific interaction of these subunits. Expression of bcyt fused to glutathione S-transferase (GST) together with the δ subunit in Escherichia coli resulted in the overproduction of these subunits in soluble form, whereas expression of the GST-bcyt fusion alone had no such effect, indicating that GST-bcyt was protected by the co-expressed δ subunit from proteolytic attack in the cell. These results indicated that the membrane peripheral domain of b subunit stably interacted with the δ subunit in the cell. The affinity purified GST-bcyt did not contain significant amounts of δ, suggesting that the interaction of these subunits was relatively weak. Binding of these subunits observed in a direct binding assay significantly supported the capability of binding of the subunits. The ATPase activity was reconstituted from the purified bcyt together with α, β, γ, δ, and ε, or with the same combination except ε. Specific elution of the ATPase activity from glutathione affinity column with the addition of glutathione after reconstitution demonstrated that the reconstituted ATPase formed a complex. The result indicated that interaction of b and δ was stabilized by F1 subunits other than ε and also suggested that b-δ interaction was important for F1-F0 interaction.

Proton-translocating ATPase (F1F0-ATPase) expressed on the membranes of mitochondria, chloroplasts, and bacteria has a key role in energy transduction (1–7). The complex subunit structure of this enzyme is basically similar among various species, but there are some differences in the membrane integral subcomplex (F0). Escherichia coli ATPase is composed of two different moieties, F1 and F0. F1, occupying the peripheral membrane portion, has the catalytic activity with five different subunits, α, β, γ, δ, and ε. F0 is the integral membrane portion with three different subunits, a, b, and c that forms a proton channel. The enzyme catalyzes ATP synthesis with a H+ gradient across the membrane formed by the respiratory chain in mitochondria and bacteria or photosynthetic electron transport in chloroplasts. In the reverse reaction, this enzyme pumps protons from the inside to the outside of bacterial cells and mitochondria coupled with hydrolysis of ATP.

Based on the three-dimensional structure of αβγ complex of the bovine F1-ATPase (8) and the results of kinetic analyses of nucleotide binding and catalysis (9), rotation of αβδ around γ was proposed to occur during catalysis (8). Data to support this hypothesis have been reported (10–12), and recently direct visualization of this rotation of the γ subunit within the αβγ complex has been performed for thermophila bacteria (13). Although evidence of the rotation within the F1 complex has been thus presented, its structural basis, especially in terms of the connecting portion between the αβγ complex and F0 is not well understood (14). The δ and ε subunits were shown to be important for this interaction (1, 2, 5, 6, 14). The atomic structure of the ε subunit and a portion of the δ subunit was resolved by NMR analysis for E. coli (14–16). The ε subunit interacts with the DELSEED region of the α and β subunits (14, 19) and also binds to the cytoplasmic region of the ε subunit (17). However, the topological arrangement of the δ subunit in the F1F0 complex is less well known than that of the ε subunit. In an early study of the biochemical properties of the purified δ subunit from E. coli (18), an elongated structure was estimated. This subunit is also known to open sealed proton channel activity of F0 during enzyme biosynthesis (20). Based on the results of hydrophathy analysis of the primary structure of the b subunit of F0 (2, 3), the majority of this subunit is estimated to be hydrophilic and possibly extruded into the cytoplasm, whereas the small amino-terminal portion is essential for its integration into membranes (22–24). The region between residues 25 and 146 of this subunit was overproduced as a soluble form and was shown to be capable of binding to the F1 complex (24). Although these previous studies strongly suggested interactions between the b and δ or ε subunits, direct evidence of b-δ or bε interaction has not been reported. The a subunit of F0 has been shown by genetic analysis to be required for F1-F0 binding (21) and its interacting partners within F1 subunits have been also determined by chemical cross-linking experiments (25). However, the precise regions involved in the binding of this subunit to a specific F1 subunit have not been elucidated.

Here, we have found a specific interaction between the b and δ subunits with two approaches; a genetic approach with the yeast two-hybrid system (26, 27) and a biochemical approach with overproduction, purification, and in vitro binding of the subunits (28). The results directly indicate that the interaction of b and δ has an important role for F1-F0 interaction.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions—E. coli strains BL21 and JM109 were used (28) for overproduction of various peptides and genetic manipulations including preparation of various plasmids, respectively. E. coli cells were cultured in a minimal medium (Tanaka me-
Position 1 corresponds to the first base (A) of the initiation codon of the a subunit gene in the unc operon. Sequences of the top two lines and the other lines correspond to the sequences of the b and a subunits, respectively. The first 5' bases of the given reading frame (sequence) are shown next to a comma, and the residue number is shown in the left column (position). The first four nucleotides in all oligonucleotides were random and the subsequent GOATTC and GAATTC sequences are recognition sites for the restriction endonucleases BamHI and EcoRI, respectively.

TABLE I
Oligonucleotide primers used for polymerase chain reaction

| Primer | Sequence | Position |
|--------|----------|----------|
| bT25-F | 5'-GCATGAAATTC, TGGCGGCCATTAAATGGCCAGC-3' | 1239 |
| bT165-R | 5'-GCATGGATCCCTA, CAGTTCAAGCGACAGTGT-3' | 1631 |
| aT60-F | 5'-GCATGAAATTC, TTCCGTAGCTGAGCAAAATAUUU | 178 |
| aT160-R | 5'-GCATGAAATTC, TCGATTCTGTTTCTACAGGT-3' | 178 |
| aT200-R | 5'-GCATGAGGCTTTA, CAGGCTTACCCCTTCAAGGAG-3' | 600 |

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The expression plasmids carrying the genes for the indicated fusion proteins were cotransfected into yeast SFY526 by the lithium acetate method as described under "Materials and Methods." Transformants were selected by plating onto SD medium lacking Trp and Leu. Transformants that contained both plasmids were grown to mid-log phase of cell growth in SD medium lacking Trp and Leu and then assayed for β-galactosidase activity, shown here in Miller units (c). The pairs in the top six lines indicate combinations $b_{\delta}$ fused to the activation domain of GAL4 (GAL4-ad) and various $F_1$, $F_2$, and $F_3$ subunits fused to the binding domain of GAL4 (GAL4-bd). Similarly, the next five lines indicate combinations of $b_{\delta}$ fused to the binding domain of GAL4 and various subunits fused to the activation domain of GAL4. In the bottom two lines, the pair of $a$ and $b$ subunits fused to the activation and binding domains, respectively, and the combination of vectors alone (−) are shown as positive and negative controls, respectively.

**Table II**

| Fusion protein | GAL4-ad | GAL4-bd | β-Galactosidase activity (c) |
|---------------|---------|---------|-----------------------------|
| $b$           | $\alpha$| 0.09    |
| $\beta$       |         | 0.12    |
| $\gamma$      |         | 0.13    |
| $\delta$      | 15.0    |         |
| $\epsilon$    | 0.08    |         |
| $b$           | $\delta$| 0.08    |
| $\epsilon$    | 0.08    |         |
| $\alpha$      | $\beta$| 18.0    |
| $\alpha$      | $\beta$| 0.08    |

**FIG. 1.** Cloned portions of the $a$, $b$, or $\delta$ subunit genes in various expression vectors. Cloned portions of the $b$ and $a$ subunits in the expression plasmids for the two-hybrid system and GST fusion proteins are shown schematically in (1) and (2), and (3), respectively. For GAL4-ad (activation domain of GAL4) and GAL4-bd (binding domain of GAL4) plasmids, pGAD424 and pGBT9 were used, respectively. pGEX-2T was used for the expression of fusion proteins. Details of plasmid construction are described under "Materials and Methods."

**Mic Domain of the $b$ Subunit**—To identify subunits capable of binding the $b$ subunit, we took two approaches; a genetic approach with the yeast two-hybrid system (27) and a biochemical approach with in vitro binding assay of subunits. For these approaches, we constructed two types of chimeric plasmids (Fig. 1). For biochemical approaches, DNA fragment spanning the membrane peripheral portion of the $b$ subunit (residues 26–156) that was deduced from hydropathy analyses was fused to the GST gene to create a fusion peptide, and for genetic analysis with the two-hybrid system, the same portion of the $b$ subunit was fused to the activation or DNA binding domain of GAL4 gene. The $b_{\delta}$ region of the $b$ subunit gene was amplified by the polymerase chain reaction and fused to the GST or GAL4 gene. Based on the previous model of membrane topology in the $a$ subunit of $F_0$ (3, 42–44), we amplified DNA of this gene corresponding to the putative peripheral membrane domain between residues 60 and 100 and 160 and 200, and also fused it to the binding or activation domain of the GAL4. To determine the binding partners of $b_{\delta}$ and portions of the $a$ subunit, we used chimeric plasmids of $F_1$ subunits ($a$, $\beta$, $\gamma$, $\delta$, and $\epsilon$) fused to the activation or binding domain of the GAL4 gene for the yeast two-hybrid system constructed in the previous study (26).

**Detection of Subunits Capable of Binding to the $b$ Subunit**—First, we examined the interaction between $b_{\delta}$ and one of the $F_1$ subunits, and then we analyzed $b_{\delta}$-$b_{\delta}$ and also $b_{\delta}$-$a$ interactions with the putative peripheral membrane domains of the $a$ subunit. The pair of $a$ and $b$ subunits gave strong expression of the reporter gene as we described previously (26), whereas the vectors alone did not (Table II). Among the various combinations tested, the combination of $b_{\delta}$ fused to GAL4-ad (activation domain of GAL4) and $\delta$ fused to GAL4-bd (binding domain of GAL4) alone resulted in strong expression of the reporter gene, whereas all other combinations showed no significant expression (Table II). These results demonstrated that the interaction between $b_{\delta}$ and $\delta$ occurred under in vitro conditions and other combinations might not cause interaction in those pairs. Although the purified soluble fraction of $b$ residues 25–146 or 26–156 was reported to form a dimer in vitro (24, 45), we did not observe this interaction in the two-hybrid system. The putative membrane peripheral region of the $a$ subunit also did not show reporter gene expression, suggesting that these portions may not interact stably with the $\delta$ subunit.

**Overproduction of the $b_{\delta}$ and $\delta$ Subunits Fused to GST**—When we induced expression of the GST-$b_{\delta}$ in *E. coli* carrying the GST-$b_{\delta}$ expression plasmid, GST alone without GST-$b_{\delta}$ was observed as shown in Fig. 2A. This observation suggested that the $b_{\delta}$ portion of the overproduced GST-$b_{\delta}$ was degraded in the cells, whereas the GST portion in the fusion protein was intact. Next, we tried to overproduce the GST-$b_{\delta}$ together with the $\delta$ subunit, because the interaction of these subunits indicated in the yeast two-hybrid system might protect the $b_{\delta}$ portion from degradation by proteases. In fact, the overproduction of GST-$b_{\delta}$ and $\delta$ were observed on SDS-polyacrylamide gel electrophoresis of cell extract by staining (Fig. 2B) and immunological detection with specific antiserum for GST and $\delta$, respectively (data not shown), consistent with the results of the two-hybrid system and our expectations. The stoichiometry of GST-$b_{\delta}$ and $\delta$ was 1.5 based on densitometric analysis of the stained bands. This result supported the notion that GST-$b_{\delta}$ interacted with $\delta$ in *E. coli*. It should be noted that the overproduced $\delta$ subunit has not been degraded, as shown immunologically (data not shown). According to our previous results regarding to GST-$\delta$ expression (28), this fusion subunit is susceptible to proteolytic attack in *E. coli* cells.

**Affinity Purification of GST-$b$ Fusion Subunit**—The soluble fraction of *E. coli* cells overproducing GST-$b_{\delta}$ and $\delta$ was subjected to glutathione-Sepharose affinity chromatography. The GST-$b_{\delta}$ fusion protein was eluted specifically with the addition of glutathione (Fig. 2B, final lane), whereas the $\delta$ subunit was not.
amounts of a munological detection with antisera against the subunits (Fig. 5). Phoresis and subsequent staining of proteins (Fig. 5) was eluted after washing the column and also with addition of glutathione (26% of the applied activity) with specific activity of 19.0 units per mg of protein at the peak fraction. The eluted activity chromatography. As shown in Fig. 4, the ATPase activity in the purified GST-bcyt and GST fractions (100 µg each) was measured, and significant activity was found for GST-bcyt (0.044 units/mg of protein) compared with GST alone (0.023 units/mg of protein). The presence of the a, b, and γ subunits in the fraction was detected by immunological assay (data not shown). These results suggested that a small portion of the GST-bcyt contained the ATPase complex with GST-bcyt formed in vivo, and also that the GST-bcyt could behave as the b subunit itself in vivo.

We tested in vitro binding of the δ and bcyt by assay of the binding product with glutathione-Sepharose affinity chromatography, but no significant binding was detected. Then we tested binding of these subunits on membrane filters, a procedure which is more sensitive than affinity chromatography. The interaction was detected by this binding assay: bcyt bound to δ and not to control GST (Fig. 3).

Reconstitution of the ATPase from the bcyt and α, β, γ, δ, and ε—Since interaction of bcyt and F1 complex was demonstrated by the two different approaches, namely in vivo and in vitro binding assays, and a trace but a significant amount of ATPase formed in vivo was further detected as described above, we tested the interaction of bcyt and F1 complex as follows. The ATPase was reconstituted from the purified α, β, γ, δ, and GST-bcyt. The reconstituted ATPase was then subjected to glutathione affinity chromatography. As shown in Fig. 4, the ATPase activity was eluted after washing the column and also with addition of glutathione (26% of the applied activity) with specific activity of 19.0 units per mg of protein at the peak fraction. The eluted materials were analyzed by SDS-polyacrylamide gel electrophoresis and subsequent staining of proteins (Fig. 5A) or immunological detection with antisera against the subunits (Fig. 5B). The a, β, γ, and GST-bcyt and these subunits together with δ and ε were detected by staining (Fig. 5A) and immunological detection (Fig. 5B), respectively. The stoichiometry of α, β, γ, δ, and GST-bcyt contained in the peak fraction based on densitometric analysis of the stained bands was 2.5:2.6:1.4:1.0:0.7:1.9, which is similar to that in the native F1Fo. When GST instead of GST-bcyt was used for reconstitution, the specific elution of the ATPase was not observed with the addition of glutathione (data not shown). These results indicated that at least one-third of the subunits were reconstituted with bcyt as a native F1Fo-like complex.

Reconstitution of the ATPase from Various Combinations of the Subunits—To determine the interacting partner of the bcyt, we tested reconstitution of the ATPase from the combination of F1 subunits except δ or ε and bcyt. As shown in Fig. 6, A and B, the combination with δ but not ε reconstituted the ATPase, the activity of which was specifically eluted with glutathione. The reconstituted activity was 38.1 units per mg of protein at the peak fraction. The subunits contained in this peak were those expected as the α, β, γ, δ, and bcyt (Fig. 7A). The combination with the ε subunit instead of δ did not yield the ATPase activity after addition of glutathione, and no subunits other than GST-
b\textsubscript{cyt} alone were observed (Fig. 7B). These results indicated that GST-b\textsubscript{cyt} did not bind a\textbeta\textgamma\textepsilon complex but to a\textbeta\textgamma\textdelta complex and that interaction of b\textsubscript{cyt} and d is more important than that of b\textsubscript{cyt} and e in F\textsubscript{1} and F\textsubscript{0} interaction.

To obtain insight into the topological arrangement of the amino terminus of the d or e, and the b\textsubscript{cyt} within the F\textsubscript{1}F\textsubscript{0}-ATPase, we tested reconstitution of the ATPase from combinations of subunits, a, b, g, e, b\textsubscript{cyt}, and a, b, g, d, b\textsubscript{cyt}, and GST-e. The ATPase activities reconstituted with 21.4 and 25.2 units per mg of protein for the complex with GST-d and GST-e, respectively, were eluted in both cases after addition of glutathione (Fig. 8, A and B) on glutathione affinity chromatography. This result indicated that the amino termini of both subunits did not exist within the ATPase complex but were exposed on the surface of the complex.

DISCUSSION

It was shown by gel filtration that the purified membrane peripheral portion of the b subunit (residues 25–146) was integrated in a complex together with F\textsubscript{1} (24). This portion also inhibited the binding of F\textsubscript{1} to the F\textsubscript{0} portion. These previous results suggest that the cytoplasmic region of the b subunit interacts with the F\textsubscript{1} subunits. However, the subunits capable of binding to this subunit have not been identified. Here, we provide the evidences of b-d interaction with the yeast two-hybrid system and in vitro binding of the purified subunits. However, the levels of the d subunit in the affinity-purified GST-b\textsubscript{cyt} fraction of E. coli extract and in a mixture of purified GST-b\textsubscript{cyt} and d in vitro binding assay were low, indicating that the interaction of the subunits might be weaker than that observed for g-e interaction reported previously (26, 28).

Here, we clearly showed the reconstitution of the active a\textbeta\textgamma\textepsilon\textdelta\textgamma\textsubscript{cyt} complex from the purified subunits, which has a similar structure to that of a native complex. Therefore, the relatively weak b-d interaction might be stabilized by complex formation with the other F\textsubscript{1} subunits. This suggests that the d subunit interacts not only with b\textsubscript{cyt} but also with other subunits of F\textsubscript{1}. We have reported previously that this subunit interacts with the a, b, and g subunits weakly based on the results of the yeast two-hybrid system (26). Dunn and co-workers (54) reported that deletion of the amino-terminal 15 residues of the a subunit caused loss of F\textsubscript{1} binding to d, suggesting a-d interaction. Aris and Simoni (25) described chemical cross-linkage between a-d, b-d, and g-d. Recently, extensive analyses in terms of subunit interaction of the d with other subunits have been reported for chloroplast F\textsubscript{1} by cross-linking between Cys introduced into the d subunit, and residues in the a or b subunits (46). This previous study showed the interaction of Ser-10 substituted by Cys in the d subunit with the amino-terminal 62 residues in the b subunit. Ser-57, -82, and -166 substituted by Cys were cross-linked within the amino-terminal 192 residues of the a subunit. Therefore, stabilization of b-d interaction requires complex interactions of most other subunits of F\textsubscript{1}.

The stoichiometry of b and d was shown to be 2 to 1 based on the purified F\textsubscript{1}F\textsubscript{0}-ATPase (18, 45, 47). According to semi-quantitative analyses of GST-b\textsubscript{cyt} and d expression in total extracts of E. coli cells in this experiment, the stoichiometry b and d was 1.5, indicating that both GST-b\textsubscript{cyt} and d subunits were protected from proteolytic attack in E. coli cells to a certain extent. The specific interaction of these subunits based on the stoichiometry (GST-b\textsubscript{cyt}:d = 1.9:1) was also observed for the reconstituted a\textbeta\textgamma\textepsilon\textdelta\textgamma\textsubscript{cyt} complex. These results suggest that GST-b\textsubscript{cyt} and d interact with each other in a manner similar to the native complex.

In marked contrast with a\textbeta\textgamma\textepsilon\textdelta\textgamma\textsubscript{cyt} complex formation, the e subunit did not reconstitute a\textbeta\textgamma\textepsilon\textdelta\textgamma\textsubscript{cyt} complex, indicating the e-b\textsubscript{cyt} interaction may be very weak even in the presence of other F\textsubscript{1} subunits. Since we have shown that purified a, b, g, and GST-e reconstitute an active complex (28), GST-b\textsubscript{cyt} may not interact with this complex. Previous biochemical and genetic studies have indicated that F\textsubscript{1} (a\textbeta\textgamma\textdelta
complex) does not bind to F₀ without the ε subunit (18, 48, 49), which is inconsistent with the present results. However, the previous conclusion was based on the experiments in vivo or in vitro with membranes as the F₀ source, which was clearly different from the present experimental conditions. This difference suggests that the α and c subunits in F₀ (membrane) may destabilize the β-δ interaction without the ε subunit. It has been shown that the ε subunit interacts with γ and c subunits (17). The γ subunit was also found to interact with the ε subunit (50). Therefore, the F₁F₀ interaction may be completed with two independent structures, interactions of γ-c and ε-c, and β-δ.

It has been shown that 20 residues of the carboxyl-terminal region of the δ subunit are more susceptible to trypsin compared with the amino-terminal region (53). Recently based on NMR analyses, the atomic structure of δ subunit between residues 1–134 has been reported (16). In this study δ was shown to be a protein with two characteristic domains, with the compact six α-helix bundle at NH₂ terminus and a less well characterized COOH-terminal domain. These two domains may be cross-linked by a disulfide bridge between residues 64 and 140 as reported previously (51). Based on the present study together with these previous observations including CF₁ δ reported by Lill et al. (46), the δ subunit within NH₂-terminal 104 residues is thought to bind the amino-terminal regions of the α and β subunits, and the rest of δ may bind to the dimer of b. Since random and site-specific mutagenesis studies suggested that no essential residues were present in the δ subunit (52), this subunit might be structurally, rather than functionally, required for catalysis. Formation of β-δ-α or β-δ-β interaction may thus be structurally important. It would be of interest to analyze whether the α, β, δ, and b interaction is involved in the rotation as has been reported very recently (13) or whether this structure forms an immobile stator.

The previous study showed that the cytoplasmic region (residues 25–146) of b subunit tagged with a few amino acids was overproduced without degradation in the absence of δ subunit coexpression (24). The same region of δ in the present study fused to GST was also reported to be overproduced (45). Although the reason for the discrepancy between our result and those of the previous studies is not clear, differences in the experimental conditions for the tagged protein and different host cells used for overproduction as well in the these studies might be a cause. For the latter case (45), GroES (chaperonin proteins) coexpressed with δ might lead to no degradation of δ. It should be noted that point mutation in the δ subunit (Asp-161 to Pro) and total deletion the δ subunit causes degradation of the b subunit (52, 55), consistent with our results. Although
the b subunit was reported to form a dimer (24, 45), we could not
detect b_{cyt-b} - b_{cyt} interaction with the yeast two-hybrid sys-
tem. Fusion of the DNA binding and activation domain of GAL4
gene product might interfere with this interaction. We also did
not observe an interaction between the b subunit and putative
peripheral membrane portions of the a subunit (residues 60–
100 and 160–200), regions that were selected based on the
previous report (15). This negative result suggests that the
interaction. Yamada et al. (44) recently reported a new topology model
of the a subunit in which residues 60 to 100 are in the exterior
surface or within the membrane. Our results in terms of the
region from residues 60 to 100 is consistent with this hypothet-
ic model of membrane topology of the a subunit (15) may not be
correct or that other subunits are involved for the b-a interac-
tion. Based on these results, we propose a new model for the
F_{1}F_{0} interaction.

In the present study, we have detected b-o interaction with
the yeast two-hybrid system. This system may be useful to
obtain mutants defective in b-o interaction. This approach was
also successfully demonstrated for α-β interaction in our pre-
vious study (26).

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