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Interactions of the F_1-ATPase subunits from *Escherichia coli* detected by the yeast two-hybrid system

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Abstract

Subunit interactions among the F_1-ATPase subunits were studied by the yeast two-hybrid system. Various pairwise combinations of genes encoding α, β, γ, δ and ϵ subunits of *Escherichia coli* H^+-ATPase fused to the DNA-binding or activation domain of the yeast GAL4 gene were introduced into yeast and expression of a reporter gene encoding β-galactosidase was detected. Combinations of the α and β subunit genes, and of the ϵ and γ subunit genes showed high levels of reporter gene expression, while those of α and δ, β and δ, γ and δ, and δ and ϵ demonstrated weak but significant reporter gene expression. However, combinations of α and γ, β and γ, α and ϵ, and β and ϵ did not induce reporter gene expression. None of the fused genes alone induced reporter gene expression. These results suggested that specific and strong interactions between the α and β, γ and ϵ, and weak interactions between the α and δ, β and δ, and γ and δ subunits occurred in yeast cells in the two-hybrid system. Effects of previouly identified mutant β subunits with Leu-40 to Pro, Glu-41 to Lys or Pro-332 to Gln subsitutions which caused defects in molecular assembly of F_1-ATPase were analyzed with regard to α-β interaction. No interaction of the α and β subunits was observed in this system using the β subunit with mutation of Pro-332 to Gln. However, for the other two mutations, α-β interactions were observed. This system may be useful for isolating mutants which have defects in interaction of F_1-ATPase subunits.

Keywords: ATPase, F_1-; Subunit interaction; Two-hybrid system

1. Introduction

The proton-translocating ATPase plays a central role in the energy transduction system in living cells [1–6]. This enzyme synthesizes ATP by utilizing proton gradients across energy transducing membranes such as those of mitochondria, chloroplasts, and bacterial cytoplasmic membranes formed by respiration or photoreaction. This enzyme from *Escherichia coli* has a similar structure to those of the higher organisms and is composed of two distinct entities, F_1 and F_0. The F_1 is a membrane peripheral portion with five subunits, α, β, γ, δ, and ϵ in 3:3:1:1:1 stoichiometry, and forms the catalytic portion. The β subunit contains the catalytic center and the $\alpha\beta\gamma$ complex has the minimum core required for catalysis in *E. coli* [7]. F_0 is a membrane integral portion with three subunits, a, b and c, and forms a proton channel. The primary structures of the whole subunits were deduced from the nucleotide sequences of the genes from *E. coli* [1–6,8] and other species [9].

Based on the complex structure of this enzyme with three sets of catalytic β subunits and extensive kinetic analyses of nucleotide binding, alternating binding of the substrate to these sites was proposed [10]. Recent X-ray crystallographic data of the $\alpha\beta\gamma$ complex from bovine mitochondria supported this proposal and further suggested that dynamic rotation of the $\alpha\beta$ portion around the γ subunit might occur [11]. Therefore, the subunit interactions among the F_1-ATPase subunits may play important roles in the catalytic mechanism. Identification of interacting residues among the subunits is a prerequisite to understand the dynamic interactions of the subunits. Further, the dynamic interactions of the subunits should be clarified at the level of amino acid residues. Although X-ray crystallographic data have been reported, information is available only for the α and β subunits and a portion of the γ

Abbreviations: F_1-ATPase, F_1 sector of the proton-translocating ATPase, F_1F_0.

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subunit, and is informative only for static relationships among the interacting residues. Genetic and biochemical approaches are still important in order to gain a complete understanding of the subunit interactions.

Chemical cross-linkage data for the F_1F_0 subunits have been reported [12–14], but these data were too complicated to allow analysis of the interactions between two subunits, and caution is required in interpretation of the results because chemical modifications of amino acid residues including cross-linkage often cause artificial distortion in the target subunits. In vitro analyses of two-subunit interactions have also been reported [15], but the procedures required for such studies are time-consuming and binding conditions are not necessarily the same as those encountered physiologically. The yeast two-hybrid system for detecting protein-protein interaction developed by Fields and Song [16] is widely applicable for analysis of biologically important protein interactions [17]. This system may be useful for basic analysis of subunit interactions between two subunits of the F_1-ATPase because the interactions are easily detected under physiological conditions and, furthermore, this system is amenable to genetic analysis. In the present study, we investigated two-subunit interactions among the F_1-ATPase subunits α, β, γ, δ and ϵ.

2. Materials and methods

2.1. E. coli and yeast strains, and culture media

E. coli DK8 (*ΔuncB-C, ilv::Tn10*) [18], JP17 (*ΔuncD, argH, pyrH, entA, recA::Tn10*) [19], KM230 (*thy, thi, asn*) [20], JM103 [21] and yeast SFY526 (*MAT*a, *gal*4-542, *gal*80-538, *URA*3::*GAL*1-*lacZ* *can*^{r}, *ura*3-52, *his*3-200, *ade*2-101, *lys*2-801, *trp*1-901, *leu*2-3, 112) [16] were used. *E. coli* cells were grown in LB medium or in minimal Tanaka medium [20] at 37°C with vigorous shaking. Yeast cells were grown in YPD medium or minimal SD medium [16].

2.2. Construction of plasmids

The coding sequences for the α, β, γ, δ and ϵ subunit genes were obtained from the previously reported expression vectors derived from pET plasmids [22–25]. The coding sequences in the plasmids were recovered after digestion with *Nco*I, end-filling with Klenow and then cleaving with *Bam*HI. The coding sequences thus generated were inserted into the unique *Sma*I and *Bam*HI sites in pGBT9 carrying the DNA-binding domain or pGAD424 carrying the activation domain, respectively, of the yeast GAL4 gene. The DNA fragments containing the mutation sites of the mutant β subunit genes, βLeu-40 to Pro, and βGlu-41 to Lys cloned on pKM06 [26] were recovered from the plasmids after digestion with *Nar*I and *Sac*I. The DNA fragments were then inserted into the *Nar*I and *Sac*I sites of the β subunit gene in a pGBT9 derivatives carrying the wild-type gene for the β subunit. For the mutant β subunit with Pro-332 to Gln substitution, the *Sty*I-*Bam*HI fragment was recoverd from the β subunit expression plasmid pRM21 and replaced by the corresponding region in pGBT9 with the wild-type β gene.

2.3. Assay of the reporter gene

The expression plasmids were introduced into yeast SFY526 by the lithium acetate method as described previously [16,36–38]. Transformants were selected by cell growth in SD medium with no Leu and/or Trp supplementation. The selected transformants were grown in 5 ml of SD medium and harvested at the logarithmic phase of growth. The harvested cells were suspended in Z buffer (60 mM sodium phosphate, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$) and disrupted by freeze-thawing once. An aliquot of this extract was used for the β-galactosidase assay using *o*-nitrophenyl β-galactoside as the substrate. The specific enzyme activity is presented in Miller units [27]; the optical density at 420 nm of *o*-nitrophenol released by the enzyme was normalized by the cell density measured photometrically at 600 nm.

2.4. ATPase assay and immunological detection of the α and β subunits

E. coli cells grown in Tanaka medium supplemented with glycerol were disrupted using the French press system and the membranes were prepared by centrifugation as described previously [26]. F_1-ATPase activities in the membrane fraction were assayed according to a colorimetric procedure as described previously [26]. Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted onto GVHP filters [23]. The α and β subunits on the filters were reacted with specific monoclonal antibodies and visualized as described previously [23].

2.5. Other procedures

In the plasmids for expression of the fusion proteins, the sequences at junctions of the two genes were confirmed by dideoxy sequencing using [α-^{35}S]dCTP [28]. Proteins were determined according to a published procedure [29]. DNA manipulations such as ligation, preparation of DNA, and agarose gel electrophoresis were performed according to published procedures [30].

2.6. Enzymes and reagents

Restriction endnucleases, T4 DNA ligase and Klenow polymerase were purchased from Toyobo Co., Takara Co., or New England Biolabs. [α-^{35}S]dCTP (37 TBq/μmol) was purchased from New England Nuclear Co. Other

reagents used were of the highest grade commercially available.

3. Results

3.1. Construction of plasmids for the yeast two-hybrid system

The genes encoding the α, β, γ, δ, and ε subunits were cloned previously between *Nco*I and *Bam*HI restriction sites in the expression plasmid pET3d. The plasmids carrying these genes were digested with *Nco*I, and the cohesive ends thus generated were filled in with the Klenow fragment of DNA polymerase I. Then, the digested plasmid DNAs were digested again with *Bam*HI, and the coding sequences of the genes were purified by agarose gel electrophoresis. These DNA fragments were inserted into unique *Sma*I and *Bam*HI sites in plasmids pGAD424 or pGBT9 carrying the activation domain or the DNA-binding domain, respectively, of the yeast GAL4 gene.

3.2. Expression of the reporter gene with various combinations of the α and β fusion genes

Formation in vitro of a stable complex of the α and β subunits of *E. coli* has not previously been reported, although these subunits interact in the F_1 complex as shown by X-ray crystallography [11] and chemical cross-linkage experiments [12]. We first investigated the interaction of these subunits in the two-hybrid system. Expression of the reporter gene, β-galactosidase, was assayed in yeast cells carrying combinations of the derivatives of the two plasmids pGAD424 and pGBT9 containing the α or β subunit genes. The reporter gene was expressed when both subunit genes were present, but not with one of the subunits alone (Table 1). When we changed the fusion partner of the α and β subunit genes, the same results were obtained (Table 1). These results indicated that interaction of both subunits occurred in yeast cells without the γ subunit.

Table 1
Interaction of the α and β fusion proteins in the yeast two-hybrid system

Fusion proteins	β-Galactosidase activity
GAL4 bd-β + GAL4 ad-α	4.4 ± 0.4
GAL4 bd-β + GAL4 ad	0.2
GAL4 bd + GAL4 ad-α	0.1
GAL4 bd-α + GAL4 ad-β	4.2 ± 0.6
GAL4 bd-α + GAL4 ad	0.1
GAL4 bd + GAL4 ad-β	0.1
GAL4 bd + GAL4 ad	0.1

The expression plasmids carrying the genes for the indicated fusion proteins were cotransfected into yeast strain SFY526 [16] by the lithium acetate method [16,36–38]. Transformants were selected by plating onto SD medium lacking Trp and Leu. Transformants which contained both plasmids were grown to the 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 [27]. The activity values are averages of three independent experiments. bd, binding domain of GAL4; ad, activation domain of GAL4.

Table 2
ATPase activities on membranes from the β subunit mutants

Plasmid	ATPase activity (%)
pST03β (wild-type)	100
pST03 (no β)	0.3
L40P	5.0
E41K	0.9
P332Q	1.2

Membranes were prepared from the transformants of β-less mutant JP 17 with the expression vector plasmid of the β subunit (pST03β) [26] carrying the various mutations. F_1-ATPase activity was assayed by a colorimetric procedure as described previously [26]. 4.0 U/mg of the membrane protein was taken as 100%. Amino acid residues are presented as single-letter abbreviations.

3.3. Interaction of mutated β subunits with the wild-type α subunit detected by the two-hybrid system

We then assessed whether mutant β subunits, which cause assembly defects in the F_1 complex on the membranes, interact with the α subunit in this system. Here, we analyzed three mutant β subunits with Leu-40 substituted by Pro, Glu-41 substituted by Lys, or Pro-332 substituted by Gln. As reported previously [26], the former two mutants showed very low ATPase activity and low levels of the α subunit on the membranes (Table 2 and Fig. 1), while the same amounts of α and β subunits as the wild-type were observed in total cell extracts [26]. The β subunit mutant with substitution of Gln for Pro-332 isolated previously (Shin and Kanazawa, manuscript in preparation) also showed low ATPase activity and very low levels of α and β subunits on the membranes (Table 2 and Fig. 1), while amounts of both subunits equivalent to those of the wild-type were observed in total cell extracts (data not shown). This mutant β demonstrated very weak interaction with the α subunit as detected by β-galactosidase expression (Table 3). However, the mutant β with Leu-40 to Pro or Glu-41 to Lys substitution exhibited

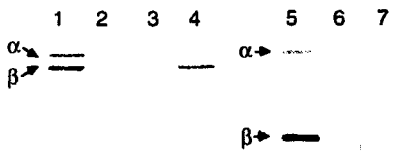

Fig. 1. Immunological detection of the α and β subunits on *E. coli* membranes. Aliquots of 5 μg of membrane proteins were applied to SDS-PAGE and blotted electrophoretically onto GVHP filters. The α and β subunits were probed with specific respective monoclonal antibodies. Lanes 1 and 5, pST03b (wild-type); lanes 2 and 6, pST03; lane 3, pST03b-L40P; lane 4, pST03b-E41K; lane 7, pST03b-P332Q. Amino acid residues are presented as single-letter abbreviations.

Table 3
Interaction of the wild-type or mutant β subunit with the α subunit in the yeast two-hybrid system

Fusion proteins	β-Galactosidase activity
GAL4 bd-β (wild-type)+GAL4 ad-α	4.5±0.4
GAL4 bd-βL40P+GAL4 ad-α	7.9±1.8
GAL4 bd-βE41K+GAL4 ad-α	8.7±1.2
GAL4 bd-βP322Q+GAL4 ad-α	0.1
GAL4 bd+GAL4 ad	0.1

The mutant β subunits were fused with the DNA-binding domain of GAL4. The wild-type or mutant β fusion plasmid and the α fusion plasmid were cotransfected into SFY526. β-Galactosidase activity was determined as described in the footnote to Table 1. The mutant β subunits fused to bd or ad alone gave the same levels of β-galactosidase expression as those for bd or ad alone shown in Table 1.

rather higher expression of β-galactosidase (Table 3), suggesting an unexpected interaction of the mutant β and the wild-type α subunit.

3.4. Interaction of two F_1 subunits

All pairwise combinations of the two subunits from F_1-ATPase were tested in terms of the subunit interaction by the two-hybrid system. Besides α and β, the combination of γ and ϵ yielded strong expression of the reporter gene, indicating that this subunit interaction is stable. A weak but significant interaction was also observed for combinations of the α and δ, β and δ, γ and δ, and δ and ϵ subunits. Interaction of the α and β subunits was detected even when the fusion partners of these subunits were changed (Table 1). However, for the weaker binding combinations, only one partner for fusion showed positive binding (Table 4).

Table 4
Subunit interactions in F_1-ATP synthase detected by the yeast two-hybrid system

Fusion protein	β-Galactosidase activity
GAL4 ad-α +GAL4 bd-β	4.0±0.4
γ	0
γ *	0
δ	1.7±0.3
δ *	0
ϵ	0
ϵ *	0
GAL4 ad-β +GAL4 bd-γ	0
γ *	0.1
δ	1.7±0.2
δ *	0.1
ϵ	0
ϵ *	0.1
GAL4 ad-γ +GAL4 bd-δ	1.8±0.2
δ *	0
ϵ	0
ϵ *	6.9±0.4
GAL4 ad-δ +GAL4 bd-ϵ	0
ϵ *	1.1±0.2

Asterisks indicate reverse combinations of the fusion partner. β-Galactosidase activity was assayed and the activities are expressed as described in the footnote to Table 1. The γ, δ, or ϵ subunit fused to ad or bd alone gave the same levels of β-galactosidase expression as those of ad or bd alone shown in Table 1.

4. Discussion

A previous study by Aris and Simoni [12] using cross-linkage analysis indicated a close association of the α-β, α-γ, α-δ, β-γ, β-δ, γ-δ, and γ-ϵ subunits within the F_1 complex. Other reports have described cross-linkages between the β and ϵ [35] and the α and ϵ subunits [33]. Interaction of the δ and ϵ subunits has also been described by Penin et al. [32] with circular dichroism and intrinsic fluorescence changes. These previous observations suggested that for any combinations of two subunits, subunit interactions occur within the F_1-ATPase complex. However, in vitro stable interactions of pairs of F_1 subunits was reported only for a combination of γ and ϵ subunits [15]. These discrepancies between the results obtained by cross-linkage experiments and in vitro binding assays raise two possibilities: (1) cross-linkage might cause artificial associations for some combinations of the F_1 subunits; (2) in vitro conditions for two-subunit binding are very different from those in vivo and interactions may not have occurred. The experimental conditions for the two-hybrid system are obviously different from those used for cross-linkage and in vitro binding experiments and have advantages in that direct interactions between pairs of F_1 subunits can be observed without any influence of other subunits.

Here, we detected in the two-hybrid system interactions between α and β, γ and ϵ, α and δ, β and δ, and γ and δ, which except for the γ and ϵ interaction were not observed by in vitro binding experiments. However, interactions with combinations of the α and γ, β and γ, α and ϵ, and β and ϵ, subunits which were observed in cross-linkage experiments were not detected in this system. Therefore, the two-subunit interactions observed in this study may provide new insights into the subunit-subunit interactions within the F_1 complex.

We observed different levels of reporter gene expression with various pairs of subunits. Although the expression levels could not be directly equated to the affinities of subunit pairs, we believe that the levels may reflect the affinities in the subunit pairs to some extent based on the following observations and discussion. The expression levels of the reporter gene decrease in the order γ-ϵ, α-β, and α-δ (also β-δ, γ-δ, and δ-ϵ) (Table 4). The highest level of expression was observed with the combination of γ and ϵ subunits. In this case, stable binding was reported in vitro [15], which result we have also confirmed recently using the ϵ subunit fused to glutathione-*S*-transferase [25].

The second highest level of expression was observed for the α-β pair. Since stable binding of the α-β pair in vitro was not observed, the binding stability may be weaker than that for γ-ϵ, which corresponds well with the expression levels of both pairs. For the third group including α-δ (also β-δ, γ-δ, and δ-ϵ) exhibiting expression levels weaker than those of the α-β pair, stable binding in vitro was also not reported for any pairs. Although we could not distinguish the in vitro stabilities of subunit binding between the second (α-β) and third groups, the α-β pair might bind more strongly than the third group pairs (α-δ, β-δ, γ-δ, and δ-ϵ).

Although an in vitro stable complex of the α and β subunits has not been reported for *E. coli* F_1 ATPase, we observed this interaction for the first time in the present two-hybrid system. For thermophilic bacteria, a stable $\alpha_3\beta_3$ complex was reconstituted from the purified subunits [31,39]. The binding affinity between these subunits might thus differ among species. For *E. coli*, addition of the γ subunit to the mixture of the α and β subunits in in vitro reconstitution experiments could restore the active F_1 ATPase activity as a single complex [7]. This observation and the present result suggest that the γ subunit stabilizes the preformed $\alpha\beta$ complex during assembly. It should be noted that relatively weak but significant binding was observed for combinations with the δ subunit. The δ and ϵ subunits were reported to form a connecting portion between the catalytic $c\beta\gamma$ core and F_0 portion [34]. However, the present results suggested that the δ subunit may have functional significance other than this connecting role, because this subunit was found to interact significantly with the catalytic α, β and γ subunits.

Establishment of the two-hybrid system for the F_1 subunits will be useful for further genetic study to identify residues involved in subunit interactions. As a first step, we analyzed three mutant β subunits in which defective assembly of the F_1 portion was observed. Three mutant β subunits with Leu-40 to Pro, Glu-41 to Lys [26], or Pro-332 to Gln substitutions exhibited decreased levels of the α subunit on the membranes, suggesting that the α and β interaction was lost. In fact, no α and β interaction was observed for Pro-332 to Gln mutation in the two-hybrid system, indicating that this system was applicable for detection of protein-protein interactions in mutants with defective F_1-subunits. Surprisingly, the other two mutations exhibited rather increased levels of α-β interaction, suggesting that the α-β interactions with these mutant subunits are stronger than with the wild-type. Leu-40 and Glu-41 are in the amino-terminal domain of the β subunit and located close to the α subunit in the tertiary structure of F_1 according to the findings of X-ray crystallographic analysis [11]. These findings and the phenotype of the β Leu-40 to Pro and Glu-41 to Lys mutants suggested that these residues are involved in the α-β interaction [26]. For the Leu-40 to Pro mutation, both the α and β subunits were lost on the membranes, while both subunits were detected in total cell extracts. Therefore, the α-β complex of the mutant may be present mainly in the cytoplasmic fraction, because the unusually tight binding of these subunits caused a defect in proper assembly with other subunits as the F_1 complex on the membranes. For the Glu-41 to Lys mutation, the mutated β subunits were observed on the membranes, in contrast to the tight binding suggested here. In this case, the other subunits such as the γ subunit may be involved in causing defective assembly. Further biochemical analyses in terms of the binding properties of the β subunits with other F_1-subunits in these mutants are required to clarify the altered assembly of the mutant F_1s.

Interactions of two subunits shown in Table 4 other than α-β were observed for single combinations of fusions but not for the reverse combinations in protein fusion. For example, β-δ interaction was observed between the β fused to the activation domain and δ fused to the binding domain but not for the reverse combination of the fusion partner. At present, the reason for this is not clear but some interactions between the subunits and the fusion-partner may occur which inhibit the subunit interaction. Although in most studies using the two-hybrid system reverse combinations of fusions have not been described, very different levels of reporter gene expression were also found for interaction between Jun and Fos, oncogene products [40].

Since binding of two subunits occurs in the nucleus of yeast cells, the binding condition may not be the same as that in *E. coli* cytoplasm. The F_1 subunits fused to the yeast transcription factor in this study might lead to some unknown steric problems in the nucleus. Therefore, for interpretation of the data, it may be necessary to take into account some limitations of the technique. However, since the mutations of the β subunits defective in the assembly of F_1 portion also caused altered binding properties in the two-hybrid system, this system may be basically reliable for the purpose described in this study.

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