In *Escherichia coli*, a parallel homodimer of identical b subunits constitutes the peripheral stalk of F<sub>1</sub>F<sub>0</sub> ATP synthase. Although the two b subunits have long been viewed as a single functional unit, the asymmetric nature of the enzyme complex suggested that the functional roles of each b subunit should not necessarily be considered equivalent. Previous mutagenesis studies of the peripheral stalk suffered from the fact that mutations in the *uncF*<sub>b</sub> gene affected both of the b subunits. We developed a system to express and study F<sub>1</sub>F<sub>0</sub> ATP synthase complexes containing two different b subunits. Two mutations already known to inactivate the F<sub>1</sub>F<sub>0</sub> ATP synthase complex have been studied using this experimental system. An evolutionarily conserved arginine, b<sub>Arg-36</sub>, was known to be crucial for F<sub>1</sub>F<sub>0</sub> ATP synthase function, and the last four C-terminal amino acids had been shown to be important for enzyme assembly. Experiments expressing one of the mutants with a wild type b subunit demonstrated the presence of heterodimers in F<sub>1</sub>F<sub>0</sub> ATP synthase complexes. Activity assays suggested that the heterodimeric F<sub>1</sub>F<sub>0</sub> complexes were functional. When the two defective b subunits were expressed together and in the absence of any wild type b subunit, an active F<sub>1</sub>F<sub>0</sub> ATP synthase complex was assembled. This mutual complementation between fully defective b subunits indicated that each of the two b subunits makes a unique contribution to the functions of the peripheral stalk, such that one mutant b subunit is making up for what the other is lacking.

F<sub>1</sub>F<sub>0</sub> ATP synthases are responsible for the production of ATP, providing the majority of cellular energy in most eukaryotes and prokaryotes (1–9). Enzymes in this family utilize the electrochemical gradient of protons across the membranes to synthesize ATP from ADP and inorganic phosphate during oxidative phosphorylation or photophosphorylation (4). In *Escherichia coli*, F<sub>1</sub> consists of the cytoplasmic subunits α<sub>1</sub>β<sub>1</sub>γ<sub>δ</sub>ε and includes the catalytic sites as well as the “rotor” or central stalk (1, 4–9). F<sub>0</sub> consists of the membrane-spanning subunits α<sub>βγδε</sub> and includes the sites responsible for proton translocation as well as a major component of the “stator” or peripheral stalk (10–13).

In the F<sub>1</sub>F<sub>0</sub> ATP synthase enzyme complex, the peripheral stalk consists of a parallel dimer of identical b subunits. Dimerization of the b subunits is thought to be an early event necessary for enzyme assembly and function (14). The two b subunits exist in an extended α-helical conformation, spanning from the periplasmic side of the membrane to near the top of F<sub>1</sub>. However, because of the asymmetric nature of the enzyme complex, the two b subunits cannot participate in identical protein-protein interactions with the other subunits. In the N-terminal membrane-spanning region, the peripheral stalk contacts a single α subunit. Similarly, at the C-terminal end of the peripheral stalk, the two b subunits interact with a single δ subunit. The b dimer has been studied by a variety of traditional biochemical approaches such as CD spectroscopy, cross-linking, and sedimentation experiments (10, 15–20). Limited structural information is available from studies of polypeptides modeling functionally defined domains of the b subunit. Dmitriev et al. (23) studied the N-terminal membrane-spanning domain by NMR using a 34-amino acid polypeptide, revealing an α-helix with a 20° bend at Pro-27 and -28. A systematic mutagenesis approach supported the model described in the NMR paper, suggesting that the extreme N termini of the two b subunits were in close contact, and then the subunits flared apart as they traversed the membrane (23, 24). X-ray crystallography of a model polypeptide reflecting residues 62–122 corresponding to the dimerization domain showed an extended highly α-helical structure (25). The structures of the tether domain contributing to the segment between the membrane surface and the bottom of F<sub>1</sub> and the F<sub>1</sub> binding domain in the C-terminal region have yet to be determined.

Previously (27, 28) we have shown that b subunits with deletions and insertions in the tether region of up to 11 and 14 amino acids, respectively, formed functional F<sub>1</sub>F<sub>0</sub> complexes. These observations suggested that flexibility is an inherent characteristic of the peripheral stalk. This apparent flexibility also extended to the dimerization of the b subunits. When two b subunits with tether domains of unequal length were expressed together, F<sub>1</sub>F<sub>0</sub> ATP synthase complexes containing heterodimeric peripheral stalks were assembled (29). F<sub>1</sub>F<sub>0</sub> complexes were able to tolerate the incorporation of two different b subunits with a size difference of at least 14 amino acids. Although activity data suggested the heterodimeric F<sub>1</sub>F<sub>0</sub> complexes were likely functional, the earlier study was not designed to rigorously demonstrate that these complexes were active.

A major problem that plagued all mutagenesis studies of the b subunit was that mutations constructed in the *uncF*<sub>b</sub> gene affected both subunits of the b homodimer. One missense mutation led to two amino acid replacements. Our ability to express two different b subunits in the same cell and detect b subunit heterodimer formation provided an approach to studying the individual functional roles of the b subunits. In the present study, two different b subunit mutations were studied. Mutation of an evolutionarily conserved arginine, b<sub>Arg-36</sub>, to an
isoascorbic or glutamate has been shown previously to result in an intact completely defective F,F_ATP synthase complex (30). Also, deletion of the last four amino acids at the C terminus has been shown to affect the ability of the b dimer to form a stable interaction with the b subunit F, resulting in a major defect in F,F_ATP synthase assembly (31, 32). Here, we demonstrate functional activity for F,F_ATP synthase complexes containing a heterodimeric peripheral stalk, with major defects occurring in the different domains of each b subunit.

**EXPERIMENTAL PROCEDURES**

**Materials—**Molecular biology enzymes and oligonucleotides were obtained from Invitrogen, New England Biolabs, and Stratagene. Reagents were obtained from Sigma, Bio-Rad, and Fisher. Western blotting reagents and high performance chemiluminescence film were purchased from Amersham Biosciences. Polyclonal antibodies against SDS-denatured b subunit (33, 34) were kindly provided by Dr. Kari heinz Altenfors (Universit-tat Osnabr,ck, Osnabr,ck, Germany). Monoclonal antibodies against the V5 epitope were purchased from Invitrogen.

**Strains and Media—**The wild type b subunit expression plasmid, pKAM14, and plasmids used to express Arg-36 mutation b subunits have been described previously (17, 30). The plasmids encoding the uncF b subunit were used to complement E. coli strain KM2 ( ) carrying a chromosomal deletion of the gene (35). All strains were streaked onto plates containing minimal A medium supplemented with succinate (0.2% w/v) to determine enzyme viability. Cells harvested for membrane preparation were grown in Luria broth supplemented with glucose (0.2% w/v). Isopropyl-1-thio-galactopyranoside (40 g/ml), am picillin (100 g/ml), and chloramphenicol (25 g/ml) were added to the medium as needed. All cultures were incubated at 37 °C for the appropriate duration.

**Recombinant DNA Techniques—**Plasmid DNA was purified with the Qiagen Mini-Prep and Maxi-Prep kits. Restriction endonuclease digests, ligations, and transformations were performed according to the recommendations of the manufacturers (New England Biolabs, Stratagene, and Invitrogen). Site-directed mutagenesis was performed either by means of a Stratagene QuikChange kit or by ligation-mediated mutagenesis. DNA fragments were separated in 0.8% agarose gel by electrophoresis and purified using a Qiagen, Inc. QiAgel quick gel extraction kit. Plasmid 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 ( , Ap) (17) was used to construct b subunits with a deletion of the last four amino acids. Plasmids pKAM14 ( , Ap), pTLC11 (, Ap), or pTLC15 (, Ap) (30) were used to construct the epitope tagged b subunits. Mutations were introduced into each of the plasmids using the Stratagene QuikChange kit. A histidine epitope tag was inserted at the N terminus by mutagenesis between the first and second codons of the gene to express b ( , His) or ( , His). All of the recombinant histidine-tagged b subunit plasmids were then digested with PstI and NdeI and subsequently ligated into a plasmid conferring the chloramphenicol resistance gene and the pACYC184 origin of replication (see Table I). A V5 epitope tag was added to the C terminus by site-directed mutagenesis before the termination codon of the gene to express b ( , V5) or b ( , V5). All of the recombinant V5-tagged b subunit plasmids included the ampicillin resistance gene and the pUC18 origin of replication. Unique restriction enzyme sites SphI and NdeI were constructed near the histidine and V5 epitope tag sequence, respectively, for an initial detection of the insertions, and then the nucleotide sequence was subsequently confirmed by automated sequencing in the Interdisciplinary Center for Biotechnology Research core facility. Throughout this paper, the mutation and the epitope tag are indicated along with the plasmid name for clarity; for example, plasmid pTAM51 (, His). Each plasmid and the control plasmids pKAM14 ( , V5) and pBR322 were expressed in the E. coli cell line KM2 ( ) for study, so that the only b subunits in the cells were the product of the plasmid genes. The two-plasmid expression system allowed expression of various combinations of histidine- and V5-tagged b subunits in the same cell (see Figs. 1C, 3C, and 5C). Appropriate antibiotics were added to the growth medium, and in the mutants and in the coexpressed plasmids, both ampicillin and chloramphenicol were added to select for cells expressing both plasmids.

**Preparative Procedures—**Inverted membrane vesicles from KM2 ( ) strains expressing the desired b subunits were prepared essentially as described previously (27). Protein concentrations were determined by the bichinonic acid assay (36). Nickel-resin purification was achieved using the High Capacity Nickel Chelate Affinity Matrix purchased from Sigma. A total of 5 mg of membrane protein was brought up to 1 ml with final concentrations of 0.2% tageminoide WS-35, 0.15 m NaCl, and 1 mM imidazole. The purification procedure was accomplished using the batch method as described by the manufacturer.

**Assay of F,F_ATPSynthase Activity—**Growth on a minimal sucinate medium was used as an initial in vivo assay for enzyme viability. ATP hydrolysis activity was assayed by the acid molybdate method (37). The membranes were assayed in buffer (50 mM Tris-HCl, 1 mM MgCl, pH 9.1) to determine the linearity with respect to time and enzyme concentration. Membrane energization was detected by the fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) (38).

**Immunoblot Analysis—**Proteins were loaded on a 15% Tris-glycine SDS gel and transferred onto nitrocellulose by electroblot. The b subunit antibody incubation was performed essentially as described by Grabar and Cain (29); see also Ref. 39 using a 1:25,000 dilution of the anti-b subunit antibodies. Secondary antibody incubation was performed with horseradish peroxidase-linked sheep anti-rabbit antibody (1:50,000), and the antibody was detected by enhanced chemiluminescence. The V5 subunit antibody incubation was performed as described by the manufacturer followed by a secondary antibody incubation with horseradish peroxidase-linked sheep anti-mouse antibody (1:10,000). Signals were visualized on high performance chemiluminescence film using a Kodak X-Omat.

**RESULTS**

**Construction and Growth Characteristics of Mutants—**To investigate the function of individual b subunits in a F,F_ATP synthase complex, plasmids expressing defective b subunits with either a histidine or a V5 epitope tag were generated by site-directed mutagenesis. A total of five plasmids were constructed expressing the b (His), b (His), b (V5), or b (V5). Signals were needed to facilitate enzyme purification and subunit detection on a Western blot, respectively. To express two different b subunits in the same cell, we used the two-plasmid expression system described previously (29). Enzyme complex expression incorporated with a histidine or V5 epitope-tagged b subunit homodimer have been studied previously (29) and were shown to result in a functional enzyme complex. E. coli strain KM2 ( ) was used as the host because the uncF gene has been deleted, eliminating any background b subunit.

Previous analyses (30–32) of F,F_ATP synthase complexes incorporated with the mutant b or b found the mutants to be completely defective. Therefore, our ability to form F,F complexes containing b heterodimers offered an approach to study activity in a complex with only a single functional b subunit protein. First, the effects of the b or b mutations for dimerization with a wild type epitope tagged b subunit to complement the E. coli strain KM2 ( ) (35). Growth on succinate minimal medium was used as an initial qualitative gauge of enzyme activity in vivo because E. coli strains lacking F,F_ATP synthase cannot derive energy from fermentable carbon sources (Table I). As expected, when any one of the mutants was expressed alone in the cells, no growth was detected. In each case, the strains coexpressing the mutant epitope-tagged b subunits with a wild type epitope tagged b subunit grew comparably to the wild type strain.

**Heterodimer Formation of b Defective Subunits with b—**Although two b subunits of unequal length formed a heterodimer in an intact F,F_ATP synthase complex (29), it was possible that a single amino acid substitution could affect dimerization, preventing assembly of a complex. To consider whether a V5 epitope-tagged b subunit, b or b or 1

1 The abbreviations used are: ACMA, 9-amino-6-chloro-2-methoxyacridine; MOPS, 4-morpholinepropanesulfonic acid.
**b Subunit Complementation**

**Table I**

| Strains          | Description | Growth | Specific activity | Wild type activity |
|-----------------|-------------|--------|------------------|-------------------|
| KM2/pKAM14 (+)  | b<sub>wt</sub>, Ap<sup>d</sup> | +++    | 1.18 ± 0.05      | 100               |
| KM2/pBR322 (-)  | Δb, Ap<sup>d</sup> | −      | 0.24 ± 0.03      | 0                 |
| KM2/pTAM37     | b<sub>wt</sub>, Cm<sup>+</sup> | +++    | 1.08 ± 0.08      | 89                |
| KM2/pTAM51     | b<sub>Arg</sub>-36<sub>−</sub>-His<sub>5</sub>, Cm<sup>+</sup> | −      | 0.22 ± 0.05      | 0                 |
| KM2/pTAM46     | b<sub>wt</sub>-V5 | +++    | 1.22 ± 0.04      | 104               |
| KM2/pTAM53     | b<sub>Arg</sub>-36<sub>−</sub>-Glu<sub>5</sub>, Ap<sup>d</sup> | −      | 0.80 ± 0.02      | 60                |
| KM2/pTAM54     | b<sub>Arg</sub>-36<sub>−</sub>-Glu<sub>5</sub>, V5 | −      | 0.75 ± 0.08      | 54                |
| KM2/pTAM37/pTAM46 | b<sub>wt</sub>-His<sup>+</sup> + b<sub>wt</sub>-V5 | +++    | 1.16 ± 0.03      | 98                |
| KM2/pTAM51/pTAM46 | b<sub>Arg</sub>-36<sub>−</sub>-His<sub>5</sub> + b<sub>wt</sub>-V5 | +++    | 1.00 ± 0.05      | 81                |
| KM2/pTAM37/pTAM53 | b<sub>wt</sub>-His<sup>+</sup> + b<sub>Arg</sub>-36<sub>−</sub>-His<sub>5</sub> | +++    | 1.12 ± 0.04      | 94                |
| KM2/pTAM53/pTAM54 | b<sub>wt</sub>-His<sup>+</sup> + b<sub>Arg</sub>-36<sub>−</sub>-Glu<sub>5</sub> | +++    | 1.10 ± 0.05      | 91                |
| KM2/pTAM51/pTAM53 | b<sub>wt</sub>-His<sup>+</sup> + b<sub>Arg</sub>-36<sub>−</sub>-Glu<sub>5</sub> | −      | 0.51 ± 0.02      | 29                |
| KM2/pTAM51/pTAM54 | b<sub>wt</sub>-His<sup>+</sup> + b<sub>Arg</sub>-36<sub>−</sub>-Glu<sub>5</sub> | −      | 0.37 ± 0.07      | 14                |

<sup>a</sup> E. coli strains were grown aerobically on succinate minimal medium. Colony size was scored after 72 h of incubation at 37 °C as: +++ ≥1.0 mm; +, <0.1 mm after 5 days of growth; −, no growth.

<sup>b</sup> ATPase activities were measured as described under “Experimental Procedures.” Units of specific activity = μmol of PO<sub>4</sub> released/mg of protein/min ± S.D. Units were calculated from the slope of the line based on three measurements with incubations for 12 minutes.

<sup>c</sup> Mutant activity less the activity of KM2/pBR322 divided by the activity of KM2/pKAM14 less the activity of KM2/pBR322 and converted to percentage.

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b<sub>Arg</sub>-36<sub>−</sub>-Glu<sub>5</sub>, could form a heterodimer with a wild type histidine epitope-tagged b subunit, b<sub>wt</sub>-His<sup>+</sup>, each pair of subunits was expressed in strains KM2/pTAM37/pTAM53 and KM2/pTAM37/pTAM54 (Δb) (Fig. 1, A and B). The b subunits were detected in membranes prepared from strains expressing a b subunit by immunoblot analysis (Fig. 1A, lanes 1–9). However, only b subunits in complexes with at least one histidine tag were retained by nickel-resin purification (Fig. 1A, lanes 10–13). Immunoblot analysis using an anti-V5 antibody was performed on the membrane preparations and nickel-purified products (Fig. 1B). The V5 epitope tag was detected only in membrane vesicles derived from KM2 strains expressing either the V5-tagged b subunit or the coexpressed V5- and histidine-tagged b subunits (Fig. 1B, lanes 1–9). When only V5-tagged b subunits were expressed alone in a cell, no F<sub>0</sub>F<sub>1</sub> complexes were recovered from the nickel-resin purification (Fig. 1B, lane 10). Finally, we investigated the ability of b<sub>wt</sub>-His<sup>+</sup> to dimerize to form intact F<sub>0</sub>F<sub>1</sub> ATP synthase complexes with b<sub>Arg</sub>-36<sub>−</sub>-His<sub>5</sub> or b<sub>Arg</sub>-36<sub>−</sub>-Glu<sub>5</sub>. Both b<sub>Arg</sub>-36<sub>−</sub>-His<sub>5</sub> and b<sub>Arg</sub>-36<sub>−</sub>-Glu<sub>5</sub> were detected with anti-V5 antibodies after nickel-resin purification (Fig. 1B, lanes 12–13). The only mechanism for the recovery of defective V5-tagged b subunits was through dimerization with b<sub>wt</sub>-His<sup>+</sup> indicating formation of heterodimeric F<sub>0</sub>F<sub>1</sub> ATP synthase complexes. Therefore, coexpression of the two different b subunits in the same cell led to three distinct interactions within an intact F<sub>0</sub>F<sub>1</sub> ATP synthase complex (Fig. 1C): 1) a homodimer of b<sub>wt</sub>-His<sup>+</sup>, 2) a homodimer of b<sub>Arg</sub>-36<sub>−</sub>-His<sub>5</sub> or b<sub>Arg</sub>-36<sub>−</sub>-Glu<sub>5</sub>, and 3) a heterodimer consisting of both the defective and wild type b subunit.

Because F<sub>1</sub> reduced affinity for the membrane in the absence of intact F<sub>0</sub>, total membrane-associated F<sub>0</sub>F<sub>1</sub> ATP hydrolysis activity was used as a test of F<sub>0</sub>F<sub>1</sub> ATP synthase complex assembly. Under conditions of high pH, F<sub>1</sub> can be released from the influence of F<sub>0</sub>; therefore, the amount of ATPase activity in the solution was used as a measure of the amount of intact enzyme complex located in the membrane vesicles. Previous data (29) indicated minimal effects on specific activity because of the epitope tags. Confirming these observations, the V5 epitope tag did not have an apparent effect on enzyme assembly. Membrane preparations with b<sub>wt</sub>-V5 incorporated into the F<sub>0</sub>F<sub>1</sub> ATP synthase complex had virtually the same specific activity of the wild type strain (Table I). A small decrease in specific activity was reproducibly observed in membrane vesicles isolated from cells when a histidine epitope tag was incorporated onto the b<sub>wt</sub> (~89% of the wild type strain). Furthermore, comparable activities were observed in samples when the histidine- and V5-tagged b<sub>wt</sub> subunits were coexpressed. Also verifying previous data (30), when b<sub>Arg</sub>-36<sub>−</sub>-His<sub>5</sub> or b<sub>Arg</sub>-36<sub>−</sub>-Glu<sub>5</sub> was expressed alone in strains KM2/pTAM53 and KM2/pTAM54, the membranes retained abundant activity (~60 and 54% of the wild type strain, respectively) (Table I), indicating considerable amounts of intact F<sub>0</sub>F<sub>1</sub> ATP synthase complexes found in the membranes. When b<sub>Arg</sub>-36<sub>−</sub>-His<sub>5</sub> or b<sub>Arg</sub>-36<sub>−</sub>-Glu<sub>5</sub> was coexpressed with b<sub>wt</sub>-His<sup>+</sup> by construction of
isolated from cells coexpressing histidine- and V5-tagged b subunits. Strains expressing either the histidine-tagged species. Membrane preparations from unCF
pared from unCF strains KM2/pTAM37/pTAM53 (wt-His/Arg-36)
pared by differential centrifugation. Membrane protein (200 
3 ml of assay buffer (50 mM MOPS, 10 mM MgCl2, pH 7.5).
The fluorescent dye ACMA was added to a final concentration of 1 
ACMA (Fig. 2). Membranes isolated from cells expressing either 
fluorescence of ACMA (Fig. 2). Membranes isolated from cells 
490 nm. ATP was added as indicated to a final concentration of 1 mM.
Level of NADH-driven fluorescence quenching was monitored 
strate that the membrane vesicles were intact and closed, the 
level of NADH-driven fluorescence quenching was directly comparable in every case (data not shown).

Subunit Complementation

\[ \text{F}_1 \text{F}_0 \]  ATP synthase-mediated ATP-driven proton pumping activity in membrane vesicles prepared from the epitope-tagged mutants was used as an indication of coupled activity. Acidification of inverted membrane vesicles was examined by fluorescence of ACMA (Fig. 2). Membranes isolated from cells expressing a V5 epitope tag incorporated onto the b subunit, KM2/pTAM46 (bwt-V5), reproducibly displayed a very small reduction in coupled activity, correlating very well with the F1, ATP hydrolysis activity. Consistent with previous observations, a larger reduction in coupled activity of about 20% was observed in membrane vesicles isolated from strain KM2/pTAM37 (bwt-His) (29). The coupled activities observed in membranes isolated from cells coexpressing histidine- and V5-tagged b subunits were intermediary between the V5-tagged species and the histidine-tagged species. Strains expressing either bArg36→Ile-V5 or bArg36→Glu-V5 displayed no ATP-dependent proton pumping activity as expected. Significantly, when either one of the bArg36 subunits was coexpressed with bwt-His, KM2/pTAM37/pTAM53 (bwt-His/bArg36→Ile-V5) or KM2/pTAM37/pTAM54 (bwt-His/bArg36→Glu-V5), the coupled activity observed was essentially the same as strain KM2/pTAM37/pTAM46 (bwt-His/bwt-V5). When expressed alone, the strains with bArg36-V5 mutants had no activity, and the strains expressing bwt-His displayed less activity than the bwt-His/bArg36-V5 mutant enzyme. As a consequence, it was most likely that the F1, ATP synthase complexes with either the bwt-His/bArg36→Ile-V5 or bwt-His/bArg36→Glu-V5 peripheral stalks were active. To demon-
strate that the membrane vesicles were intact and closed, the level of NADH-driven fluorescence quenching was monitored for all membrane preparations. The levels of NADH-driven fluorescence quenching were strongly and directly comparable in every case (data not shown).

**Heterodimer Formation of F1 F0 ATP synthase containing a b subunit C-terminal truncation.** Membrane preparation, nickel-resin purification, and Western blot analysis were accomplished as described in the legend to Fig. 1. Proteins were transferred to a nitrocellulose membrane and probed using a polyclonal anti-b antibody (A) or an anti-V5 mouse monoclonal antibody (B). C, line diagram of the different b dimers found in the cell.
pumping activity in membrane vesicles prepared from the epitope-tagged mutants (Fig. 4). Membranes containing only the \textsubscript{A153-end-His} subunit exhibited no coupled activity as expected. Membranes isolated from cells coexpressing pTAM51 (b\textsubscript{A153-end-His}) and pTAM46 (b\textsubscript{wt-V5}) displayed a slight reduction in coupled activity of about 20% compared with membranes from cells coexpressing b\textsubscript{wt-V5} and b\textsubscript{wt-His}. It is feasible that the observed reductions in enzymatic activities were entirely because of formation of the mutant homodimeric species. This could not be directly demonstrated by these methods due to background activity from b\textsubscript{wt-V5} homodimeric F\textsubscript{1}F\textsubscript{0} ATP synthases present in the membranes.

**Mutual Complementation**—To look for mutual complementation of b subunits with defects in different functional domains, b\textsubscript{Arg-36-Ile-V5} and b\textsubscript{Arg-36-Glu-V5} were coexpressed with b\textsubscript{A153-end-His} in the absence of a functional wild type b subunit. Significantly, when b\textsubscript{A153-end-His} and b\textsubscript{Arg-36-Ile-V5} were coexpressed in the same cell (KM2/pTAM51/pTAM53), small colonies were present after 5 days of growth. Expression of either b\textsubscript{Arg-36-Ile-V5} or b\textsubscript{A153-end-His} alone in KM2 (Δb) resulted in a completely defective enzyme complex; any F\textsubscript{1}F\textsubscript{0} ATP synthase activity must necessarily have come from a heterodimer formed from two defective b subunits. Therefore, the b\textsubscript{Arg-36-Ile} and b\textsubscript{A153-end} mutations qualified as intergenic second site suppressor mutations.

To determine whether there was a direct protein-protein interaction between two mutant subunits, we investigated the ability of b\textsubscript{A153-end-His} to form an interaction with b\textsubscript{Arg-36-Ile-V5} or b\textsubscript{Arg-36-Glu-V5} by the standard immunoblot (Fig. 5, A and B). All of the controls provided on the first immunoblot were included and displayed similar results. An additional control was included to ensure complete solubilization of membrane vesicles was achieved and nonspecific aggregation of the b subunits was not a concern (Fig. 5, A and B, lanes 10 and 11). Two independent membrane preparations were mixed together. Membrane vesicles derived from strains KM2/pTAM51 (b\textsubscript{A153-end-His}) and KM2/pTAM53 (b\textsubscript{Arg-36-Ile-V5}) or KM2/pTAM54 (b\textsubscript{Arg-36-Glu-V5}) were mixed, and the membranes were solubilized with 0.2% octylglucoside WS-35 prior to nickel-resin purification. Immunoblot analysis with anti-b antibodies showed normal levels of histidine-tagged b subunit upon nickel-resin purification (Fig. 5A, lanes 10 and 11). No V5 epitope was present after nickel-resin purification, indicating that neither nonspecific aggregation nor incomplete solubilization was a factor in the observed results (Fig. 5B, lanes 10 and 11). Therefore, in cells expressing two different b subunits, any observed interactions of b subunits must have been because of heterodimers integrating into an intact F\textsubscript{1}F\textsubscript{0} ATP synthase complex. Importantly, immunoblotting using the anti-V5 antibody clearly detected V5 epitope-tagged b subunits in nickel-resin purified samples when two different b subunits, b\textsubscript{A153-end-His} and b\textsubscript{Arg-36-Ile-V5} or b\textsubscript{Arg-36-Glu-V5}, were coexpressed (Fig. 5B, lanes 12 and 13). The data indicated that dimerization between the two defective b subunits could occur, and the F\textsubscript{1}F\textsubscript{0} ATP synthase complex accepted incorporation of a b heterodimer with mutations affecting two different domains.

Expression of two different defective b subunits yielded three distinct F\textsubscript{1}F\textsubscript{0} ATP synthase complexes including the b\textsubscript{Arg-36-Ile-V5} or b\textsubscript{Arg-36-Glu-V5} homodimeric F\textsubscript{1}F\textsubscript{0} (a partially assembled b\textsubscript{A153-end-His} homodimer complex) and the heterodimeric F\textsubscript{1}F\textsubscript{0} ATP synthase complexes. Total membrane-
**b Subunit Complementation**

**Fig. 6.** ATP-driven energization of membrane vesicles incorporated with F$_{1}$F$_{0}$ ATP synthase containing complementing defective b subunits. Membrane preparation and ATP-driven proton pumping was accomplished as described in the legend to Fig. 2. The samples for each trace have been labeled according to the subunit mutation and the epitope tag. The strains used as the sources of the samples were as follows: $\Delta b$, KM2/pBR322; b$_{153}$, KM2/pKM14; $b_{153}$/end-His/KM2/pTM51; b$_{Arg-36}$/Ile-V5/KM2/pTM53; b$_{Arg-36}$/Glu-V5/KM2/pTM54; b$_{Arg-36}$/Ile-V5/KM2/pTM37/pTM46; $b_{153}$/end-His/ b$_{Arg-36}$/Ile-V5/KM2/pTM51/pTM53; and $b_{153}$/end-His/b$_{Arg-36}$/Glu-V5/KM2/pTM51/pTM54.

Associated F$_{1}$-ATP hydrolysis activity was used as a test of F$_{1}$F$_{0}$ ATP synthase complex assembly (Table I). Membrane vesicles isolated from strains KM2/pTM51/pTM53 ($b_{153}$/end-His/b$_{Arg-36}$/Ile-V5) and KM2/pTM51/pTM54 ($b_{153}$/end-His/b$_{Arg-36}$/Glu-V5) yielded membrane-associated specific activities of about 29 and 14%, respectively, which was intermediate between the specific activities if either of the mutants were expressed alone in the cell. An indication of coupled activity was obtained by F$_{1}$F$_{0}$ ATP synthase-mediated ATP-driven proton pumping activity in membrane vesicles prepared from the epitope-tagged mutants (Fig. 6). As expected, membranes from all three strains expressing defective b subunits by themselves showed no proton pumping activity. Membranes isolated from cells expressing $b_{153}$/end-His/b$_{Arg-36}$/Ile-V5 or $b_{153}$/end-His/b$_{Arg-36}$/Glu-V5 (KM2/pTM51/pTM53 or KM2/pTM51/pTM54, respectively) displayed coupled ATP-driven proton pumping of about 20 and 16%. Because either homodimer leads to a completely defective enzyme, any coupled activity observed necessarily came from an intact heterodimeric F$_{1}$F$_{0}$ ATP synthase. The data indicated that F$_{1}$F$_{0}$ ATP synthase complexes incorporated with heterodimers of two mutant b subunits were indeed functional. Furthermore, the results demonstrated that the roles of the two b subunits in the peripheral stalk were not equivalent. Protein-protein contacts made by one b subunit cannot be made by the other. In the present work, a unique expression system that facilitates the production, purification, and detection of F$_{1}$F$_{0}$ ATP synthase complexes incorporated with a b subunit heterodimer was utilized to study the functional roles of the two b subunits. The experiments involved a two-plasmid expression system that directed the production of two different b subunits in the same cell and an epitope tag system that allowed detection of a b subunit heterodimer F$_{1}$F$_{0}$ ATP synthase species. Two regions of the b subunit were considered. An evolutionarily conserved arginine, b$_{Arg-36}$/end-His located near the interface between the membrane and tether domains had been found to be crucial for F$_{1}$F$_{0}$ ATP synthase function (30). Enzyme complexes incorporated with a $b_{Arg-36}$/Ile-V5 or $b_{Arg-36}$/Glu-V5 were found to be intact yet functionally defective. Also, the C-terminal last four amino acids had been shown to be essential for the F$_{1}$ binding domain (31, 32). Enzyme complexes with a $b_{Arg-36}$/end-His were found to be only partially assembled. However, the $b_{Arg-36}$ missense mutations in combination with the C-terminal deletion proved to be mutually suppressing mutations.

Dimerization was observed in membrane preparations from cells expressing both $b_{153}$/end-His/b$_{Arg-36}$/Ile-V5 and $b_{153}$/end-His/b$_{Arg-36}$/Glu-V5. More importantly, enzyme complexes incorporated with the mutant heterodimers were functionally active, suggesting that each of the b subunits was complementing the other to form an intact and functional enzyme complex. This observation demonstrated unambiguously that F$_{1}$F$_{0}$ ATP synthase complexes containing b heterodimers were active and provided evidence that each of the individual b subunits provides specialized functions within the peripheral stalk. Clearly, each of the mutant b subunits compensates for what the other is lacking.

This raises a question concerning the relative positions of the individual b subunits of the peripheral stalk. For F$_{1}$F$_{0}$ ATP synthase containing the two different mutant b subunits to be intact and functional, it is likely that the $b_{Arg-36}$/Ile-V5 (or $b_{Arg-36}$/Glu-V5) subunit must be positioned such that its extreme C terminus forms the appropriate contacts with the $\delta$ subunit.

**DISCUSSION**

Historically the b subunit dimer has been viewed as a single functional unit. However, the asymmetric nature of the F$_{1}$F$_{0}$ ATP synthase enzyme complex suggested that the functional role of each b subunit should not necessarily be considered equivalent. Protein-protein contacts made by one b subunit cannot be made by the other. In the present work, a unique expression system that facilitates the production, purification, and detection of F$_{1}$F$_{0}$ ATP synthase complexes incorporated with a b subunit heterodimer resulted in a functional enzyme complex. The $b_{Arg-36}$/Ile-V5 subunit provides an intact F$_{1}$ binding domain for interaction with the $\delta$ subunit, and the $b_{153}$/end-His subunit carries out the interaction with the a subunit.
subunit of F$_1$. Similarly, the $b_{\Delta 153}$–end-His subunit must be positioned so that its $b_{\text{Arg-36}}$ makes the appropriate contacts with the F$_0$ subunits (Fig. 7). Incorrect positioning of the mutant $b$ subunits during assembly might be expected to lead to an inactive or partially assembled enzyme complex.

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