Insertion Scanning Mutagenesis of Subunit a of the F$_1$F$_0$ ATP Synthase near His$^{245}$ and Implications on Gating of the Proton Channel

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Subunit a of the E. coli F$_1$F$_0$ ATP synthase was probed by insertion scanning mutagenesis in a region between residues Glu$^{219}$ and His$^{245}$. A series of single amino acid insertions, of both alanine and aspartic acid, were constructed after the following residues: 225, 229, 233, 238, 243, and 245. The mutants were tested for growth yield, binding of F$_1$ to membranes, dicyclohexylcarbodiimide sensitivity of ATPase activity, ATP-driven proton translocation, and passive proton permeability of membranes stripped of F$_1$. Significant loss of function was seen only with insertions after positions 238 and 243. In contrast, both insertions after residue 225 and the alanine insertion after residue 245 were nearly identical in function to the wild type. The other insertions showed an intermediate loss of function. Missense mutations of His$^{245}$ to serine and cysteine were nonfunctional, while the W241C mutant showed nearly normal ATPase function. Replacement of Leu$^{162}$ by histidine failed to suppress the 245 mutants, but chemical rescue of H245S was partially successful using acetate. An interaction between Trp$^{241}$ and His$^{245}$ may be involved in gating a “half-channel” from the periplasmic surface of F$_0$ to Asp$^{61}$ of subunit a.

The F$_1$F$_0$ ATP synthase from Escherichia coli is typical of the ATP synthases found in mitochondria, chloroplasts, and many other bacteria (for recent reviews, see Refs. 1–4). It comprises an F$_1$ complex, which contains the nucleotide-binding subunits b and c, form F$_0$ with a likely stoichiometry of 1:2:9–12 (5). The other membrane proteins by single-amino acid insertions have been described, including bacteriorhodopsin (23) and lac permease (24). In the present study, we have extended the analysis of subunit a from residue 229 to 245. The results indicate a single region of disruption, between residues 238 and 245, but insertions after 245 have little effect.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs, Inc. Materials for silver sequencing and plasmid minipreps were obtained from Promega Corp. Synthetic oligonucleotides were obtained from Operon Technologies, Inc. or National Biosciences. Urea was from International Biotechnologies, Inc. Oligonucleotides were obtained from Operon Technologies, Inc. or National Biosciences. Urea was from International Biotechnologies, Inc. ACMA$^-$ was obtained from Molecular Probes. Dicyclohexylcarbodiimide (DCCD) and octyl glucoside were obtained from Sigma. Immunoblotting reagents and detergent-compatible protein assay materials were obtained from Bio-Rad. Nickel-nitrotriacetic acid-agarose was obtained from Qiagen. ATP and anti-HA antibody were obtained from Boehringer Mannheim.

Bacterial Strains—Strain XL1-Blue (recA1, endA1, gvpA96, thi, hsdR17(hsr-,mkr+), supE44, relA1, λlac, F+, proAB, lacIq ΔM15, Tn10 Tet$^+$) was obtained from Stratagene and was used for subclonings and mutagenesis. Strain RH305 (uncB205, recA56, srl::Tn10, bgIR, the oligomeric structure of c subunits and how they interact with subunits a and b. Atomic force microscopy (12, 13) and electron spectroscopic imaging (14) have provided some evidence for a ring of 9–12 c subunits adjacent to the subunits a and b.

Subunit a is the largest of the F$_0$ subunits (271 residues). Mutagenesis has revealed that in addition to Asp$^{61}$ of subunit c, three residues in subunit a seem to be important in proton translocation: Arg$^{210}$, Glu$^{219}$, and His$^{240}$ (15–19). Of these three, only Arg$^{210}$ is strictly conserved among all known sequences, which suggests a unique role for this residue. No substitutions at this position permit ATP synthesis, but an alanine substitution allows limited passive proton permeability (20). The positions of the Glu and His residues are found to be reversed in some organisms, such as human and bovine mitochondria. Other sequences lack an ionizable residue at one of these positions, such as Bacillus subtilis and spinach chloroplast. Correspondingly, some amino acid substitutions at positions 219 and 245 in E. coli have been found to be partially functional (16, 21). Therefore, these two residues can be considered of secondary importance.

In a previous study (22), we probed subunit a by a series of single amino acid insertions at seven distinct locations between residues 187 and 222. Alanine insertions after residues 212, 217, and 222 were highly disruptive of function, consistent with the importance of this region in proton translocation. Studies of other membrane proteins by single-amino acid insertions have been described, including bacteriorhodopsin (23) and lac permease (24). In the present study, we have extended the analysis of subunit a from residue 229 to 245. The results indicate a single region of disruption, between residues 238 and 245, but insertions after 245 have little effect.

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1 The abbreviations used are: ACMA, 9-amino-6-chloro-2-methoxyacridine; DCCD, N,N′-dicyclohexylcarbodiimide; octyl glucoside, n-octyl-β-D-glucopyranoside; HA, the hemaglutinin HA2 epitope (YPDYVPDYA); anti-HA, mouse monoclonal antibody raised against the HA2 epitope; bp, base pair(s); MOPS, 4-morpholinopropanesulfonic acid.
thi-1, rel-1, Hfr PO1) (25) was used to characterize mutations in uncB. It produces an a subunit that is truncated near Pro240 (26) and is complemented by plasmids containing a wild type uncB gene. Cultures were grown at 37 °C, and cell density was monitored by optical density at 600 nm using a Milton Roy 1061 spectrophotometer. Rich medium was LB supplemented with 0.2% glucose, and minimal medium was A salts supplemented with succinate (0.2%) or with glucose, as indicated (27). Media were supplemented with chloramphenicol (34 mg/liter) or tetracycline (12.5 mg/liter) as appropriate. Growth yields were determined in minimal A supplemented with 7 mM glucose.

Construction of Plasmids and Insertions—Plasmids used in this study are shown in Fig. 1. The uncB mutations analyzed in this study were constructed using the cassette mutagenesis technique, as described previously (28). To construct pSBV18, plasmid pSBV16 (21) was digested with BstHI and BamHI to excise a 686-bp fragment. It was replaced in a two-step procedure. First, a synthetic 69-bp fragment (Fig. 2A) was ligated to the remaining large fragment of pSBV16. This construct retained the BstHI site, but the BamHI site was lost. However, a BstYI site was created at the same position, which can generate compatible cohesive ends. Second, the new plasmid was digested with BstYI, producing a single fragment, and was ligated to the 617-bp BamHI fragment from pSBV11 (29). This produced pSBV18, shown in Fig. 1. Plasmid pBJA1018 was constructed from pSBV10 and pSBV18 by digesting each with BstHI and BamHI. The 686-bp fragment from pSBV18, containing most of uncB, was ligated to the 3222-bp fragment of pSBV10 to produce pBJA1018. Insertion mutations (Fig. 3A) and missense mutations (Fig. 3B) at position 245 were constructed in pSBV18 and moved to pBJA1018 in the same way. Insertion mutations at positions 225, 229, and 233 (Fig. 3A) were constructed using the cassette mutagenesis technique, as described previously (28). To construct pSBV18, plasmid pSBV16 (21) was digested with BstHI and BamHI to excise a 686-bp fragment. It was replaced in a two-step procedure. First, a synthetic 69-bp fragment (Fig. 2A) was ligated to the remaining large fragment of pSBV16. This construct retained the BstHI site, but the BamHI site was lost. However, a BstYI site was created at the same position, which can generate compatible cohesive ends. Second, the new plasmid was digested with BstYI, producing a single fragment, and was ligated to the 617-bp BamHI fragment from pSBV11 (29). This produced pSBV18, shown in Fig. 1. Plasmid pBJA1018 was constructed from pSBV10 and pSBV18 by digesting each with BstHI and BamHI. The 686-bp fragment from pSBV18, containing most of uncB, was ligated to the 3222-bp fragment of pSBV10 to produce pBJA1018. Insertion mutations (Fig. 3A) and missense mutations (Fig. 3B) at position 245 were constructed in pSBV18 and moved to pBJA1018 in the same way. Insertion mutations at positions 225, 229, and 233 (Fig. 3A) were constructed in pBJA1018.

Plasmid pBJA46 was constructed from pBJA1018 by digesting with DraI and PstI, which excised a 125-bp fragment, followed by ligation to a synthetic fragment of 127 bp (Fig. 2B), as described previously (28). This introduced several unique restriction sites via silent mutagenesis, such as AarII, and an EcoRV site that was created by introducing two extra bases, G(ATT)ATC, in the region coding for Ile245. Therefore, pBJA46 does not code for a full-length subunit a; but since EcoRV generates blunt ends by cleaving between the ATs, it can be used in the construction of the L162H mutant and to regenerate a gene coding for the wild type subunit a (Fig. 3B).

Construction of pTW1HisHA is described in an accompanying paper (30). Its features include a unique BamHI site (as in pSBV18) and a hemagglutinin epitope (HA) near the COOH terminus followed by a hexahistidine tag. The HA consists of the sequence YPYDVPDYA and can be recognized by a monoclonal antibody raised against that peptide (Boehringer Mannheim). Insertion mutations (Fig. 3A) at positions 238 and 243 and missense mutation (Fig. 3B) at 241 were constructed in pTW1HisHA.

Preparation of Cell Fractions and Assays—Fractionation of cells and isolation of membranes and stripped membranes were carried out as described previously (15). Protein concentrations were determined by a detergent-compatible protein assay (Bio-Rad) using bovine serum albumin as standard. ATP hydrolysis assays and fluorescence quenching assays were performed essentially as described previously (28). ATP hydrolysis was measured in 50 mM Tris-HCl (pH 9.1), 1 mM MgCl2, 3 mM ATP at 37 °C, and fluorescence assays were measured using 400 mg/liter of membrane protein in a solution of 50 mM MOPS (pH 7.3), 10 mM MgCl2, 1 μM ACMA, and either 0.5 mM NADH or 0.1 mM ATP, as appropriate. The excitation wavelength was 410 nm, and the emission wavelength was 490 nm. Inhibition of ATP hydrolysis by DCCD was measured as described previously (28).

Immunoblotting—Membranes were prepared as above from 250-ml cultures. The membrane pellets were suspended in 1.5 ml of a detergent solution containing 0.1% deoxycholate, 0.5% cholate, 1.5% octyl glucoside, and 50 mM Tris-HCl, pH 7.5, and incubated for 1 h at 25 °C. The solubilized membranes were centrifuged for 10 min at 16,000 × g in a microcentrifuge, and the supernatant fraction was mixed with 0.4 ml of detergent-compatible protein assay (Bio-Rad) using bovine serum albumin as standard. ATP hydrolysis assays and fluorescence quenching assays were performed essentially as described previously (28). ATP hydrolysis was measured in 50 mM Tris-HCl (pH 9.1), 1 mM MgCl2, 3 mM ATP at 37 °C, and fluorescence assays were measured using 400 mg/liter of membrane protein in a solution of 50 mM MOPS (pH 7.3), 10 mM MgCl2, 1 μM ACMA, and either 0.5 mM NADH or 0.1 mM ATP, as appropriate. The excitation wavelength was 410 nm, and the emission wavelength was 490 nm. Inhibition of ATP hydrolysis by DCCD was measured as described previously (28).

RESULTS

A series of single amino acid insertions, both alanine and aspartic acid, were constructed after residues 225, 229, 233,
238, 243, and 245 in the a subunit of the F₁F₀ ATP synthase, as described under “Experimental Procedures.” These sites span the two residues of secondary importance in proton translocation, Glu²¹⁹ and His²⁴⁵. Growth yields of E. coli strains carrying these mutations were determined and are shown in Tables I and II. The four mutants with the lowest growth yields, those at positions 238 and 243, were all unable to grow on succinate minimal medium, indicating a deficiency in oxidative phosphorylation.

Membrane vesicles were prepared from cells carrying each of the 12 insertion mutations. The membrane and supernatant fractions were tested for ATPase activity to determine the relative binding of the F₁ sector to the membranes. The results are reported in Tables I and II. Alanine insertions (Table I) had little effect on F₁ binding after residues 225, 243, and 245, but after residue 238 significant loss of F₁ binding occurred. The ATPase activities of the membrane fractions were also tested for sensitivity to DCCD, a reagent that reacts specifically with the subunit c of the F₀ complex (31). These results are also presented in Tables I and II. Alanine insertions (Table I) after residues 225 and 245 had no detectable effect, while those after residues 238 and 243 caused a loss of sensitivity.

Membrane vesicles from all 12 insertion mutants were also tested for ATP-dependent proton translocation activity, using the fluorescent dye ACMA, and the results are shown in Fig. 4. Nearly normal ATP-driven proton translocation was seen with the mutants 225VA, 225VD, and 245VA. Little or no proton translocation was seen with mutants 238VA, 238VD, 243VA, and 243VD. The other insertion mutants showed intermediate levels of proton translocation that were at least 50% of the wild type level. The membranes were also stripped of F₁ and assayed for passive proton permeability, using NADH to generate a proton gradient. These results are presented in Table 1. In general, the same trends were observed, but the aspartate insertions had a noticeably more significant effect on passive proton permeability than did the alanine insertions. Membranes from the four mutants unable to grow on succinate minimal medium (238VA,D, and 243VA,D) were tested for the level of subunit a by immunoblotting. The results, presented in Fig. 6, indicate that subunit a is present at somewhat reduced levels in the 238VA,D mutants and at even lower levels in the 243VA,D mutants.

The residue His²⁴⁵ was replaced by Gly, Ser, and Cys to see if small or polar residues at that position would retain any function. None of the three mutants was able to grow on succinate minimal medium after 2 days of growth at 37 °C. Some growth was seen when the H245S strain was supplemented with 10–100 mM sodium acetate. This had no effect on the inability of the uncB strain RH305 to grow on succinate minimal medium. Attempts to demonstrate enhanced ATP-dependent proton translocation, by preparing membranes or performing assays in the presence of 10–100 mM acetate, all failed to
show significant differences. In contrast, membranes prepared from W241C showed normal rates of proton translocation, although passive proton permeability was somewhat reduced from the wild type level (results not shown).

In a previous study (21), residue Leu\(^{162}\) of subunit a in \textit{E. coli} was identified as corresponding to a histidine that is conserved among all known \textit{Bacillus} species. Three double mutants were constructed to see if relocating His\(^{245}\) to position 162 would retain function: L162H/H245G, L162H/H245S, and L162H/H245C. It was determined that none could grow on succinate minimal medium.

**DISCUSSION**

All subunits of the \textit{E. coli} F\(_1\)F\(_0\) ATP synthase are essential for ATP synthesis. Subunit a has been implicated in proton translocation through F\(_0\), but its precise role remains largely unknown. Single amino acid insertion-scanning mutagenesis has been applied to a region of subunit a between the important residues Glu\(^{219}\) and His\(^{245}\). The goal was to detect amino acid residues in subunit a that are important in proton translocation and regions of subunit a that are in close contact with other subunits. This study is a continuation of a previous study (22) in which insertions of alanine and aspartate were made after residues 187, 193, 198, 202, 212, 217, and 222 of subunit a. In that study, it was found that insertions of both alanine and aspartate after residues 212, 217, and 222 reduce or abolish ATP-driven proton translocation. In particular, the 222 insertions were the most severe and showed disruption of F\(_1\) binding to membranes. Interestingly, this region of subunit a had also been found to be the location of several second-site suppressors of mutations in subunit c (32). Most of the rest of the insertions had little or no effect on function; therefore, no other residues were implicated in function.

In this study, a similar pattern of results was obtained. Insertions of alanine or aspartate after residues 238 or 243 disrupted oxidative phosphorylation such that the strains could not grow on succinate minimal medium. Assays of the membrane fractions indicated that F\(_1\) binding was decreased significantly in the case of 238VA, while some ATP-driven proton translocation remained. In contrast, membranes from the 243VA mutant had normal F\(_1\) binding but no ATP-driven proton translocation. In both cases, the DCCD sensitivity of ATPase activity was very low. These results indicate two different types of disruption by the insertions of alanine at 238 and at 243.

The phenotype of 238VA is consistent with a disruption of a-b or a-c interactions. In a previous study, Kumamoto and Simoni (33) discovered that Pro\(^{240}\) was the site of two suppressor mutations of subunit b mutation G9D. This subunit b mutation had been isolated and characterized by two groups (34, 35) and was further analyzed by immunoprecipitation (36). Its properties include inability to grow on succinate minimal medium, reduced binding of F\(_1\) to membranes, and greatly diminished ATP-driven proton translocation and sensitivity of ATPase activity to DCCD. Two virtually identical partial suppressors were identified as P240A and P240L in subunit a (33). The suppressors permitted growth on succinate minimal medium and significant ATP-driven proton translocation but had little effect on F\(_1\) binding or DCCD sensitivity. Detergent solubilization and immunoprecipitation studies of the original mutant, G9D, indicated very low levels of subunit a in F\(_1\)F\(_0\) complexes, suggesting that the mutation affects a-b interactions. In the presence of the suppressor mutation, a normal complex of subunits a, b, and c was detected (36). The results presented here, including loss of F\(_1\) binding and DCCD sensitivity, are also consistent with an important interaction between subunit a in the region of Pro\(^{240}\) and either the membrane-spanning NH\(_2\) terminus of subunit b or subunit c.

The phenotype of 243VA was quite different, in that F\(_1\) remained bound to the membranes, but ATP-driven proton translocation and sensitivity to DCCD were completely missing. The immuno blot using anti-HA showed at least a low level of subunit a, which presumably is necessary for normal F\(_1\)
strictly conserved among all species, is also shown in boldface type

The best candidate would be Trp 241, for the following two reasons. First, a tryptophan is found at this position in all V. cholerae (43), and the residue Gln252, which is strictly conserved among all species, is also shown in boldface type.

binding. Therefore, the loss of function is not consistent with a large scale disruption of subunit interactions but rather suggests a more specific effect. Further insight into the 243-VA,D mutants may be gained by considering the insertions after residue 245 and the conservation of residues 241–245. Although His245 seems to be a functionally important residue from the results of mutagenesis (15, 19), the insertion of alanine following it has very little effect on function. One interpretation is that His245 does not interact with other residues toward the carboxyl terminus of subunit a but that the insertion at 243 disrupts an important interaction between the side chain of His245 and a nearby residue toward the amino terminus. The best candidate would be Trp241, for the following two reasons. First, a tryptophan is found at this position in all subunit a sequences that have a histidine at position 245 (Fig. 7). Second, a recent study (37) of model peptides has revealed a pH-dependent stabilization of an a-helical conformation mediated by Trp241 and His245. This stabilization was not seen for peptides with Trp241/His245 or for His245/Trp241. An analysis of proteins of known three-dimensional structure has revealed a significant number of instances of interactions in a-helices between Trp241 and His245. The side chains were found to be oriented for an interaction between the protonated imidazolium of His and the planar indole ring of tryptophan. Such an interaction at the periplasmic surface of F0 could function as a proton gate, regulated by the pH of His245. In this way, at sufficiently low pH, the imidazole of His245 would be protonated, allowing an interaction with Trp241, and thereby stabilizing the a-helix. This interaction might, through conformational or pH shifts, permit access of protons to Asp61 of subunit c. The half-channel from the second subunit a to the cytoplasmic surface is drawn accessible to solvent, and this would keep the aspartate unprotonated. Glu196, a conserved residue, is located near the cytoplasmic surface of this half-channel as a facilitator of proton transport. Mutagenesis of this residue (28) showed a charge- and polarity-dependent effect on rates of proton translocation. High resolution structural information about F0 subunits will be necessary for further insight into the mechanism of proton translocation.

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