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Residues interacting with serine-174 and alanine-295 in the β-subunit of *Escherichia coli* H^+-ATP synthase: possible ternary structure of the center region of the subunit

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Abstract

The mutation of serine-174 to phenylalanine that causes a defect in the *Escherichia coli* F_1-ATPase β-subunit is suppressed by further mutations; Gly-149 to Ser, Ala-295 to Thr, Ala-295 to Pro, or Leu-400 to Gln (Miki, J., Fujiwara, K., Tsuda, M., Tsuchiya, T. and Kanazawa, H. (1990) J. Biol. Chem. 265, 21567–21572). We analyzed the effects of these second site mutations and of a newly identified Asn-158 to Tyr mutation on the activities of the ATPase without the original Ser-174 to Phe mutation. The β-subunit with each amino acid replacement was expressed in the mutant strain JP17, which does not have a β-subunit. Cells transformed with the plasmid carrying Ala-295 to Pro mutation alone did not grow on minimal medium agar supplemented with succinate as the sole carbon source, and showed 3% of the wild-type ATPase activity, suggesting that this mutation caused structural alterations affecting the catalytic function of the enzyme. Conversely transformants with other mutations grew well and had higher ATPase activities, suggesting that these mutations did not cause extensive structural alterations. From the transformants with the plasmid carrying the Ala-295 to Pro mutation, seven revertants capable of cell growth on succinate plates were isolated and reversion mutations were identified at residues 140, 159, 166, 171, 172 and 184 of the β-subunits. The results suggested that Ser-174 and Ala-295 do not necessarily interact directly, but that the regions including these suppression mutation sites close to Ser-174, and Ala-295 interact with each other for the proper functioning of the ATPase. The ternary structure of the region surrounded by the residues which were identified as the reversion mutation sites for Ser-174 to Phe and Ala-295 to Pro mutations is important for the catalytic function of this enzyme.

Key words: ATP synthase, F_1F_0-; Beta subunit; Suppression mutation

1. Introduction

The proton translocating F_1F_0 ATPase has a central role in oxidative phosphorylation and photophosphorylation (for review, see Refs. [1–4]). The catalytic portion, F_1, has five different subunits, α, β, γ, δ, and ε in *Escherichia coli*. The α-, β-, and γ-subunits form the catalytic complex, and the β-subunit or the interface of the α- and β-subunits contains the active center of the ATPase. The entire amino acid sequences of the subunits of the F_1F_0 ATPase are known, and the role of each subunit has been described in detail [1,4].

Residues in the β-subunit important for the catalytic functions of the enzyme have been studied by random and site-directed mutagenesis, and by chemical modifications [1,4]. The region between residues 140 and 340 may form the catalytic domain, since the functionally important or essential residues are located in this region [5]. The glycine-rich flexible loop sequence between residues 149 and 156, which is the consensus sequence of nucleotide binding proteins, lies in this region [6,7]. Since the three-dimensional struc-

Abbreviations: F_1F_0, 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 [6]. 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.

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ture of the *E. coli* β-subunit has not yet been described, it is not known how the region is folded. We have approached this issue using pseudo-reversion mutations of the mutant strain KF11 defective in the β-subunit. Serine-174 of this mutant subunit is substituted by phenylalanine, and its ATPase activities are about 10% those of the wild-type enzyme [8,9]. Pseudo-reversion mutants were isolated from this mutant and second mutations were identified at residues 149, 295, and 400 [10]. The reversion mutations were also identified at residues 158 and 167, as shown in the present report. Accordingly, it was proposed that these residues interact with the original mutation site at residue 174 near the catalytic site, and that a second mutation could recover the conformation important for the catalytic activity.

In the present study, we investigated whether the second mutations by themselves cause a defect in function of the β-subunit. This information will provide insight into the function of the residues near the catalytic site. We also identified pseudo-reversion mutations against the mutation of alanine to proline at residue 295 which causes defective catalysis. The functional interaction between the regions containing residues 174 and 295 is discussed.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli wild-type strain KY7230 (*thy*, *thi*, *asn*), β-subunit-defective mutant KF11 (*uncD11*, *thy*, *thi*) [8], and β-less mutant JP17 (*ΔuncD*, *argH*, *pyrE*, *entA*, *recA::Tn10*) [11] were used. JP17 was kindly donated by Dr. A.E. Senior, University of Rochester School of Medicine and Dentistry. Minimal medium [12] containing required supplements plus a carbon source (0.5% succinate or 0.2% glucose), and rich medium (L-broth) [12] were used. For preparation of membranes, cells were grown in minimal medium with 0.5% glycerol. Plasmid transformants were selected by resistance for the antibiotics ampicillin (50 μg/ml) or tetracycline (20 μg/ml).

2.2. Isolation of revertants from KF11 and from a mutant with substitution of Ala-295 by Pro in the β-subunit

Revertants (RE101, 102, 104, 105, 107, and 108) from KF11 capable of growth on the selecting agar plates containing minimal medium including succinate as a sole carbon source were isolated as described previously [10]. Revertants from the mutant with the βA295P substitution in the β-subunit were isolated as follows: 1 μg of total DNA, prepared by the published procedures [13] from wild-type KY7230, was used as a polymerase chain reaction (PCR) template. DNAs of the entire region of the β-subunit gene were amplified by PCR with primers, β1 and β2 (Fig. 1), using the procedure originally described by Saiki et al. [14]. In the reaction, 0.4 μM of dCTP (lower than the standard concentration of 0.2 mM) was used with 0.2 mM dATP, dGTP, and dTTP to cause misreading by *Taq* polymerase. The reaction mixture was incubated for 5 cycles (1 min at 94°C, 2 min at 45°C, and 2 min at 72°C) and for a further 25 cycles after increasing the concentration of dCTP to 0.2 mM. The amplified DNAs were then used as the template in a second PCR using *VENT* polymerase with 0.2 mM substrates and 50 pmoles of the primers BEF-N3 and BR6 (Fig. 1). The

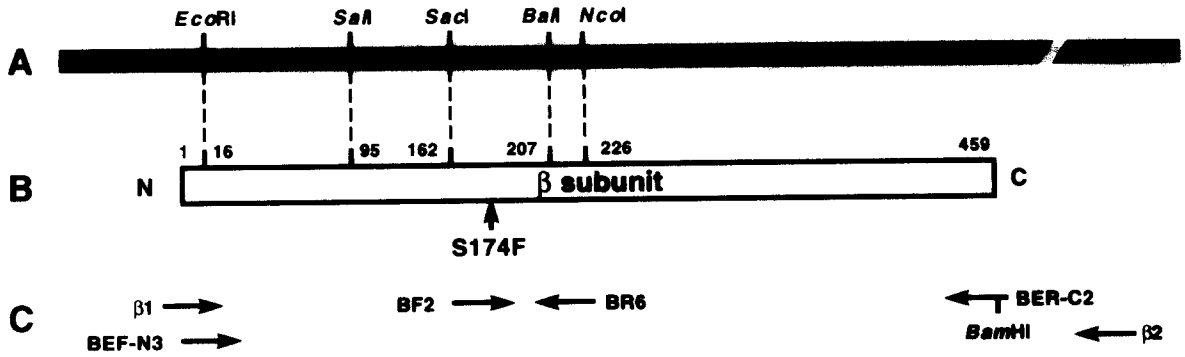

Fig. 1. Restriction map and primers used to amplify the *E. coli uncD* gene. (A) Recognition sites of restriction enzymes used in the present study. (B) Location of restriction sites on the reading frame of the β-subunit. As the amino terminal Met is missing from the isolated β-subunit, amino acid residues are numbered from the second codon. An arrow indicates the position of a mutation of Ser for Phe at residue 174 in KF11. (C) The locations of the oligonucleotides are indicated. The sequences of oligonucleotides, β1 (5′-ACAGGTTATTTCGTAGAGGATTT-3′), BEF-N3 (5′-TACTCCATGGCTACTGGAAAGATTG-3′), and BF2 (5′-TGCGGGTGTAGGTAAAACCG-3′), and BR6 (5′-CGTCACGGAATTT CTCAGCC-3′), and BER-C2 (5′-TACTGGATCCGATTAAGGCGTT AAAG-3′), correspond to that of the antisense and sense strands, respectively. BER-C2 has a recognition sequence for *Bam*HI at the 5′ terminus. The sequence of β2 (5′-TAACACCGGCTTGAAAAGC-3′) corresponds to that of the antisense strand downstream from the *uncC* gene coding for the ε-subunit.

amplified DNAs were digested with the restriction endonucleases *Eco*RI and *Nco*I, for which there are unique sites in the β-subunit gene (Fig. 1), and the fragments were ligated into the expression plasmid pST03β-A295P of the β-subunit carrying the βA295P mutation. The β-less strain JP17 was transformed with the ligated DNAs, and viable colonies on minimal agar with succinate were selected as revertants.

2.3. Construction of the expression plasmids of the β-subunit carrying the point mutation from KF11 revertants

Construction of the expression plasmid pST03β of the β-subunit gene was described previously [15]. For RE17 (βG149S) and RE102 (βN158Y), the DNA segments between *Eco*RI and *Sac*I sites (Fig. 1) were prepared by digesting the DNA amplified by PCR from total RE17 and RE102 DNA using oligonucleotide β1 and BR6 as primers (Fig. 1). These were replaced with the wild-type sequence in pST03β. For RE10 (βA295T), RE20 (βA295P), and RE18 (βL400Q), the DNA segments between *Nco*I and *Bam*HI (Fig. 1) were integrated into pST03β after amplification of the DNA from total DNA of each strain by PCR using oligonucleotides BF2 and BER-C2. The *Bam*HI site was created by PCR using BER-C2 containing an additional *Bam*HI recognition sequence. Thus, the point mutation βS174F was omitted from these expression plasmids. Treatment with restriction endonucleases and DNA ligase, and the isolation and cloning of the DNA fragments into vectors were performed as described previously [16].

2.4. Genetic mapping of the reversion mutations

Genetic mapping of the newly identified reversion mutants RE102 and 108 was performed as described previously [10]. For reversion mutations of the βA295P mutation, the DNA segments between *Sac*I and *Nco*I sites were purified from the recombinant plasmids isolated from the revertant strains, with the exception of RV8 from which the *Sal*I-*Nco*I fragment was prepared, since the plasmid DNA could not be digested with *Sac*I. These fragments were substituted with corresponding regions of the β-subunit gene on the pST03β-A295P. Cells transformed with these ligated DNAs were analyzed in terms of growth on minimal agar plates containing succinate as the sole carbon source.

2.5. Sequencing

Single-stranded DNAs corresponding to the region encoding the β-subunit were amplified from the chromosomal DNAs of RE101, 102, 104, 105, and 107 by asymmetric PCR with β1 and β2 as primers [10]. Amplified single-stranded DNAs were used as templates for dideoxynucleotide chain-termination sequencing reactions [17] with ^{32}P-labeled primers as described previously [10]. [α-^{35}S]dCTP was used for labeling in the reaction with plasmid DNA as a template.

2.6. Preparation of membranes and the ATPase assay

Cells harvested in the late-logarithmic phase of growth were passed through a French Pressure Cell, and membranes were isolated as previously described [12]. ATPase activity and protein concentration were assayed by the reported procedures [18,19].

2.7. Materials

Restriction endonucleases, T4 polynucleotide kinase, and T4 DNA ligase were purchased from Life Technologies (USA). *Tth* DNA polymerase was purchased from Toyobo (Japan). *VENT* DNA polymerase was purchased from New England Biolabs (USA). [γ-^{32}P]ATP (259 TBq/μmol) and [α-^{35}S]dCTP (37 TBq/μmol) were purchased from ICN (USA) and DuPont/NEN Research Products (USA), respectively. Other materials were of the highest grade commercially available.

3. Results

3.1. Effects of substitutions at residues neighboring βS174 in the β-subunit

We have previously reported four reversion mutations of the βS174F mutation [10]. More revertants were isolated from the same original mutant in the present study, and their suppression mutations were determined. The revertant strains RE104 and 107 showed a suppression mutation at codon 295 with replacement of alanine by threonine, which was also found in the revertant strain RE10 [10]. RE101 and RE105 showed replacement of Gly-149 by serine which was also described for RE17 [10]. However, RE102 and 108 had novel second mutations at codon 158 with replacement of asparagine (AAC) by tyrosine (TAC), and at codon 167 with replacement of alanine (GCG) by threonine (ACG), respectively.

The suppression mutations identified in the revertants RE10 (βA295T), 17 (βG149S), 18 (βL400Q), 20 (βA295P), and 102 (βN158Y) were analyzed in terms of their own effects without the original mutation upon cell growth and ATPase activity. The entire sequence of the β-subunit gene from each revertant was amplified and then a portion of the gene including the

suppression mutation alone was integrated into the β-subunit expression plasmid pST03β after digestion by the appropriate restriction endonucleases. Thus, the second mutation alone was present in these plasmids. With RE108 (βA167T), since suitable restriction sites to separate codon 174 from codon 167 were not available, further analysis was not performed. Expression plasmids carrying the second mutations (pST03β-G149S, N158Y, A295T, A295P, L400Q) were introduced into strain JP17, which does not carry the β-subunit gene. The growth rate of cells transformed with pST03β-A295P was 27% of the wild-type carrying the pST03β in JP17, but other mutants showed significantly higher rates of growth (Table 1). The ATPase activities in the membranes of transformants carrying βN158Y, βA295T, and βL400Q mutations (Table 1) were close to those from wild-type cells. For the βG149S mutation, the ATPase activity decreased to 71% of the wild-type, corresponding to the lower growth rate of the transformant cells. The βA295P mutation resulted in defective ATPase activity to a level equivalent to that of KF43 [20], another defective β-subunit mutant.

3.2. Isolation and characterization of revertants from βA295P mutant

We isolated revertants of βA295P mutation using PCR-induced mutagenesis. The mutagenized DNAs were inserted into pST03β-A295P using the restriction endonucleases *Eco*RI and *Nco*I (Fig. 1). Constructs were introduced into JP17, and viable clones on minimal agar supplemented with succinate as the sole carbon source were isolated. The transformed JP17 cells with pST03β-A295P did not form colonies on the plates, although this transformant showed a growth rate of 27% of the wild-type in liquid medium (Table 1). The plasmids isolated from these clones were re-introduced into JP17, and the transformants were then tested for their ability to grow on succinate plates. Seven plasmids which conferred positive growth on JP17 tranformants on succinate plates were isolated, and named pRV1, 2, 4, 5, 8, 9, and 10.

Table 1
Effects of suppression mutations of the βS174F mutation on ATPase activity and growth dependent on oxidative phosphorylation

Plasmid	Growth rate [a] (relative)	ATPase activity [b] (relative)
pST03β (wild-type)	100	100
pBR322	0	0.3
pRE17 (βG149S)	67	71
pRE102 (βN158Y)	72	115
pRE10 (βA295T)	69	91
pRE20 (βA295P)	27	2.9
pRE18 (βL400Q)	73	118

[a] An aliquot (0.2 ml) of each strain grown in L-broth was transferred to synthetic medium (15 ml) containing succinate as the sole carbon source. Growth of the cells was monitored by measurement of the optical density at 650 nm, and growth rates defined as a reciprocal number of doubling time of cells were calculated from the logarithmic phase of growth. The wild-type value was 0.935/h.
[b] Membrane vesicles were prepared as described previously [12]. ATPase activity was assayed in the presence of Mg^{2+} as previously reported [18]. 100% ATPase activity was 3.28 units/mg protein.

Table 2
Growth and ATPase activities of revertant strains isolated from the βA295P mutant

Strain	Growth rate (relative)	ATPase activity (relative)
JP17/pST03β (wild-type)	100	100
JP17/pRE20 (βA295P)	24	3.2
RV1 (JP17/pRV1)	41	9.9
RV2 (JP17/pRV2)	48	14
RV4 (JP17/pRV4)	60	13
RV5 (JP17/pRV5)	51	17
RV8 (JP17/pRV8)	38	2.1
RV9 (JP17/pRV9)	59	18
RV10 (JP17/pRV10)	39	28

Growth rate dependent on oxidative phosphorylation and membrane ATPase activities were measured as described in Materials and methods and in Table 1.

Derivative strains of JP17 with these plasmids, named RV1, 2, 4, 5, 8, 9, and 10, were analyzed in terms of their growth rates dependent on succinate and membrane ATPase activities. Growth rates of the revertants in minimal medium containing succinate as the sole carbon source increased up to 40–60% of the wild-type, although the growth rate of the βA295P mutant was 24% of the wild-type (Table 2). Thus, it was suggested that ATP synthesis was recovered in these revertants to some extent. The membranes from the revertants had lower ATPase activities than the wild-type, although they were higher than that of the original βA295P mutant, except for RV8 (Table 2). The ATPase activities of the membranes from the RV8 were similar to that of the βA295P mutant. The restoration of the growth rates and the membrane ATPase activities were not necessarily coincident with each other.

3.3. Characterization of the reversion mutations

To localize the second mutation sites, we recovered a DNA fragment between *Sac*I and *Nco*I sites (Fig. 1) from the isolated plasmid, and introduced it into pST03β-A295P. DNA from plasmid pRV8 could not be digested by *Sac*I, suggesting that the enzyme recognition site in this plasmid is mutated. The *Sal*I-*Nco*I DNA fragment (Fig. 1) from pRV8 was then introduced into pST03β-A295P. The resultant construct for each revertant was introduced into JP17, and transformants were tested for their ability to grow on succinate

Table 3
Suppression mutations of βA295P

Plasmid	Suppression mutation
pRV1	Gly-172 (GGT) → Asp (GAT)
pRV2	Ala-140 (GCT) → Thr (ACT)
pRV4	Ile-166 (ATC) → Thr (ACC)
pRV5	Ile-166 (ATC) → Thr (ACC) Ala-177 (GCG) → Val (GTG)
pRV8	Arγ-184 (CGT) → Cys (TGT)
pRV9	Met-159 (ATG) → Thr (ACG)
pRV10	Ser-171 (TCC) → Pro (CCC)

The nucleotide sequences between *Sac*I and *Nco*I sites were determined as described in Materials and methods. For RV8, the sequence between *Sal*I and *Nco*I was determined. Replaced codons are shown in parentheses.

plates. All transformants were capable of growth on succinate plates, indicating that the reversion mutations of all revertants except for RV8 occurred within the region between *Sac*I and *Nco*I, and that of RV8 was in the region between *Sal*I and *Nco*I.

The entire nucleotide sequences of the mapped regions were determined. Single point mutations at residues 140, 159, 166, 171, and 172 were found in RV2, 9, 4, 10, and 1, respectively (Table 3). Double mutations at residues 166 and 177 were found in RV5, and the mutation at residue 166 was identical to that in RV4. A silent mutation at residue 161 in the recognition sequence for *Sac*I was found together with a mutation at residue 184 in RV8. The presence of the mutation at residue 161 is consistent with the inability of *Sac*I to digest RV8.

4. Discussion

The ternary structure of the β-subunit is important for understanding the molecular mechanisms of F_1-ATPase because this subunit is believed to contain the catalytic center of the enzyme [1–4]. Since the ternary structure of the β-subunit is not known, other approaches are required to determine the interaction of residues necessary for the proper functioning of the ATPase. We showed possible ternary interactions of βS174 in the *E. coli* β-subunit with residues 149, 295, and 400 in our previous report [10], and with residues 158 and 167 in the present study by analyzing revertants from a mutant carrying the βS174F mutation. Our findings suggested that residues 149, 158, 167, 174, 295, and 400 are located close together and form a ternary structure important for catalysis. The suppression may occur by interactions of the side chains of these amino acid residues and βF174. We noted that reversion mutations seem to compensate for the original mutation of Ser to Phe in terms of hydrophilicity, and that the environment of a ternary structure surrounded by Ser-174 and the reversion sites important for catalysis would be hydrophilic [10]. Two novel mutations identified here, βN158Y and A167T, also have a hydroxyl group, supporting this observation.

To determine whether the second mutations by themselves caused defects in the function of the β-subunit, each second mutation, βG149S, βN158Y, βA295T, βA295P, or βL400Q, was introduced into pST03β, and then these mutant subunits were expressed in the β-less mutant JP17. The mutations βN158Y and L400Q alone did not cause significant defects in ATPase activities, suggesting that these mutations suppressed the defect caused by the S174F mutation without significant structural alteration. The results also suggested that these residues are not essential for catalysis by themselves, although residue 158 is very close to the essential residue, βK155 [21]. Iwamoto et al. [23] reported that the βG149S mutation did not affect ATPase activity. However, we found a significant reduction in activity caused by the βG149S mutation. Since our experimental system differed from that used in this previous study, the reason for this discrepancy is not clear at present. The βA295P mutation may cause significant structural changes at the level of the main chain of the peptide, leading to a loss of ATPase activity. As discussed above, we feel that the mechanism of suppression of the defect caused by the S174F mutation is recovery of the hydrophilic environment in the domain important for catalysis surrounded by Ser-174 and residues found as suppressive residues here. The βA295P mutation suppressed the defect caused by the S174F mutation possibly by the same mechanism. In this case, this could be achieved in an indirect manner such as by conformational changes around residue 295 caused by replacement with Pro and subsequent structural changes of the main chain around Ala-295. At present we have not determined in detail the possible conformation responsible for this hypothetical hydrophilic environment, but some other residues besides Ala-295 close to the replaced Pro-295 may be involved in its formation. Since a point mutation, βS292F, causes a defect in the assembly of the F_1-ATPase as described previously [22], mutation of βA295P may induce a similar effect.

We investigated suppression mutations of βA295P to determine which residues interact with the region containing residue 295. Since the βA295P mutation was found as a second site mutation of S174F, Ser-174 itself or residues around it may be suppressive residues. In fact, residues 140, 159, 166, 171, 172, and 184 were identified as sites of second mutations capable of suppressing the defect caused by A295P. The amino acid substitutions at these residues may compensate for the structural changes induced by the βA295P mutation, suggesting that the two regions including these suppression mutation sites or residue 295 are located close

to each other. Although S174F did not represent a second site mutation capable of suppressing the effects of A295P, we may find such a mutation by more extensive survey of suppression mutants of A295P. Analysis of pseudo-reversion mutations to obtain understanding of the ternary structure of a protein sometimes yields results which are controversial in terms of the reality of residue interactions. In the present study, we have shown that second site mutations of A295P originally found as second site mutations of S174F are again localized close to the original Ser-174, like Ala-140 to Arg-184 (Table 3). Therefore, we believe that discussions of the topological arrangement of residues around Ser-174 based on analyses of suppression mutations [10] are reasonable.

Iwamoto et al. [24] identified residues which form a domain near the γ-phosphate of ATP. Some of the residues (βG149, βG172, and βS174) described in the present paper overlapped with them, suggesting that the domain including βA295 which is important for catalysis is near the γ-phosphate-binding domain.

The ATPase activities of all revertants of the A295P mutation were relatively low, while their growth rates were higher (Table 2). These discrepancies may reflect functional instability of the revertant ATPase. Another possibility is that the F_1 portion becomes detached from the membranes during preparation.

Acknowledgments

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