Mutations in the Dimerization Domain of the b Subunit from the Escherichia coli ATP synthase

DELETIONS DISRUPT FUNCTION BUT NOT ENZYME ASSEMBLY*

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The b subunit dimer of Escherichia coli ATP synthase serves essential roles as an assembly factor for the enzyme and as a stator during rotational catalysis. To investigate the functional importance of its coiled coil dimerization domain, a series of internal deletions including each individual residue between Lys-100 and Ala-105 (\(b_{\text{AK100}}\)-\(b_{\text{A105}}\), \(b_{\text{AK100-A103}}\), and \(b_{\text{AK100-Q106}}\) as well as a control \(b_{\text{K100A}}\) missense mutation were prepared. All of the mutants supported assembly of ATP synthase, but all single-residue deletions failed to support growth on acetate, indicating a severe defect in oxidative phosphorylation, and \(b_{\text{AK100-Q106}}\) displayed moderately reduced growth. The membrane-bound ATPase activities of these strains showed a related reduction in sensitivity to dicyclohexylcarbodiimide, indicative of uncoupling. Analysis of dimerization of the soluble constructs of \(b_{\text{AK100}}\) and the multiple-residue deletions by sedimentation equilibrium revealed reduced dimerization compared with wild type for all deletions, with \(b_{\text{AK100-Q106}}\) most severely affected. In cross-linking studies it was found that F1-ATPase can mediate the dimerization of some soluble b constructs but did not mediate dimerization of \(b_{\text{AK100}}\) and \(b_{\text{AK100-Q106}}\); these two forms also were defective in F1 binding analyses. We conclude that defective dimerization of soluble b constructs severely affects F1 binding in vitro, yet allows assembly of ATP synthase in vivo. The highly uncoupled nature of enzymes with single-residue deletions in b indicates that the b subunit serves an active function in energy coupling rather than just holding on to the F1 sector. This function is proposed to depend on proper, specific interactions between the b subunits and F1.

Oxidative or photophosphorylation, occurring in bacteria, chloroplasts, and mitochondria, utilizes the enzyme F1-F0-ATP synthase for the synthesis of ATP. The enzyme, which uses the energy of the transmembrane proton gradient developed through cellular respiration or light-driven H\(^+\) pumping, is composed of two sectors, both of which act as molecular motors. In the prototypical enzyme from Escherichia coli, the membrane-integral portion, F0, has three subunits in the stoichiometry \(a_3b_3\gamma_6\delta_2\), and translocates protons through the membrane via rotation of the \(c\) subunit ring. The membrane-peripheral sector, F1, is comprised of five subunits, \(\alpha_3\beta_3\gamma_6\delta_2\). This portion of the enzyme houses the three catalytic nucleotide binding sites and couples rotation of the centrally located \(\gamma\) to the synthesis or hydrolysis of ATP at catalytic sites found at \(\alpha/\beta\) interfaces (1–5).

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3 The abbreviations used are: RHCC, right-handed coiled coil; BS\(^2\), bis(sulfosuccinimidyl)suberate; DCCD, dicyclohexylcarbodiimide.
of the hydrophobic interface of the \( b \) dimer is more important than the strength of dimerization and furthermore that \( b \) has a function beyond simply acting as a stator holding \( F_1 \) and \( F_0 \) together.

**EXPERIMENTAL PROCEDURES**

**General Methods**—Recombinant DNA techniques were performed as described by Sambrook et al. (21). Protein concentrations were determined according to the method of Bradford (22) for all soluble proteins except for \( b \) which was determined using the Advanced Reagent from Cytoskeleton Inc., or membranes that were determined using the method of Lowry et al. (23). SDS-PAGE was carried out using 15% separating gels according to the method of Laemmli (24) and stained with Coomassie Brilliant Blue R-250. Western blotting was performed as described previously (25) using antibodies that were labeled with \(^{125}\text{I} \text{IODO-GEN method} \text{ (26).} \) Polyclonal anti-\( b \) antibodies were raised in a rabbit and affinity-purified from serum. The anti-\( \alpha \) monoclonal antibody (\( \alpha-II \)) was a generous gift from Drs. Robert Aggerel and Rod Capaldi of the University of Oregon. Electrospary ionization mass spectrometry was carried out at the Dr. Don Rix Protein Identification Facility at the University of Western Ontario.

**Enzyme Assays**—Membranes were prepared and assayed for ATP hydrolysis and ATP-dependent proton pumping as described (27). Membranes were labeled with 50 \( \mu \text{M DCCD in 50 mM Tris-HCl, pH} \ 8, \ 5 \text{ mM MgCl}_2, \ 300 \text{ mM KCl and incubated for 15 min at room temperature.} \) Samples were then diluted into that same buffer to determine the membrane bound activity or into 2 mM Tris-HCl, pH 8, 50 \( \mu \text{g/ml bovine serum albumin for the determination of the released activity.} \)

**Plasmid Construction and Mutagenesis**—Plasmid pDM8 (10) encoding a synthetic form of the \( b \) subunit was the parent plasmid for all full-length \( b \) constructs. Similarly, all soluble forms of \( b \) were derived from pSD114 (10) encoding residues 34–156 of \( b \) with an SYW leader sequence. PCR with mutagenic primers was used to create the sequences shown in Fig. 1. Following initial cloning and sequencing, the mutations were moved into pDM8 and pSD114 using unique restriction sites in the synthetic \( b \) sequence (10).

**Minimal Media Growth Tests**—To address the in vivo effect of \( b \) mutations, full-length \( b \) constructs were expressed in the \( uncF \) deletion strain KM2 (28). Oxidative phosphorylation was tested by growth on M9 media plates supplemented with 0.2% acetate as the sole carbon source.

**Protein Purification**—\( b \) was purified as described (11). All other \( b \) proteins were purified as follows. MM294 cells (29) bearing plasmids encoding the soluble constructs were grown in 2X YT medium (16 g of bacto-tryptone, 10 g of bacto-yeast, and 5 g of NaCl per liter) at 37 °C with vigorous shaking. Cultures were induced with isopropylthiogalactoside when \( A_{600} \) reached 0.8 and harvested when cultures reached stationary phase. Cells were washed once, and suspended in a volume corresponding to 10 times their packed wet weight in 50 mM Tris-HCl, pH 8, 10 mM MgCl\(_2\). Phenylmethylsulfonyl fluoride was added to 1 mM, and cells were broken by passage through a French pressure cell at 20,000 p.s.i. The lysate was centrifuged at 38,000 rpm in a Beckman Ti-45 rotor for 90 min. The soluble fraction was precipitated with 45% ethanolamine-HCl, pH 7.5, 1 mM EDTA, 5 mM MgCl\(_2\) (TEA buffer), and loaded onto a Q-Sepharose XL-A analytical centrifuge equipped with absorption optics. A four-hole An-60Ti rotor and six-channel Epon charcoal centerpieces were used. Samples were dialyzed into 10 mM sodium phosphate, pH 7, 1 mM EDTA, 100 mM NaCl. Initial protein concentrations were 0.1 mg/ml. Samples were equilibrated for 24 h at 20 °C and 25,000 rpm. Equilibrium was judged to be attained when scans separated by 4 h were identical. Data were fitted using Microcal Origin 4 with the sedimentation routines supplied by Beckman. Partial specific volumes were determined by the method of Cohn and Edsall (30), and the buffer density was calculated from published tables. Data were analyzed using models for either a single ideal species or else a monomer–dimer equilibrium using the monomer molecular mass determined by mass spectrometry.

**Bis(sulfosucinimidyl)suberate (BS\(_2\)) Cross-linking Studies**—1 \( \mu \text{g of soluble } b \) protein, previously dialyzed into 50 mM triethanolamine HCl, pH 7.5, 1 mM EDTA, 5 mM MgCl\(_2\) (TEA buffer), was diluted into 20 \( \mu \text{l of } b \) TEA buffer supplemented with 1 mM BS\(_2\) (Pierce). The reaction proceeded for 10 min at room temperature and was terminated by addition of 20 \( \mu \text{l of 1 M ethanolamine-HCl, pH 7.5.} \) Protein samples containing 0.02 \( \mu \text{g of } b \) were analyzed by SDS-PAGE under reducing conditions, blotted to polyvinylidene difluoride membranes, and probed with an \(^{125}\text{I-labeled antibody raised against the } b \) subunit of ATP synthase. To test for \( F_1 \) induced dimerization of \( b, 20 \mu \text{g of } F_1 \) was also added to the cross-linking mixture. Control samples without \( F_1 \) or BS\(_2\) were brought up to the experimental total volume using TEA buffer. \( F_1 \) was moved into TEA buffer immediately prior to the experiment using a 5-mL centrifuge column containing Bio-Gel P-10 (Bio-Rad) resin.

**Competitive \( F_1 \) Binding Assays**—The ability of soluble \( b \) constructs to compete with a \( b \) affinity resin for the binding of \( F_1 \) was performed essentially as described by McLachlin et al. (31). 31.3 \( \mu \text{g of } F_1, 0.8 \mu \text{g of } \delta, \) and 20 \( \mu \text{l of the affinity resin} \) were mixed in a final volume of 250 \( \mu \text{l of } 50 \text{ mM Tris-HCl, pH} 7.4, 100 \text{ mM NaCl, } 2 \text{ mM ATP, } 10 \% \text{ glycerol, and } 0.15 \text{ mg/ml bovine serum albumin.} \) Gels were incubated at room temperature for 1 h. Where indicated, soluble \( b \) subunit was added to either 1 or 10 \( \mu \text{M prior to the incubation.} \) Samples were then centrifuged to pellet the resin, the supernatant was discarded, and the pellets were boiled in 50 \( \mu \text{l of SDS-PAGE sample buffer containing DTT.} \) After centrifugation, 10 \( \mu \text{l of the supernatant was analyzed by SDS-PAGE.} \)

**RESULTS**

**Plasmid Construction and Mutagenesis**—To investigate the importance of the coiled coil in the dimerization domain of the \( b \) subunit of ATP synthase, a series of \( b \) deletion mutants were constructed (Fig. 1). Single-residue deletions in the heart of this domain would be expected to interfere with normal helix-helix interactions in the \( b \) subunit dimer. To ensure that the observed effects were not due to loss of specific interactions of any particular residue, we constructed a series of single-residue deletions encompassing residues 100–105 as well as multiresidue deletions \( b_{ \text{K100-A101} } \) and \( b_{ \text{A100-C105} } \), and a control \( b_{ \text{K100} } \) substitution. The deletions of 4 and 7 amino acids remove one or two turns of the \( \alpha \)-helix, respectively, and were expected to have less severe effects on the coiled coil interactions than the single-residue deletions. All mutants were made in the \( b \) subunit expression plasmid pDM8 (10) and expressed in the \( uncF \) deletion strain KM2 (28). The mutations were also moved into the expression plasmid pSD114 (10), which encodes a soluble form of the \( b \) subunit consisting of residues Glu-34 to Leu-156 of wild type \( b \) preceded by a MSYW leader sequence.

**Mutations in the \( b \) Subunit of ATP Synthase**
Mutations in the b Subunit of ATP Synthase

Effect of b Mutations on Oxidative Phosphorylation—The b mutants were tested for their ability to support oxidative phosphorylation by growth on M9 agar plates containing acetate as the sole carbon source. All of the single-residue deletions prevented growth on acetate, indicating an inability to establish an adequate phosphorylation potential (Table 1). The other mutations did support growth on acetate indicating a functional ATP synthase, although the ΔbK100-Q106 mutant showed slow growth giving rise to colonies that were about half the size of the wild type, implying partial loss of function.

Effect of b Mutations on ATP Synthase Assembly—To address the effect the b mutants might have on the assembly and stability of ATP synthase, membranes were prepared and Western blotting was performed, using antibodies raised against the b and α subunits of ATP synthase (Fig. 2). There was no b and extremely little α detected in the membranes prepared from the Δb deletion strain, while all strains carrying mutant b subunits with single residues or groups of residues deleted produced membranes containing amounts of b and α similar to those seen in the wild type, indicating that enzyme assembly was essentially normal.

Effect of b Mutations on Enzymatic Activity—The rate of ATP hydrolysis was determined for both membrane-bound F1F0 and released F1. The single-residue deletions all gave similar results in the ATP hydrolysis assay (data not shown). Since deletion of each individual residue between Lys-100 and Ala-105 also led to virtually identical effects on oxidative phosphorylation and enzyme assembly, these results imply that the effects are not due to specific roles of individual residues but rather to the proper coiled coil structure. Based on these results the single ΔbK100 deletion, for which there is a bK100A control, was chosen as a representative for further detailed analysis.

As seen in Table 2, the Δb mutant had almost no activity, showing 0.07 unit/mg of bound activity and 0.1 unit/mg of released activity. Among the b mutants presented here, there was some small variation in the released activities, with released activities ranging from 1.08 to 1.62 units/mg, suggesting modest differences in expression or assembly that were not detectable by Western blotting. To correct for differences in the amount of ATP synthase present in the membrane vesicles, the normalized rates of ATP hydrolysis (27, 32) were determined by taking the ratio of bound:released activities. In no case was there a substantial difference in the normalized ATPase activities between membranes carrying the b mutations and the wild type, indicating that the b mutations had little effect on the turnover number of the membrane-bound enzyme. There were, however, large differences in the DCCD sensitivity. Of the three deletion mutants shown in Table 2, the ΔbK100-Q106 mutant had almost no activity, showing little sensitivity, membranes carrying the ΔbK100-A103 with 60% sensitivity. The other single-residue deletions gave between 0.34 and 0.47 normalized activity units and showed between 19–34% DCCD sensitivity (data not shown). These results suggest that although the b deletions had little effect on the assembly or activity of ATP synthase, they did result in significant uncoupling of the enzyme. It is important to note that the bK100A mutation had no effect on the rate of ATP hydrolysis nor the DCCD sensitivity showing that the lysine residue at position 100 is not essential.

To further test for coupling of ATP hydrolysis to movement of protons through the membrane, 9-amino-6-chloro-2-methoxyacridine quenching assays were performed (Fig. 3). Membranes prepared from the Δb control strain showed no proton pumping activity, while those from the wild type b and bK100A control strains showed a very strong quenching response bringing the relative fluorescence to less than 20% within 1 min of adding ATP. Despite their marked differences in DCCD sensitivity, membranes carrying the ΔbK100, bK100-A103, and ΔbK100-Q106 all gave similar levels of proton pumping. This level is reduced in comparison with the wild type, confirming that these enzymes are partially uncoupled.

Dimerization Interactions in Soluble b Mutants—To determine the effect of deletions on the dimerization of b, the mutations were moved into a soluble form of the protein consisting of residues 34–156

### Table 1

| b construct            | Plasmid/strain | Average colony size |
|------------------------|----------------|---------------------|
| Δb                     | pSD80/KM2      | No growth*          |
| Wild type b            | pDM18/KM2      | 1.3 mm              |
| bK100A                 | pDC19/KM2      | 1.2 mm              |
| bΔK100                 | pSD295/KM2     | No growth           |
| bΔA101                 | pSD296/KM2     | No growth           |
| bΔV102                 | pSD297/KM2     | No growth           |
| bΔA103                 | pSD298/KM2     | No growth           |
| bΔQ104                 | pSD299/KM2     | No growth           |
| bΔA105                 | pDC29/KM2      | 0.9 mm              |
| bΔK100-A103            | pDC28/KM2      | 0.6 mm              |

* Colony sizes are averages of five determinations

### Notes

1. Colony sizes are averages of five determinations.
2. Membrane samples containing 2 μg of membrane protein were analyzed by SDS-PAGE followed by Western blotting. Blots were probed with 125I-labeled antibodies raised against α and b.
3. The ratio of bound:released activities. In no case was there a substantial difference in the normalized ATPase activities between membranes carrying the b mutations and the wild type, indicating that the b mutations had little effect on the turnover number of the membrane-bound enzyme. There were, however, large differences in the DCCD sensitivity. Of the three deletion mutants shown in Table 2, the DCCD sensitivity followed the same trend as the in vivo growth tests, with the worst being the ΔbK100 mutant at 34% sensitivity, followed by the ΔbK100-Q106 with 56% sensitivity, and the ΔbK100-A103 with 60% sensitivity. The other single-residue deletions gave between 0.34 and 0.47 normalized activity units and showed between 19–34% DCCD sensitivity (data not shown). These results suggest that although the b deletions had little effect on the assembly or activity of ATP synthase, they did result in significant uncoupling of the enzyme. It is important to note that the bK100A mutation had no effect on the rate of ATP hydrolysis nor the DCCD sensitivity showing that the lysine residue at position 100 is not essential.

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TABLE 2
ATPase activity of membrane vesicles

Membranes were suspended to 2 mg/ml in 50 mM Tris-HCl, pH 8, 5 mM MgCl$_2$, 300 mM KCl in the presence or absence of 50 μM DCCD and incubated for 15 min at room temperature. Membranes were then diluted into ATPase assays as described under “Experimental Procedures.” Data shown are the average of triplicate assays ± S.D. 1 unit of activity releases 1 μmol of product per min.

| ATP synthase type | Membrane-bound ATPase activity | DCC sensitivity | Released ATPase activity | Normalized activity$^a$ |
|------------------|-------------------------------|-----------------|--------------------------|------------------------|
|                  | units/mg                      | %               | units/mg                 |                        |
| Δb               | 0.07 ± 0.02                   | N/A$^b$         | 0.10 ± 0.03              | N/A                    |
| Wild type b      | 0.60 ± 0.01                   | 75.7$^b$        | 1.62 ± 0.05              | 0.37 ± 0.01            |
| b$_{K100}$       | 0.60 ± 0.01                   | 78.7$^b$        | 1.52 ± 0.05              | 0.39 ± 0.02            |
| b$_{K100}$       | 0.43 ± 0.01                   | 33.8$^b$        | 1.08 ± 0.03              | 0.40 ± 0.02            |
| b$_{K100-A103}$  | 0.45 ± 0.01                   | 60.1$^b$        | 1.31 ± 0.02              | 0.34 ± 0.01            |
| b$_{K100}$-Q106  | 0.47 ± 0.02                   | 56.4$^b$        | 1.16 ± 0.02              | 0.41 ± 0.02            |

$^a$ Membrane-bound activities were normalized to the activity released from membranes and expressed as the fraction of units bound per units released.

$^b$ N/A, not applicable.

TABLE 3
Sedimentation equilibrium analysis of soluble b mutants

Sedimentation equilibrium experiments were carried out at 20 °C in 10 mM sodium phosphate, 100 mM sodium chloride, 1 mM EDTA, pH 7.0, and analyzed using the Origin 4 software package. $K_d$ values were calculated from the average molecular mass and the calculated monomer molecular mass.

| Polypeptide | Plasmid | Monomer molecular mass$^a$ | Average molecular mass$^a$ | $K_d$ |
|-------------|---------|---------------------------|----------------------------|--------|
|             |         |                           |                            | Da     |
| Soluble b$^b$ | pSD114  | 13,323.58                  | 24,000 ± 1640              | 0.65   |
| Soluble b$_{K100}$ | pWX1  | 13,267.19$^b$              | 21,600 ± 1120              | 3.3    |
| Soluble b$_{K100}$ | pDC18  | 13,889.96                  | 15,600 ± 820               | 78     |
| Soluble b$_{K100-A103}$ | pDC32 | 13,606.66                  | 16,700 ± 903               | 36     |
| Soluble b$_{K100}$-Q106 | pDC31 | 13,279.15                  | 13,900 ± 1000              | 310    |

$^a$ Membrane-bound activities were normalized to the activity released from membranes and expressed as the fraction of units bound per units released.

$^b$ N/A, not applicable.

$^c$ Monomer molecular masses are based on mass spectrometry results, which indicated the removal of the N-terminal methionine residue. In the cases of wild type b and b$_{K100}$, there was cleavage of the first five residues at the N terminus (SYWEK) during the protein purification procedures.

$^d$ Average molecular mass determined for a single ideal species, presented as the average ± S.E. for three determinations.

FIGURE 3. ATP-dependent proton pumping by membrane vesicles carrying F$_{1}$F$_{0}$ bearing the different b mutations. 500 μg of membranes were suspended in 2 ml of 50 mM HEPES-KOH, pH 7.5, 5 mM MgCl$_2$, 150 mM KCl (HMK buffer) and 9-amino-6-chloro-2-methoxyacridine was added to 0.3 μg/ml. Excitation and emission wavelengths were 410 and 490 nm, respectively. At the times indicated ATP and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) were added to 2 mM and 2 μM, respectively. Traces represent Δb (curve a), wild type b (curve b), b$_{K100}$ (curve c), b$_{K100}$ (curve d), b$_{K100}$-Q106 (curve e), and b$_{K100}$-A103 (curve f).

(Δb$_{67–156}$), purified, and analyzed by sedimentation equilibrium analytical ultracentrifugation (Table 3). To distinguish these soluble forms of b from their full-length counterparts, they will be referred to as “soluble b mutation.” At 20 °C, soluble b showed an average molecular mass of 24,000 Da, almost twice the calculated monomer molecular mass of 13,324 Da. Analysis of the same data using a monomer–dimer model yielded a dissociation constant ($K_d$) of 0.65 μM. Soluble b$_{K100}$ showed similar results with an average molecular mass of 21,600 Da or a $K_d$ of 3.3 μM. While the dissociation constants may seem significantly different, it should be noted that a limitation of this method is the high degree of error involved in calculating a $K_d$ near the extremes of either complete monomer or complete dimer. With this limitation in mind, it is clear that the deletion mutants show clear deficiencies in their ability to form a dimer in solution. Soluble b$_{K100}$ showed an average molecular mass of 15,600 Da, and a much elevated $K_d$ of 78 μM. Similarly, soluble b$_{K100}$-A103 and soluble b$_{K100}$-Q106 showed average molecular masses of 16,700 and 13,900 Da, giving $K_d$ values of 36 and 310 μM, respectively. Given the failure of soluble b$_{K100}$ to support oxidative phosphorylation in vivo, and the greater expected disruption of the hydrophobic surface, it was surprising that the b$_{K100}$-Q106 mutant led to the most drastic destabilization of the soluble dimer.

To further characterize the effects that these mutations have on the dimerization of soluble b, and to determine whether F$_{1}$ might mediate the dimerization, BS$^3$ cross-linking was conducted both in the presence and absence of F$_{1}$ (Fig. 4). The effect of added F$_{1}$ was initially addressed using b$_{67–156}$ (A), a soluble form of b consisting of residues 67–156 previously shown to be defective in dimerization (7). While wild type soluble b readily formed cross-linked dimers either with or without F$_{1}$ present, cross-linking of b$_{67–156}$ was strongly stimulated by F$_{1}$, indicating F$_{1}$-mediated dimerization (A). Soluble b$_{K100}$ readily formed a dimer that was slightly enhanced by the addition of F$_{1}$. Neither soluble b$_{K100}$ nor soluble b$_{K100}$-Q106 were cross-linked (C), indicative of a failure to dimerize, supporting the sedimentation equilibrium results, and even the addition of F$_{1}$ failed to promote dimerization, indicating a more severe disruption of interaction. The soluble b$_{K100}$-A103 mutant showed modest cross-linking that was moderately enhanced by the addition of F$_{1}$. Overall, these cross-linking results were consistent with the sedimentation equilibrium studies regarding the strength of dimerization in solution and furthermore indicate that dimerization of some b constructs may be mediated by F$_{1}$.

F$_{1}$ Competition Binding Assays—To further investigate whether b mutants could bind to F$_{1}$, competition binding assays (Fig. 5) were performed as described by McLachlin et al. (31). In this method, soluble b mutants compete with b conjugated to an agarose resin for the binding of F$_{1}$. Experiments conducted using a control resin with no conjugated b had only small amounts of F$_{1}$ trapped within the resin, whereas the affinity resin with no added competitor, labeled “No b,” bound substantial F$_{1}$. The specific binding of F$_{1}$ to the affinity resin was greatly reduced by addition of soluble wild type b to 1 μM and eliminated by addition of b to 10 μM. Similar results were obtained with soluble b$_{K100}$, while soluble b$_{K100}$-A103 showed slightly reduced competition with the affin-
Mutations in the β Subunit of ATP Synthase

In a coiled coil model for β subunit dimerization and assembly of ATP synthase, deletion of four or seven residues would remove one or two turns of the helix, respectively, maintaining the hydrophobic face of the amphipathic α-helix and should show only minor effects on assembly and function. Consistent with this expectation, cells expressing the bΔK100 and bΔK100-Q106 mutations were capable of oxidative phosphorylation, but cells expressing a single-residue deletion between residues Lys-100 and Ala-105 were not. Recently, Bhatt et al. (20) have shown that 4 residues can be inserted into the dimerization domain at positions 90 or 102 with minimal effect on growth and enzyme assembly.

It was particularly interesting that although the bΔK100-Q106 mutant was able to perform oxidative phosphorylation, it did show a reduced growth rate on acetate media. This deletion would remove an entire heptad repeat and would be expected to interrupt the hydrophobic surface on the helical face, preventing β subunit dimerization and assembly of ATP synthase. Deletion of four or seven residues would remove one or two turns of the helix, respectively, maintaining the hydrophobic face of the amphipathic α-helix and should show only minor effects on assembly and function. Consistent with this expectation, cells expressing the bΔK100 and bΔK100-Q106 mutations were capable of oxidative phosphorylation, but cells expressing a single-residue deletion between residues Lys-100 and Ala-105 were not. Recently, Bhatt et al. (20) have shown that 4 residues can be inserted into the dimerization domain at positions 90 or 102 with minimal effect on growth and enzyme assembly.

Dimerization of b Is Required to Bind F1-ATPase in Vitro but Is Not Essential to Assembly of ATP Synthase—Previously it was shown that dimerization of the b subunit is well correlated with its ability to bind to F1-ATPase (17), and b dimerization was suggested to be required for the assembly of F1 onto F0. Our results generally confirm the relationship between b dimerization and F1 binding in vitro, as the bΔK100 and bΔK100-Q106, which showed the weakest dimerization (Table 3), also gave the weakest competition to the resin-bound b in the F1 binding assay (Fig. 5) and the least cross-linking in either the presence or absence of F1 (Fig. 4).

There was, however, a discrepancy between results obtained with soluble b in vitro and the in vivo properties of the full-length constructs in that despite the drastically reduced tendency of soluble bΔK100 and soluble bΔK100-Q106 mutants to form dimers or to bind to F1-ATPase, the full-length version of these mutants still allowed assembly of an ATP synthase complex. Thus, significant in vitro dimerization does not appear to be obligatory for assembly in vivo. It is noteworthy that the presence of the N-terminal membrane-spanning domain will bring the two b subunits into close proximity on the membrane and could thus favor dimerization by making it an essentially uni-molecular reaction. A
second discrepancy is that, while $b_{LD100-Q106}$ was the most weakened of the constructs in dimerization, it retained sufficient function to allow growth on acetate, while the single-residue deletions did not. Clearly, additional factors besides dimerization strength affect the severity of the mutations in vivo.

Mediation of b Dimerization by F1—Although to the best of our knowledge it has not been previously demonstrated, one would expect that if dimerization of b is essential for binding to F1, then the addition of F1 should stimulate dimerization of weakly interacting b mutants as we have seen (Fig. 4). This effect was strong for $b_{67–156}$, but moderate for $b_{AK100-A103}$ and hardly apparent for $b_{AK100}$ or $b_{LD100-Q106}$. We suggest that the explanation for this difference is related to effects of the mutations on the interactions of b with F1. Based on previous mapping of the $b_{2}-F_1$ interaction by cross-linking analysis (35), those residues removed by the N-terminal deletion in the $b_{67–156}$ construct are not located proximal to F1. Thus, if this construct were made dimeric by some means, one would expect that its interactions with F1 would be normal, so it could be considered an ideal candidate for F1-mediated dimerization. In contrast, the deletions in the Lys-100 region are proximal to F1 and therefore much more likely to affect the interaction. The deleted residues may interact with F1 directly; if not, the disruption of the hydrophobic surfaces of the helices would still be expected to alter the dimeric $b_{2}$ structure in a way that would compromise the normal interaction with F1. Consistent with these considerations, the single-residue deletions, which would be expected to have the largest structural effect for reasons described above, also caused the greatest effect in vivo.

Role of the b Subunit in Energy Coupling—It is generally accepted that b must play a crucial role in ATP synthase by acting as a stator that holds the $\alpha_3\beta_3\gamma_0$ complex stationary with respect to the a subunit of F0 during the rotation of $\gamma_{\epsilon 10}$. The intrinsic asymmetry associated with having a 3-stepping $F_0$, and 10-stepping $F_0$ motor suggests that the connections between them must have some elastic properties and cannot be rigid unyielding structures. Nevertheless, the generally accepted function of the b subunit is to hold on to F1.

Here we have described single-residue deletions in the dimerization domain of b that assemble into an ATP synthase that cannot support growth by oxidative phosphorylation. These mutants had relatively normal levels of membrane-bound ATPase activity that showed an extremely reduced sensitivity to the F0-specific inhibitor DCCD. This reduced sensitivity implies that the ATP synthase is uncoupled and thus provides biochemical evidence that b plays a critical role in energy coupling. We suggest that this function requires not simply that b binds to F1 but rather depends on specific and proper interactions, both between the two b subunits and with F1. The moderately reduced proton pumping seen with the deletion mutations (Fig. 3) indicate some level of residual coupled activity, at least in ATP-dependent proton pumping. It will be interesting to determine in the future whether the single-residue deletions completely abolish ATP synthesis or simply reduce the phosphorylation potential that can be achieved in the cell.

It has been suggested that the b subunit could provide a mechanism for elastic energy storage (36), and that the RHCC could be an important part of this mechanism (6). In a RHCC, the two helices interact through the relatively flat hydrophobic surface created by the small residues in the a and h positions, a feature that may allow them to slide relative to one another. In support of this idea, b dimerization is relatively weak (7). In contrast, a LHCC conformation with the typical knobs-into-holes packing would prevent such movements. Thus, movement of the helices in the direction parallel to the superhelical axis could be an important part of energy coupling and would provide an explanation for the occurrence of the unusual structure.

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