Lengthening the Second Stalk of F_{1}F_{0} ATP Synthase in Escherichia coli

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In *Escherichia coli* F_{1}F_{0} ATP synthase, the two b subunits dimerize forming the peripheral second stalk linking the membrane F_{0} sector to F_{1}. Previously, we have demonstrated that the enzyme could accommodate relatively large deletions in the b subunits while retaining function (Sorgen, P. L., Caviston, T. L., Perry, R. C., and Cain, B. D. (1998) *J. Biol. Chem.* 273, 27873–27878). The manipulations of b subunit length have been extended by construction of insertion mutations into the uncF(b) gene adding amino acids to the second stalk. Mutants with insertions of seven amino acids were essentially identical to wild type strains, and mutants with insertions of up to 14 amino acids retained biologically significant levels of activity. Membranes prepared from these strains had readily detectable levels of F_{1}F_{0} ATPase activity and proton pumping activity. However, the larger insertions resulted in decreasing levels of activity, and immunoblot analysis indicated that these reductions in activity correlated with reduced levels of b subunit in the membranes. Addition of 18 amino acids was sufficient to result in the loss of F_{0}F_{1} ATP synthase function. Assuming the predicted a-helical structure for this area of the b subunit, the 14-amino acid insertion would result in the addition of enough material to lengthen the b subunit by as much as 20 Å. The results of both insertion and deletion experiments support a model in which the second stalk is a flexible feature of the enzyme rather than a rigid rod-like structure.

F_{1}F_{0} ATP synthases are multimeric enzymes that function through a complex mechanism similar to a rotary motor (1–3). Most of the mass of the enzyme is organized into two sectors, referred to as F_{1} and F_{0}, which are linked by two thin stalk structures (4). A channel composed of the a and c subunits in the F_{0} sector conducts protons across the membrane down the electrochemical gradient (5). Energy derived from proton translocation results in the propagation of a conformational change through the stalks driving ATP synthesis at distant catalytic sites in the F_{1} sector. A series of elegant experiments have clearly established that this conformational change takes the form of a rotation of the central stalk (6–10). The central stalk consists of the F_{1} γ and ε subunits, which appear to be in direct contact with a ring of 12 c subunits in the F_{0} sector (11–15). During catalysis, rotation of the c_{12}γε subunits sequentially alters the properties of the three catalytic sites located at subunit interfaces in the F_{1} αβγ hexamer. Differing interactions between the γ subunit and each of the three β subunits accounts for the asymmetry observed in the high resolution structure of bovine F_{1} and the differing conformations and nucleotide binding affinities of the catalytic sites (16).

The apparent function of the peripheral second stalk is to serve as a stator holding the αβγ hexamer in place against the rotation of the central stalk. Thus, the emerging view is that the second stalk is the primary structural feature maintaining the integrity of the stator elements in F_{1}F_{0} ATP synthase. A b_{2} dimer forms the second stalk making contact with the b subunit and at least one aβ subunit pair (17–21). Based on a positioning of the b subunit atop the F_{1} sector (22), it appears that the b_{2} subunits contribute all of the material visualized in the second stalk. From the electron micrographs and composite images of the *Escherichia coli* enzyme produced by Capaldi and colleagues (23), it is clear that the second stalk spans a distance of 40–45 Å from the surface of the membrane to the bottom of F_{1}. The stalk appeared to be bent at an angle of about 20° in micrographs of single complexes (4). Recently, the structure of peptides modeling the single transmembrane domain of the b subunit dissolved in organic solvents have been determined using nuclear magnetic resonance spectroscopy (24). The evidence suggests that Trp-26 may be at the hydrocarbon-polar interface on the cytoplasmic leaflet of the bilayer, so the region of the b subunit that constitutes the second stalk visible in the electron micrographs starts in the vicinity of Ala-32 and extends to at least Gln-85 and probably a few amino acids beyond. This segment of the b subunit is predicted to be in an a-helical conformation, and substantial experimental evidence supports this secondary structure (25–27).

Previously, we performed a deletion analysis in the second stalk segment of the b subunit to determine the minimum length of the second stalk necessary to form a productive F_{1}F_{0} ATP synthase (28). Surprisingly, the b subunits could be shortened by as much as 11 amino acids or approximately 16 Å with the retention of function. Losses of activity resulting from the deletions were largely attributable to defective assembly of the enzyme complex. Once assembled, the F_{1}F_{0} ATP synthase would shorten b subunits were functional. These observations suggested that the b_{2} subunit dimer has an inherent flexibility and that the deletions removed slack present in the second stalk of the wild type enzyme. This interpretation appeared to be incompatible with models in which the second stalk was a rigid structural feature of F_{1}F_{0} ATP synthase.

In the present work, we have further tested the flexibility hypothesis by inserting additional amino acids into the second stalk regions of the b subunits. The prediction was that if the b_{2} dimer was indeed a flexible unit, then insertions of substantial length could be accommodated in functional F_{1}F_{0} ATP synthase complexes. The additional material would not produce a
devastating distortion in the enzyme as might be expected with a rigid structural unit. Insertion mutations were constructed in the uncF(b) gene duplicating segments of the b subunit sequence. Because the goal of these experiments was to determine the maximum extension of the b subunit second stalk region allowing F₁F₀ ATP synthase function, the most important issue was retention of detectable enzymatic activity. We show that insertion mutants in which the second stalk region of the b subunits has been lengthened substantially and retained biologically and biochemically measurable levels of activity.

Experimental Procedures

Strains and Media—The E. coli uncF(b) deletion strain KM2 (29) and the wild type b subunit expression plasmid pKAM14 have been described previously (30). Plasmid pDS9 (b₃₃₇) was the generous gift of Dr. Stanley Dunn (University of Western Ontario) (25). Growth of strains on a nonfermentable carbon source was scored using Minimal A medium containing succinate (0.2% w/v). Cells for membrane preparation were grown in LB supplemented with 0.2% (w/v) glucose at 37 °C. Isopropyl-1-thio-β-D-galactoside (IPTG) (40 μg/ml), ampicillin (100 μg/ml), and chloramphenicol (30 μg/ml) were included as needed. Culture medium components were purchased from Difco, and antibiotics were obtained from Sigma.

Recombinant DNA Techniques—Molecular biology enzymes were purchased from Life Technologies, Inc. and New England BioLabs, and the oligonucleotides were synthesized and purified by Gemini Biotech. Restriction endonuclease reactions, ligation reactions, and transformations were performed according to the recommendations of the manufacturers. Plasmid DNA was purified with the Qiagen Mini and Maxi-Prep kits from Qiagen, and DNA fragments were separated by agarose gel electrophoresis and purified using the Qiagen QIAquick kit. Site-directed mutagenesis was performed either by cassette mutagenesis (31) or by using a Stratagene QuikChange kit. Nucleotide sequences were determined by automated sequencing in the core facility of the University of Florida Interdisciplinary Center for Biotechnology Research.

Mutagenesis and Strain Construction—Plasmid pKAM14 (b) was used to construct the insertion mutations in the stalk region of the b subunit. All of the insertion plasmids were constructed by ligation of synthetic double-stranded oligonucleotides into the PpuMI and PvuII sites in plasmid pKAM14 (b) (see Fig. 1). Except for the insertions, the final products of all constructions were identical to the positive control plasmid pKAM14 (b) placing the recombinant uncF(b) genes under control of the lac promoter. Presence of the inserted sequences was initially detected by digestion of recombinant plasmid DNA with either SstI or HindIII, and then the nucleotide sequences were directly confirmed. For clarity, the numbers of amino acids inserted are indicated behind the plasmid name in parentheses throughout the paper, such as plasmid pAUL19 (+7). Plasmids encoding the b subunit insertions and control plasmids pKAM14 (b) and pBR322 were transformed into E. coli strain KM2 (Δb) for study. Each strain was also transformed with the plasmid pKAM16 (lacF) (29) to provide improved regulation of expression of the uncF(b) genes.

Selected mutations were transferred to plasmid pDS9 (b₃₃₇) for expression of model b₃₃₇ polypeptides. Plasmid pAUL49 (b₃₃₇,I) was constructed by replacing the 541-base pair PpuMI/BarII fragment of pDS9 (b₃₃₇) with the comparable segment of pAUL19 (+7) containing the lac promoter. Two base substitutions were generated by using the Stratagene QuikChange kit on plasmid pDS9 (b₃₃₇) at uncF gene nucleotides 96 and 99; this resulted in silent mutations in the codons for b₃₃₇ Ala-32,Ile33 while generating the unique site and the existing EII site were then used to facilitate transfer of the uncF(b) gene deletion in pAUL 3 (ΔI7) (28) to pAUL49 (b₃₃₇,I) generating plasmid pAUL50 (b₃₃₇,I). In each case, the nucleotide sequence of each recombinant plasmid was verified, and the expression of b₃₃₇ polypeptides was confirmed by immunoblot analysis using the b subunit-specific antiserum.

Protein Expression—Inverted membrane vesicles were prepared from 500-ml cultures of strain KM2 (Δb) carrying the recombinant uncF(b) gene expression plasmids according to methods described previously (25). Membrane vesicles stripped of F₁ were prepared essentially as described previously (32). E. coli strain 1100 was transformed with b₃₃₇ expression plasmids pAUL49 and pAUL50 and inoculated into 500 ml of LB supplemented with glucose medium. Preparation of b₃₃₇ polypeptides was performed as described earlier (27).

Analytic Procedures—Protein concentrations were determined by a modified Lowry procedure (33). Membrane energization was detected by the fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) (34), and ATP hydrolysis activity of membrane fractions was assayed by the acid molybdate method (35). Membranes were assayed in buffer (50 mM Tris-HCl, 1 mM MgCl₂, pH 9.1) for determinations of linearity with respect to both time and enzyme concentration. Immunoblots analyses using the anti-b subunit antibodies were performed as described previously (28). The anti-ribosomal immunoblot horseradish peroxidase-linked whole antibody (from donkey), electrochemiluminescence Western blotting detection reagents, and Hyperfilm were obtained from Amersham Pharmacia Biotech. Anti-b subunit antibodies were kindly provided by Dr. Karlheinz Altendorf (Universität Osnabrück) (36). Chemical cross-linking of b₃₃₇ peptides with bis(sulfosuccinimidyl) suberate and analytic ultracentrifugation experiments were conducted according to procedures published in earlier work (27).

Results

Construction and Growth Characteristics of Mutants—To determine the maximum length of a b subunit that can be incorporated into a functional F₁F₀ ATP synthase, a collection of insertion mutations were generated within the stalk region of the b subunit by site-directed mutagenesis. The three options available for design of the insertion sequences were strings of alanines likely to generate a-helical segments, sequences of amino acids known to form a-helices in other proteins, and duplication of a segment of the b subunit. We elected to take the last approach because it seemed to hold the most promise in terms of establishing appropriate interactions between the two b subunits necessary for dimerization (Fig. 1). The duplicated segments started at F₆₃₄₋₆₄₋₆₅ and extended in the direction of the carboxyl terminus because this area of the b subunit had proven to be the least sensitive to the effects of deletions (28). The lengths of the insertion mutations were designed to model, as closely as practicable, full turns of an a-helix. The earlier deletion experiments suggested that helix orientation effects resulting from less than complete turns reduced assembly of the enzyme complex (28). In all, four insertion plasmids were constructed ranging from addition of 7–18 amino acids modeling insertions of from 2–5 a-helical turns (Fig. 1).

The effects of the insertion mutations were studied by complementation of uncF(b) gene deletion strain KM2 (Δb). E. coli strains defective for F₁F₀ ATP synthase cannot derive energy from nonfermentable carbon sources, so growth on succinate minimal medium served as a convenient qualitative measure of enzyme function in vivo. Because high levels of expression of altered b subunits can in some instances overcome the effects from assembly defects (30), experiments were conducted under conditions of both high and low level expression of the mutated uncF(b) genes under control of the lac promoter. In experiments using low expression conditions, strain KM2/pAUL47 (+11) harbored the largest b subunit insertion capable of supporting visible colony formation on solid succinate-based medium (Table I). In the presence of saturating concentrations of IPTG, slow growth of strain KM2/pAUL48 (+14) was observed indicating biologically significant levels of F₁F₀ ATP synthase activity (Table I). Strain KM2/pAUL92 (+18) failed all growth tests, indicating that the insertion exceeded the maximum length allowable for the enzyme.

Assembly of F₁F₀ ATP Synthase—Membrane vesicles were prepared, and immunoblots were performed using an anti-b antibody to detect production and incorporation of the b insertion subunits into the membrane. Although a small reduction in the level of b subunit was observed from the low induction conditions cells, membranes from strain KM2/pAUL19 (+7)
grown in the presence of IPTG possessed levels of b subunit proteins approaching that of the wild type control membranes (Fig. 2). However, reductions in levels of b subunits were readily apparent in membranes from cells with longer insertions. As might be expected for a mutation resulting in a total assembly defect, no b subunit protein was found in the KM2/pAUL48 (28) grown in the presence of IPTG. As anticipated from the slow growth of strain KM2/pAUL48 (+14) on succinate minimal medium, membranes prepared from this strain under high expression growth conditions possessed readily detectable levels of proton pumping activity (Fig. 3B). ATP-driven proton pumping was enhanced in vesicles derived from IPTG-induced KM2/pAUL47 (+11) relative to membranes from uninduced cells, and KM2/pAUL19 (+7) membranes appeared to be essentially indistinguishable from membranes from wild type control KM2/pKAM14 membranes with respect to coupled activity. In general, the reductions in activity observed in membranes from the insertion strains reflected the reduced amounts of assembled F1F0 ATP synthase. Most importantly, the data established that intact F1F0 ATP synthase complexes containing the b subunits lengthened by as many as 14 amino acids possessed coupled activity.

Effects of Deletions and Insertions on Dimerization—The properties of model b subunit polypeptides with either a deletion or an insertion of 7 amino acids were characterized to consider whether altering the length of the second stalk region of the b subunit affected interactions between the two b subunits in the hydrophilic domains. Plasmids pAUL49 (b\_{del-7}) and pAUL50 (b\_{del+7}) were used to direct expression of a recombinant b\_{del} polypeptide, and the recombinant b\_{ins} polypeptides were purified to homogeneity. The yield of recombinant b\_{del} polypeptides obtained from approximately 2.0 g of cells (wet weight) was approximately 5 mg of pure protein. Chemical cross-linking using the irreversible agent bis(sulfosuccinimidyl) suberate was employed to look for dimer formation by the b\_{ins} proteins (Fig. 5). Cross-linking was observed for both the b\_{del-7} and the b\_{del+7} polypeptides, indicating the presence of dimers. The cross-linked bands had the expected mobility for dimers in SDS-PAGE, and an immunoblot analysis using the anti-b subunit antibody confirmed that the 34-kDa cross-linked product was indeed b\_{ins} polypeptide (data not shown). Sedimentation equilibrium experiments were also performed to study dimer formation of the b\_{del-7} and the b\_{del+7} polypeptides (Fig. 6). Like the b\_{del} control, both the insertion and deletion polypeptides were largely in the dimeric form, and the samples contained less than 2% monomer. Concentrations of tetrameric polypeptides observed in the b\_{del-7} and the b\_{del+7} preparations were...
MEMBRANE PROTEIN (300 \text{mM}) was added to a final concentration of 1 mM. ATP was added as indicated to a final concentration of 1 mM. ATPase activities were measured as described under "Experimental Procedures." Units of specific activity = \text{\textmu}mol of PO4 released per mg of protein/min ± S.D. Units were calculated from the slope of the line based on five independent measurements with incubations for 15 min.

**TABLE I**

| Strains and b subunit insertions | Amino acids added | Growtha | Specific activityb |
|---------------------------------|------------------|---------|------------------|
| KM2/pKAM14 (+)                  | Wild type        | ++      | 0.93 ± 0.04 1.20 ± 0.06 |
| KM2/pBR322 (−)                  | Deletion         | −−      | 0.20 ± 0.02 0.21 ± 0.02 |
| KM2/pAUL19 (7)                  | Leu-54-Ser60     | ++      | 0.72 ± 0.06 1.06 ± 0.03 |
| KM2/pAUL47 (7)                  | Leu-54-Gln64     | +       | 0.57 ± 0.02 0.88 ± 0.03 |
| KM2/pAUL48 (14)                 | Leu-54-Lys87     | −       | 0.48 ± 0.03 0.70 ± 0.05 |
| KM2/pAUL52 (18)                 | Leu-54-Glu71     | −       | 0.23 ± 0.03 0.27 ± 0.02 |

a E. coli strains were grown aerobically on succinate minimal medium with antibiotics. IPTG was added as indicated. Colony size was scored after 72-h incubation at 37 °C as: ++ +, ≥ 1.0 mm; ++, 0.3–0.5 mm; +, 0.1 mm; −, no growth.

b ATPase activities were measured as described under "Experimental Procedures." Units of specific activity = \text{\textmu}mol of PO4 released per mg of protein/min ± S.D. Units were calculated from the slope of the line based on five independent measurements with incubations for 15 min.

**FIG. 2. Immunoblot analysis of uncF(b) gene mutant membranes.** Membrane proteins were separated using a 15% polyacrylamide Tris-Glycine-SDS gel. A, lanes were loaded with 40 \text{\mu}g of total membrane protein from uninduced cells, and the signals were visualized on Hyperfilm-HCl for 5 min. Strengths of signals shown in the bar graphs were determined using a Kodak image digitizing system (DC120) on 30-s exposures. B, lanes were loaded with 20 \text{\mu}g of total membrane protein from IPTG-induced cells. Signals were visualized on Hyperfilm-HCl for 1 min, and image analysis was performed on 15-s exposures. The samples in each lane have been labeled according to the length of the insertions, so the strains used as the sources of the samples were as follows: +, pKAM2/pKAM14; −, KM2/pBR322; +7, KM2/pAUL19; +1, KM2/pAUL47; +14, KM2/pAUL48; +18, KM2/pAUL52.

**FIG. 3. ATP-driven energization of membrane vesicles prepared from uncF(b) gene mutants.** Uninduced (A) or IPTG-induced (B) cell membrane vesicles were prepared by differential centrifugation (see "Experimental Procedures"). Membrane protein (300 \text{\mu}g) was suspended in 3 ml of assay buffer (50 mM MOPS, 10 mM MgCl2, pH 7.3). The fluorescent dye ACMA was added to a final concentration of 1 \text{\mu}M, and fluorescence was recorded with excitation at 410 nm and emission at 490 nm. ATP was added as indicated to a final concentration of 1 mM. The b subunit insertion is labeled next to each trace as described for Fig. 2.

**FIG. 4. Proton permeability of stripped membrane vesicles prepared from uncF(b) gene mutants.** Membrane vesicles were prepared from uninduced (A) or IPTG-induced (B) cells, and F1 was stripped by gentle agitation in a low ionic strength buffer (1 mM Tris-HCl, 0.5 mM EDTA, 2.5 mM 2-mercaptoethanol, 10% glycerol, pH 8.0). Stripped vesicles (100 \mug of protein) were suspended in TM buffer. ACMA fluorescence was recorded following addition of NADH to a final concentration of 0.5 mM. The b subunit insertion is labeled next to each trace as described for Fig. 2.

**DISCUSSION**

The segment of the b subunit thought to form the portion of the F1F0 ATP synthase visualized as the second stalk in electron micrographs extends from about Ala-32 to at least Ser-85. In the present work, we have characterized the effects of insertion mutations designed to lengthen the second stalk region of the b subunit. Insertion of 7 amino acids resulted in an essentially normal phenotype. Abundant fully assembled and functional F1F0 ATP synthase was observed in the membranes, and experiments using the model bsub 1 polypeptide indicated a propensity for dimer formation similar to wild type bsub. As the sizes of the insertions were increased to 11 and 14 amino acids, the amounts of intact F1F0-ATP synthase present in the membranes fell, but significant levels of enzymatic activity remained. The losses of function observed in the insertion mutants represented assembly defects rather than functional failures in intact F1F0 ATP synthase complexes. The assembly defect resulting from insertion of 18 amino acids was so severe that F1F0 ATP synthase activity was completely lost and no b subunit could be detected in the membrane fractions.

The most important parameter in these studies was the retention of coupled F1F0 ATP synthase function with addition...
of up to 14 residues. This insertion amounts to approximately four full turns of an α-helix corresponding to the capacity to extend the second stalk by up to 20 Å. Although we did not rigorously exclude the possibility of a change in secondary structure resulting from the insertions, the simple duplication of b subunit segments in these experiments and computer analysis using secondary structure prediction algorithms argue against this idea. Therefore, the maximum functional extension of the second stalk segment of the b subunit was roughly 50% longer than that normally used to span the distance between F₁ and F₀ in the wild type enzyme. If the second stalk were a rigid rod-like structure, such a large insertion would be expected to result in a dramatic distortion of the complex leading to failure of the stator and loss of F₁F₀ ATP synthase function. The insertion mutations studies reported here and the deletions characterized earlier (28) point to an interpretation that the second stalk of F₁F₀ ATP synthases have considerable flexibility to accommodate large changes in length. The second stalk seen in the electron micrographs of Wilkens and Capaldi (4) was bent. Viewing the second stalk as a flexible feature of the complex allows shortening and lengthening the b subunit to be accomplished, at least in part, by straightening or accentuating the bend. In simplistic terms, the second stalk seems much more likely to be a flexible rope that can be pulled taut, providing the tensile strength required to function as the major structural feature of the stator, rather than an inherently stiff rod. In this way, the results can be rationalized as compatible with the transient elastic storage of energy hypothesized for F₁F₀, ATP synthase described by Junge and colleagues (2, 40).

The two structurally and functionally constrained residues, Arg-36 and Ala-79, conserved in nearly all bacterial F₁F₀ ATP synthase b subunits are separated by exactly 43 amino acids (29, 41). All of the insertions reported here, as well as the earlier deletions (28), were constructed between these two sites disturbing this apparently conserved spacing. Changes of up to 7 amino acids in either direction made very little difference in the activity of intact F₁F₀ ATP synthase complexes containing the altered b subunits. The remaining question is, why does there appear to be conservation of the length bacterial b subunits in the second stalk segment? The answer probably lies in optimization of assembly of the oligomeric enzyme complex during evolution. The length of the b subunit may have remained under selective pressure for positioning the b subunit for interactions with F₁ needed for efficient formation of the F₁F₀ ATP synthase complex. Even a small decrease in the efficiency of enzyme assembly might have constituted a selective disadvantage for procaryotic organisms competing by a strategy based on rapid proliferation during evolution.

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