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Intergenic suppression in a β subunit mutant with defective assembly in *Escherichia coli* F_1ATPase

Second-site mutation in the α subunit

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Abstract

Substitution of Leu-40 by Pro in the β subunit (βL40P) of *Escherichia coli* F_1-ATPase caused a decrease in the amount of the α and β subunits on the membranes. A revertant strain, Re50, carrying no suppression mutations in the *uncD* gene encoding the β subunit, was isolated from the βL40P mutant. The *uncA* gene from this revertant was amplified by PCR, and cloned into an expression plasmid. The expression plasmid carrying the *uncA* gene from the revertant was used for genetic suppression assays. The suppression mutation in Re50 was in the α subunit, and it recovered the assembly of the α and β subunits into the F_1F_o complex and the ATPase activity to 50% that of the wild type. In Re50, Leu-111 was substituted by Gln in the α subunit. These results suggest that the regions including Leu-40 in the β subunit and Leu-111 in the α subunit are located close together and interact with each other, either directly or indirectly.

Key words: ATP synthase; F_1F_o; Assembly mutant; Suppression mutation; *E. coli*; β-Subunit

1. Introduction

The proton translocating ATPase F_1F_o of *Escherichia coli* catalyzes the synthesis and hydrolysis of ATP as the key enzyme of biological energy transduction [1,2]. This enzyme consists of peripheral and integral membrane parts. The peripheral part, F_1, has five non-identical subunits, α, β, γ, δ, and ε and has the catalytic activity. The integral part, F_o, has proton channel function. The purified α, β, and γ subunits reconstitute the ATPase activity [3]. The primary structures of all subunits have been determined, and essential and important residues of the subunits for the catalytic function and molecular assembly have been investigated [4,5]. However, the topological arrangement of the subunits within the enzyme complex, which arrangement is important for understanding the molecular mechanisms of this enzyme, has not yet been elucidated.

The β subunit has nucleotide binding activity and is believed to contain the catalytic center [1,2]. Although it is known that the central portion of the subunit between residues 140 to 340 provides the binding site for nucleotide [5], the function of other portions, such as the amino and carboxyl terminal regions, has not been established. We have shown that a βL40P mutation caused defective molecular assembly of the α and β subunits on the membrane, suggesting that the region around the residues is important for the integration of the α and β subunits into the F_1F_o complex [6]. Here we provide evidence that a second-site mutation at Leu-111 in the α subunit could suppress the defective molecular assembly caused by the original mutation of βL40P, suggesting that both regions including, αL111 or βL40, are important for the interaction between the α and β subunits.

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Abbreviations: F_1F_o, proton translocating ATPase for oxidative phosphorylation, PCR, polymerase chain reaction; bp, base pair(s). Codons are numbered starting from the second codon of the gene coding for the β subunit, as amino terminal methionine is missing in the isolated β subunit [18]. Residues and mutations are referred to as follows: βG149 represents normal F_1 β subunit residue Gly-149. βG149S represents a substitution of Ser at residue 149 of the β subunit instead of Gly. The residue numbers used in this paper refer to the *E. coli* enzyme.

2. Materials and methods

2.1. Bacterial strains and growth conditions

An *E. coli* mutant strain, JP17 (*ΔuncD, argH, pyrE, entA, recA::Tn10*), carrying the deletion mutation of the *uncD* gene encoding the β subunit [7], donated by A.E. Senior, University of Rochester School of Medicine and Dentistry, was used. Minimal medium [8] containing required supplements plus a carbon source (0.5% succinate or 0.2% glucose), and rich medium (L-broth) [8] were used for genetic assays and biochemical analyses. For membrane preparation, cells were

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grown in minimal medium with 0.5% glycerol. Transformants by plasmids were selected by resistance to ampicillin (50 μg/ml) or chloramphenicol (50 μg/ml).

2.2. Expression plasmids for the α and β subunits

In this study, we constructed the α subunit expression plasmid pRM17 (Fig. 1A), using pBR322 [9] as a vector . The restriction sites of *Eco*RI, *Cla*I, and *Hin*dIII on pBR322 were modified by digestion with the appropriate restriction enzymes and by subsequent endfilling, using the Klenow fragment of DNA polymerase I. This plasmid carries DNA fragments containing the promoter region of the *unc* operon and *uncA* gene encoding the entire α subunit as *Hae*II–*Hin*dIII and *Hin*dIII–*Bam*HI fragments, respectively. The *Hin*dIII–*Bam*HI fragment carrying the *uncA* gene was prepared as follows: the entire coding region of the α subunit was amplified with PCR [20] and digested with *Eco*RV and *Bam*HI. These sites were then introduced into the 5′- and 3′- end of the α subunit gene by PCR with the respective synthetic primers. The synthetic primers, AEF-N10 (5′-TACTGATATCTAGACGTCTTGCAGTCTT-3′) and AER-C2 (5′-GCCTGGATCCTCAATGCCTTGCGG-3′), are carrying the recognition sequences of *Eco*RV and *Bam*HI flanked by the 5′- ends of the primers, respectively. The *Eco*RV–*Bam*HI fragment carrying the entire *uncA* gene was introduced between the *Hin*cII and *Bam*HI sites of pUC19 [10]. The *Hin*dIII–*Bam*HI fragment carrying the *uncA* gene was recovered from this recombinant plasmid.

The β subunit expression plasmid pRM25 (Fig. 1B) was constructed using pACYC184 [11] as a vector. This plasmid carries DNA fragments containing the same promoter region as the pRM17 and *uncD* gene encoding the entire β subunit in the *Hae*II–*Bam*HI region. The *Hin*dIII–*Bam*HI fragment carrying the entire *uncD* gene was recovered from another recombinant plasmid pST03β [6], and introduced into this plasmid. The *Hin*dIII–*Bam*HI fragment carrying the βL40P mutation, recovered from a plasmid, 31–134, which had been isolated previously [6], was introduced into the pRM25. The resulting plasmid was named pRM25-βL40P. The *Cla*I–*Hae*II fragment in this plasmid was obtained from the modified pBR322 in which the *Eco*RI and *Hin*dIII sites were modified by digestion and subsequent endfilling, as described above.

Treatment with restriction endonucleases and DNA ligase, and the isolation and cloning of the DNA fragments into vectors were performed as described previously [12].

2.3. Isolation of the revertants and suppression assay of cloned uncA *gene*

Spontaneous revertants were selected as colonies capable of growth on the minimal medium agar supplemented with succinate from JP17 carrying the pST03β-L40P, which is the expression plasmid of the β subunit with the βL40P mutation [6]. The plasmids were recovered from these revertants, and introduced again into JP17 to test whether the plasmids carried reversion mutations. Total DNA, which was prepared by procedures already described [19] from the revertant that did not carry the reversion mutations on the plasmid, was used for the PCR template. The DNA fragments encoding the α subunit were amplified by PCR with oligonucleotides AEF-N10 and AER-C2. The amplified DNA was digested with *Eco*RI and *Bam*HI (Fig. 1A). The fragments were ligated with pRM17 digested with *Eco*RI and *Bam*HI, and resultant plasmids were introduced into the JP17 carrying pRM25-βL40P. We tested whether the transformants could grow on succinate to assess the suppression activity of the cloned *uncA* gene.

2.4. Sequencing

The nucleotide sequences in which suppression mutations were mapped (*Xho*I–*Cla*I) (Fig. 1A) were determined using the isolated plasmids as the template for the dideoxinucleotide chain-termination reaction [13]. [α-^{35}S]dCTP (37 TBq/μmol) was used for labeling.

2.5. Other methods

Cells harvested in the late logarithmic phase of growth were passed through a French Pressure Cell and the membranes were isolated as described previously [8]. The ATPase activity and protein concentration were assayed, using reported procedures [14,15]. The α and β subunits on the membranes were detected immunologically by Western blotting analysis, as described previously [16].

2.6. Reagents and chemicals

We purchased the restriction endonucleases, the Klenow fragment of DNA polymerase I, and T4 DNA ligase from Life Technologies, Inc., and *Tth* DNA polymerase from Toyobo Co., Tokyo, Japan. [α-^{35}S]dCTP was purchased from DuPont/NEN Research Products. Other materials were of the highest grade commercially available.

3. Results

3.1. Isolation of the intergenic suppression mutants from the βL40P mutant and mapping the mutations

Revertants were isolated as clones, capable of growing on the agar containing succinate as the sole carbon source, from a defective mutant with the βL40P substitution. As the parent mutant cells, we used transformants of the β-less mutant JP17 with a plasmid, pST03β-L40P, that expressed the defective β subunit gene with the βL40P mutation. One hundred and twenty six independent clones were isolated as the revertants from 1.2×10^{11} cells of the transformants. The plasmids isolated from 13 revertants were introduced again into JP17. The complementation test showed that a plasmid derived from revertant strain, RE50, did not exhibit the revertant phenotype, while the other plasmids did. These results suggested that the suppression mutations occurred in genes other than the β subunit gene in RE50. Reversion mutations in the other revertants were found in the β subunit gene, and their altered nucleotide sequences were reported previously [6].

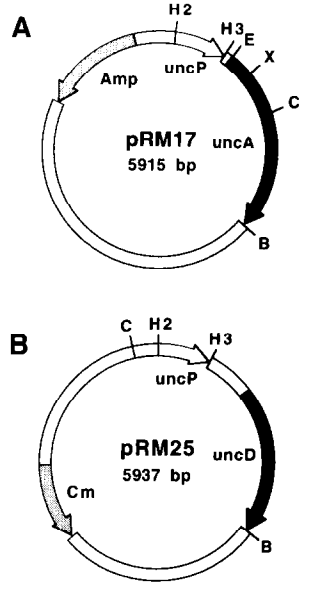

Fig. 1. Expression plasmids of the *uncA* and *uncD* gene, encoding *E. coli* α and β subunits, respectively. (A) The 470-bp (*Hae*III–*Hin*dIII) fragment carrying the promoter region (uncP) of the *unc* operon and the 1,638-bp (*Hin*dIII–*Bam*HI) fragment carrying the *uncA* gene were cloned into modified pBR322 as described in section 2. (B) The same promoter region of pRM17 and the *Hin*dIII–*Bam*HI fragment (1,785-bp) carrying the *uncD* gene were cloned into pACYC184 as described in section 2. The *Cla*I–*Hae*II region was obtained from the modified pBR322, in which the *Eco*RI and *Hin*dIII sites were modified. Restriction sites are abbreviated as follows: H2, *Hae*II; H3, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; C, *Cla*I; X, *Xho*I. Regions including the ampicillin-resistant gene (Amp) and chloramphenicol-resistant gene (Cm) are shown.

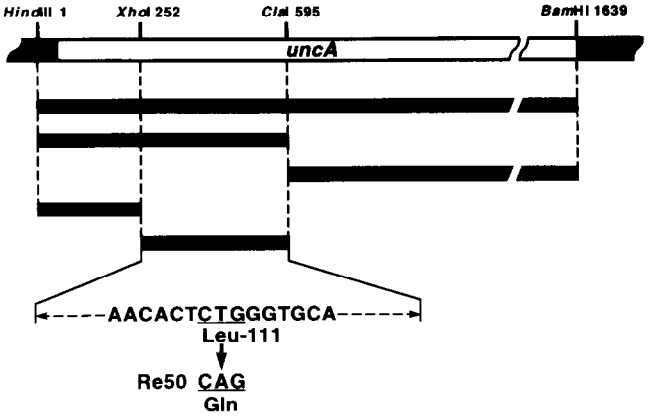

Fig. 2. Mapping and DNA sequencing of the suppression mutation in the *uncA* gene from RE50. DNA segments carried by recombinant plasmids are shown with a base substitution in the revertant strain RE50 identified from the DNA sequence. (A) Recognition sites of restriction enzymes used in this study. Nucleotides are numbered starting from the *Hin*dIII site. (B) The DNA fragments carried by recombinant plasmids used for the mapping of the suppression mutation. (C) Base substitution and amino acid replacement in RE50 determined from the DNA sequence.

3.2. Fine mapping and determination of the altered sequence

We speculated that the suppression mutation may have occurred in the α subunit. To test this possibility, we cloned the α subunit gene from the revertant and tested the suppression activity of the gene in the parent mutant with the defective β subunit. The DNA fragment encoding the α subunit was amplified using the PCR, and the *Eco*RI–*Bam*HI fragment was substituted for the corresponding region in the pRM17 (Fig. 1A), which is the expression plasmid for the α subunit gene. The DNA fragment from the revertant DNA lacked the sequence coding the first 3 residues of the α subunit from the amino terminal. The constructed plasmid was then introduced to JP17 carrying the pRM25-βL40P. The plasmid derived from RE50 suppressed phenotype of the JP17 carrying pRM25-βL40P, which did not grow on the succinate plate, indicating that the suppression mutations were in the α subunit gene. The pRM17 carrying the wild type α subunit gene did not show the suppression activity. The DNA encoding the α subunit of RE50 in the plasmid was divided into two portions, between the *Hin*dIII and *Cla*I sites (594 bp) and between the*Cla*I and *Bam*HI sites (1,044 bp) (Fig. 1A), and each fragment was introduced into pRM17. Suppression activity in the JP17 carrying pRM25-βL40P was observed within the DNA fragment between *Hin*dIII–*Cla*I encoding the amino terminal 179 residues. Within this region, the suppression activity was located in the DNA fragment between *Xho*I and *Cla*I (343 bp), using the same procedures described above. The entire sequence of this fragment derived from RE50 was determined. For RE50, substitution of single base thymine by adenine was observed at the Leu-111 codon, causing a change from Leu to Gln (Fig. 2).

3.3. Characterization of the revertants

The parent mutant with the βL40P mutation exhibited 4% of the wild type F_1F_0 ATPase activity on the membranes and decreased amounts of the α and β subunits on the membranes [6]. RE 50 had 42% of the wild type activity (Table 1) and recovered the α and β subunits on the membranes to the wild type level (Fig. 3). Thus, it was concluded that the assembly defect caused by the βL40P mutation could be recovered extensively by the substitution of Leu-111 by Gln in the α subunits.

4. Discussion

We have previously shown that βL40 and βE41 are involved in the epitope residues for the monoclonal antibody β31 [6]. The binding of these antibodies to the β subunit was lost after assembly of the F_1-ATPase complex, suggesting that the region around residues 40 and 41 is exposed to the surface of the β subunit and concealed in the F_1-ATPase complex [16]. Mutations of β L40P and βE41K caused a defect in the molecular assembly of the α and β subunits on the membranes. Although these residues are located side by side, we found that the phenotypes of the defective assembly of the α and β subunits were different. The βL40P mutation caused a decrease in both the α and β subunits, while the E41K mutation decreased the α subunit only, with a normal amount of the β subunit. It has been demonstrated that the wild type β subunit binds to the membranes without the α subunit [22]. We observed that the α subunit did not bind to the membranes without the β subunit in a β-less mutant, JP17 [6]. These findings suggested that integration of the α and β subunits into the F_1F_0 complex requires two steps: (i) the binding of the β subunit to the F_0 portion, possibly with some F_1 subunits such as the δ subunit, and (ii) the subsequent binding of the α subunit to the β subunit. The βL40P mutation seems to affect mainly the binding of the β subunit to the F_0 portion, and the E41K mutation alters the binding of the β subunit to the α subunit. We have no direct evidence to show whether or not the βL40P mutation affects the

Table 1
ATPase activity on the membranes of RE50, isolated from the βL40P mutant

Plasmid	ATPase activity (units/mg)
pST03β (Wild)	4.3 (100)
pST03β-L40P	0.2 (4)
pRE50	1.8 (42)

Membrane vesicles were prepared as described previously [20]. ATPase activity was assayed in the presence of Mg^{2+} ions, as previously reported [27]. Figured in parentheses indicate relative activity.

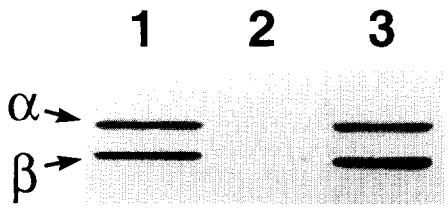

Fig. 3. Western blot analysis of the α and β subunits on the membranes with the α subunit carrying the reversion mutation. Proteins (2.5 μg) in the cytoplasmic membranes were resolved by 12.5% SDS-polyacrylamide gel electrophoresis. The proteins were blotted electrophoretically onto GVHP membranes, and the α and β subunits were probed with the monoclonal antibodies, α108 and β208 (manuscript in preparation), respectively. The monoclonal antibody β208 recognizes a region different from that recognized by β12 and β31. Lanes: 1, JP17 carrying pST03β (wild type); 2, JP17 carrying pST03β-L40P; 3, RE50.

binding of the β subunit to the α subunit. However, since these two residues are located side by side, it is conceivable that the βL40P may cause some alteration in this binding.

In this study, we have shown that the second site mutation at Leu-111 in the α subunit suppressed the defect of the β subunit carrying the βL40P mutation. This finding suggested that βL40P affected the interaction between the β and α subunits. Therefore, it is possible that βL40 may be involved in the α–β interaction, either directly or indirectly. It is difficult to assess the contribution of βL40P in the interaction of the β subunit to the F_0 portion and to the α subunit. The α subunit carrying the αL111Q mutation may bind to the β subunit, leading to the restoration of an essential conformation in the binding of the β subunit with the βL40P mutation to the F_0. The αL111Q mutation itself, or the region containing this suppression mutation, could be involved in the binding domain of the α subunit to the β subunit. It should be noted that the regions containing βL40 and βE41, and αL111 have similar sequences to those from *Bacillus* strain PS3 and that those regions of PS3 are looped out to the surface of the β and α subunits, respectively [23,24].

The membrane vesicles from JP17 carrying pRM25-βL40P had 20% of the wild type α and β subunits, and the revertant carrying the αL111Q mutation in its chromosomal DNA had the same amounts of these subunits as the wild type. However, they had only 4% and 42% of the wild-type ATPase activity, respectively, lower than would be expected from the amounts of α and β subunits on the membranes. These results suggest that an interaction between the α and β subunits altered by the βL40P mutation caused the defect in the catalytic mechanisms; this finding also supports the notion that βL40 is involved in the α–β interaction.

The region between residues 345 and 375 in the α subunit may be involved in the signal transmission between the α and β subunits required for steady-state catalysis [5]. As shown by the three-dimensional arrangement of the α and β subunits in the F_1-ATPase complex obtained on X-ray crystallographic analysis [17], these subunits have three pairs in one complex and they occupy the alternating positions. Therefore, there are at least two different sites in each β subunit that interact with the next α subunit. The αL111 may be involved in one of these interacting sites, with or without the region between residues 345 and 375.

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