Identification of an uncoupling mutation affecting the *b* subunit of F_1F_0 ATP synthase in *Escherichia coli*

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Abstract **A specific *b* subunit arginine, b_{Arg-36} in *Escherichia coli*, displays evolutionary conservation among bacterial F_1F_0 ATP synthases. Site-directed mutagenesis was used to generate a collection of mutations affecting b_{Arg-36}. The phenotype differed depending upon the substitution, and the $b_{Arg-36 \rightarrow Glu}$ and $b_{Arg-36 \rightarrow Ile}$ substitutions virtually abolished enzyme function. Although the total amounts of F_1F_0 ATP synthase present in the membranes prepared from mutant strains were reduced, the primary effect of the b_{Arg-36} substitutions was on the activities of the intact enzyme complexes. The most interesting result was that the $b_{Arg-36 \rightarrow Glu}$ substitution results in the uncoupling of a functional F_0 from F_1 ATP hydrolysis activity.**

Key words: F_1F_0 ATP synthase; Proton translocation

1. Introduction

Proton translocating adenosine triphosphate (F_1F_0 ATP) synthases are located in the inner mitochondrial membrane, the chloroplast thylakoid membrane and bacterial cytoplasmic membranes. The enzymes function by using the electrochemical gradient of protons across these energy transducing membranes to drive the synthesis of ATP [1,2]. Each of the F_1F_0 ATP synthases has homologous subunits and shares a common molecular architecture. The F_1 sector consists of the $\alpha_3\beta_3\gamma\delta\varepsilon$ subunits. High resolution structural analysis of the bovine F_1 showed that the catalytic sites in the three β subunits exist in differing conformations due to their positions relative to the γ subunit [3]. Each of the β subunits cycles through the different conformations as a result of rotation of the γ subunit relative to the $\alpha_3\beta_3$ hexamer [4–6]. The F_0 sector conducts proton translocation, and in *Escherichia coli*, consists of the ab_2c_{10} subunits. The *c* and *a* subunits are thought to participate directly in proton conduction through the membrane.

The dynamic nature of the catalytic mechanism has recently focused attention on the F_0-F_1 boundary [7]. Movement of the γε rotor either originates as a rotation within F_0 which is propagated to F_1 through the interactions between the F_1 and F_0 subunits, or alternatively, occurs at the boundary between F_1 and F_0 as a property of the interactions between F_0 and the rotor. The *c* subunit appears to have direct contact with both the γ and the ε subunits of the rotor [8,9]. A second discreet area of interaction between F_1 and F_0 is through the hydrophilic domains of the *b* subunits. The *b* subunit hydrophilic domains form a dimer [10,11], and formation of the dimer is necessary for interaction with F_1 [12]. The *b* subunit appears to be in direct contact with F_1 through the δ subunit [13,14]. The observation that the δ subunit can be fixed to a specific β subunit without loss of activity has led to the suggestion that the *b*-δ interaction is the basis of a 'stator' which holds the $\alpha_3\beta_3$ hexamer in place, allowing the γε rotor to turn within the complex [15]. Many different mutations in the hydrophilic domain of the *b* subunit [16,17] and the δ subunit [18,19] affect F_1F_0 ATP synthase function. Typically, the *uncF*(*b*) gene mutations which alter the hydrophilic segment of the subunit result in reduced levels of intact F_1F_0 ATP synthase complex in the membrane [20]. These mutations have been correlated with a failure in *b* subunit dimerization [12,13].

In this paper we continue to assess the role of the *b* subunit in the 'stator' by introducing mutations affecting the conserved b_{Arg-36}. This residue is located at the base of the *b*-δ stalk, proximal to the lipid bilayer. The site occupied by b_{Arg-36} proved to be very sensitive to substitution by other amino acids, and the phenotypes differed markedly from previously reported *uncF*(*b*) mutations. Although the amount of enzyme complex formed was altered, the major effect of several b_{Arg-36} mutations was on the activity of the intact F_1F_0 ATP synthase. Included in the collection is the first *uncF*(*b*) mutation which uncouples catalytic activity from proton translocation.

2. Materials and methods

2.1. Strains and media

Growth media were either Luria broth supplemented with 0.2% (w/v) glucose (LBG) or minimal medium consisting of A salts supplemented with 0.2% (w/v) succinate or 5 mM glucose [21]. Chemicals for mixing of the media were obtained from Difco Labs (Detroit, MI) and Sigma Chemical Corporation (St. Louis, MO). Isopropyl-1-thio-β-D-galactoside (IPTG) (0.4–40 μg/ml), ampicillin (Ap) (100 μg/ml), and chloramphenicol (Cm) (30 μg/ml) were added to media as appropriate. Growth of aerobic liquid cultures was performed with constant agitation and monitored using a Klett-Summerson colorimeter. Anaerobic conditions were achieved using a BBL 'Gas Pak' (Beckton Dickinson Microbiology Systems, Cockeysville, MD) anaerobic jar which had been flushed with N_2 prior to establishing the anaerobic atmosphere. Unless otherwise indicated, incubations of cultures were at 37°C.

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Abbreviations: LBG, Luria broth supplemented with glucose; IPTG, Isopropyl-1-thio-β-D-galactoside; Ap, ampicillin; Cm, chloramphenicol; PCR, polymerase chain reaction; ACMA, 9-amino-6-chloro-2-methoxyacridine; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone

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2.2. Recombinant DNA techniques

Plasmid DNA was purified by CsCl-ethidium bromide gradient ultracentrifugation or with the Qiagen Mini-Prep kit (Qiagen Inc., Santa Clarita, CA). Synthesis of oligonucleotides and determination of nucleotide sequences were conducted by the core facilities of the University of Florida Interdisciplinary Center for Biotechnology Research. Restriction endonuclease reactions, ligation reactions and transformations were performed according to the recommendations of the suppliers (New England Biolabs, Beverly, MA, and Gibco-BRL, Gaithersburg, MD). DNA fragments and polymerase chain reaction (PCR) products were separated by agarose gel electrophoresis, eluted from the agarose and purified using the QIAquick kit (Qiagen Inc.).

2.3. Mutagenesis and strain construction

Plasmids pRPG51(b,δ) [22] and pKAM14(b) [20] were used to construct mutations of the b subunit at Arg-36. Mutations were generated using PCR mutagenesis essentially as described previously [23]. The forward and reverse primers used to amplify a segment of DNA using pRPG51(b,δ) as a template were AAAATAAACAAATAGGGGTTCCGCG located 5′ with respect to the *uncF*(b) gene in the vector sequence and CACGTTTACGCTCGGCTTCAATTT which annealed within the *uncF*(b) coding sequence, respectively. The mutagenic primers used to replace the Arg-36 codon with codons specifying amino acid substitutions were as follows: for Ile and Lys GCAGCAATTGAAAAA(AG)(ACT)ACAAAAAG and CTTTTTGT(AGT)(CT)TTTTTCAATTGCTGC; for Ser, Cys, and Tyr GCAGCAATTGAAAAAT(GCA)CCAAAAAG and CTTTTTGG(CGT)ATTTTTCAATTGCTGC; and for Glu GCAGCAATTGAAAAAGAGCAAAAAG and CTTTTTGCTCTTTTTCAATTGCTGC. In each case, a silent mutation generating a *Mun*I site was included in each mutagenic oligonucleotide for screening purposes. The PCR reactions were carried out in a Coy TempCycler II model for 30 cycles. The PCR products were digested with *Aat*II and *Ppu*MI and then ligated to the 3 kilobase pair *Aat*II/*Ppu*MI fragment of plasmid pKAM14(b). Plasmid DNA was prepared from transformants, and recombinant plasmids were selected for the presence of a *Mun*I site. The mutations were identified by nucleotide sequence determination.

Plasmids encoding the b_{Arg-36} mutations in pKAM14(b) plasmids, as well as control plasmids pKAM14(b) and pBR322, were transformed into *E. coli* strain KM2(Δb) which has been deleted for *uncF*(b) in the chromosome [16]. The strains were also transformed with plasmid pKAM16(*lac*I^q) to provide improved regulation of the plasmid encoded *uncF*(b) genes.

2.4. Preparation of cell fractions

Inverted membrane vesicles were prepared from cultures grown in 500 ml LBG-Ap-Cm medium containing IPTG (40 μg/ml). Cells were harvested and washed in STEM buffer (0.1 M *N*-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid, 20 mM magnesium acetate, 0.25 M sucrose, 0.25 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, 40 mM ε-amino-*n*-caproic acid, pH 7.0), and then suspended in STEM buffer containing 5 mM *p*-aminobenzamidine, 1 mM dithioerythritol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml DNaseI. Cells were disrupted using a French Press at 12 000 psi. Unbroken cells and debris were pelleted by two successive centrifugations (8000×g, 10 min), and the membranes were collected by ultracentrifugation (150 000×g, 1.5 h). Membranes were washed in B_2 buffer (50 mM Tris-HCl, 5 mM $MgSO_4$, 1 mM dithioerythritol, 6 mM *p*-aminobenzamidine, 10% (v/v) glycerol, pH 7.5). The final membrane pellet was resuspended in B_2 buffer and stored at 4°C. Membrane vesicles stripped of F_1 were prepared essentially as described previously [24]. Treatment of membrane fractions with 50 μM dicyclohexylcarbodiimide was performed by incubation at 37°C of the membrane fractions for 15 min. Protein concentrations were determined by a modified Lowry procedure [25].

2.5. Assays of F_1F_0 ATP synthase activity

Membrane energization of vesicle preparations was detected by the fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) [26]. ATP hydrolysis activity of membrane fractions was assayed by the acid molybdate method [27] or by the method of Taussky and Shorr [28]. Membranes were assayed in buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, pH 9.1) for determinations of linearity with respect to both time and enzyme concentration. Rates of $\Delta\mu H^+$-driven ATP synthesis were determined as described by Al-Shawi et al. [29].

2.6. Immunoblot analysis

Membrane fractions were resuspended to a concentration of 1 mg/ml in buffer (62.5 mM Tris-HCl, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 3% SDS, pH 6.8), incubated for 4 min at 95°C, and subjected to polyacrylamide gel electrophoresis on 15% Tris-Glycine BioRad Ready Gels (BioRad, Hercules, CA). Proteins were electroblotted onto nitrocellulose. Antibody incubation was performed essentially as described by Tamarappoo et al. [30] using b subunit-specific antibodies, a kind gift of K. Altendorf and G. Deckers-Hebestreit. Secondary antibody incubation was performed with horseradish peroxidase-linked donkey anti-rabbit antibody, and antibody binding was detected by chemiluminescence using the ECL system (Amersham Corp., Arlington Heights, IL). Signals were visualized on Hyperfilm-HCL (Amersham Corp.). Strength of signal was quantified using a Kodak image digitizing system (DC120).

3. Results

3.1. Construction of uncF(b) gene mutations

Analysis of the primary amino acid sequences of a diverse list of b subunits found that Arg-36 was conserved in bacterial F_1F_0 ATP synthases (Fig. 1). In order to test the importance of this site for assembly and function of F_1F_0 ATP synthase, a collection of mutations were constructed at b_{Arg-36} of the *E. coli* subunit by PCR mutagenesis in plasmid pKAM14(b). The effects of the b_{Arg-36} replacements were studied by complementation of strain KM2(Δb) [16].

3.2. Growth characteristics of mutants

E. coli strains which are defective in ATP synthase cannot derive energy from nonfermentable sources, so growth on succinate minimal medium was used as a qualitative measure of F_1F_0 ATP synthase activity in vivo. Strain TC212($b_{Arg-36\rightarrow Lys}$) supported growth indistinguishable from that of KM200(b) at both low and high IPTG concentrations (Table 1). Reduced growth was observed for strains TC216($b_{Arg-36\rightarrow Ser}$), TC217($b_{Arg-36\rightarrow Cys}$) and TC218($b_{Arg-36\rightarrow Tyr}$). Strains TC211($b_{Arg-36\rightarrow Ile}$) and TC215($b_{Arg-36\rightarrow Glu}$) failed to support growth on succinate

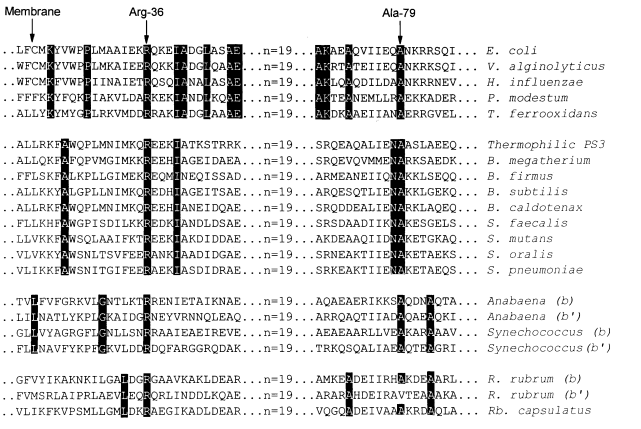

Fig. 1. Amino acid sequence alignment of bacterial b and b' subunits. Protein sequences of b and b' subunits of bacterial F_1F_0 ATP synthases were aligned with respect to where the protein is predicted to exit the membrane. The bacterial sequences are grouped based on phylogeny. Conserved amino acids are indicated in black. Sequences were plotted consecutively without omission of amino acids. The positions of *E. coli* b_{Arg-36} and b_{Ala-79} are indicated. Sequence data were obtained from GenBank.

minimal medium regardless of inducer concentration. However, under conditions of lower incubation temperature (30°C), strain TC215($b_{Arg-36\rightarrow Glu}$) produced very small colonies. No conditions were found in which strain TC211($b_{Arg-36\rightarrow Ile}$) could grow using succinate as a carbon source. Since high levels of IPTG yielded the largest cell mass, 40 μM IPTG was added to media for all subsequent experiments.

3.3. Assembly of F_1F_0 ATPase

The steady-state level of the *b* subunit can also be interpreted as indicative of incorporation of the *b* subunit into the F_1F_0 ATP synthase complex, since the subunit is not stable free of the complex [7,19]. An immunoblot analysis using anti-*b* subunit antibody was performed on membrane vesicles. Membranes derived from all of the Arg-36 mutants had reduced levels of *b* subunit present (Fig. 2). Densitometry revealed that the *b* subunit levels ranged from 25% to 60% of wild-type levels. For the strains with altered growth characteristics, the *b* subunit levels were 25% of wild-type levels for TC215($b_{Arg-36\rightarrow Glu}$) membranes to 53% for the TC211($b_{Arg-36\rightarrow Ile}$) samples.

Total membrane-associated F_1 ATP hydrolysis activity is a good test of F_1F_0 ATP synthase complex assembly, since F_1 has little affinity for the membrane in the absence of the F_0 subunits. F_1 ATPase activity was determined under high pH conditions to remove the influence of F_0 [31]. Reductions in the levels of *b* subunit observed in the immunoblot studies should be reflected in the amount of total F_1 ATPase activity in the membrane. Within the limits of experimental error, the predicted correlation between the steady-state levels of *b* subunit and total membrane-associated F_1 ATPase activity was confirmed. The membranes derived from strain TC212($b_{Arg-36\rightarrow Lys}$) had the highest level of F_1 ATPase of the mutants tested (85% of wild-type KM200(*b*)), and all of the other membranes had between 35% and 52% of activity relative to the strain KM200(*b*) preparations (Table 1).

In every case, the levels of *b* subunits and the amounts of F_1 ATPase in membranes prepared from the Arg-36 mutants should have been sufficient to yield substantial growth if the F_1F_0 ATP synthases containing the altered *b* subunits were fully active. The decrease in F_1F_0 ATP synthase in strain TC212($b_{Arg-36\rightarrow Lys}$) was apparently not sufficient to reduce the capacity of the cells to generate ATP to the rate-limiting threshold for growth. The threshold was surpassed in strains TC216($b_{Arg-36\rightarrow Ser}$), TC217($b_{Arg-36\rightarrow Cys}$) and TC218($b_{Arg-36\rightarrow Tyr}$). The more interesting observation was that the loss of in vivo F_1F_0 ATP synthase function did not correlate with the amount of assembled enzyme complex detected in the membranes of strains TC215($b_{Arg-36\rightarrow Glu}$) and TC211($b_{Arg-36\rightarrow Ile}$). Therefore, the losses in F_1F_0 ATP synthase function in the two strains were not simply attributable to a reduction in enzyme levels.

Table 1
In vivo and in vitro activities of b_{Arg-36} mutants

Strain	Mutation	IPTG (μM)[a]					ATP hydrolysis[b]
		30°C		37°C			
		0.4	40	0	0.4	40	(μmol/min/mg)
KM200	Wild-type	++	+++	++	++	+++	0.90 ± 0.11
KM322	Control	−	−	−	−	−	0.15 ± 0.05
TC211	$b_{Arg-36\rightarrow Ile}$	−	−	−	−	−	0.53 ± 0.14
TC212	$b_{Arg-36\rightarrow Lys}$	++	+++	++	++	+++	0.79 ± 0.17
TC215	$b_{Arg-36\rightarrow Glu}$	−	+	−	−	−	0.45 ± 0.05
TC216	$b_{Arg-36\rightarrow Ser}$	+	+	−	+	++	0.41 ± 0.08
TC217	$b_{Arg-36\rightarrow Cys}$	+	+	−	+	++	0.50 ± 0.07
TC218	$b_{Arg-36\rightarrow Tyr}$	+	++	+	+	++	0.54 ± 0.06

[a] *E. coli* strains were grown aerobically on solid succinate minimal media with antibiotics. IPTG was added as indicated. Colony size was scored after 72 h incubation at either 30°C or 37°C as: +++, ≥0.4 mm; ++, 0.1–0.4 mm; +, <0.1 mm; or −, no growth.
[b] ATPase activities were measured as described in Section 2 on membranes obtained from strains grown in LBG-AP-Cm, 40 μM IPTG at 37°C.

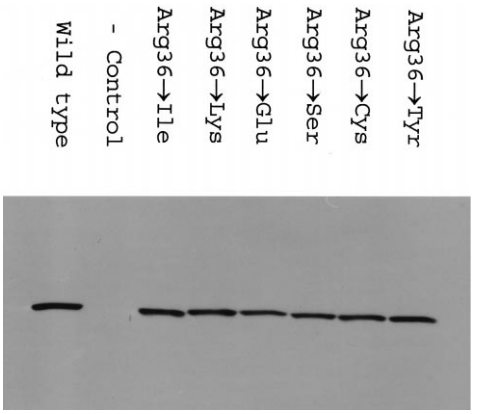

Fig. 2. Immunoblot analysis of *uncF*(*b*) gene mutants. Membrane fractions were separated on a 15% polyacrylamide Tris-Glycine SDS gel. Lanes were loaded with 15 μg total membrane protein. The blot was incubated with *b* subunit-specific antibodies. The secondary antibody was horseradish peroxidase-linked donkey anti-rabbit antibody, and antibody binding was detected by chemiluminescence. The *b* subunit substitutions are indicated above each lane.

3.4. Coupled activities

F_1F_0 ATP synthase-mediated ATP-driven proton pumping

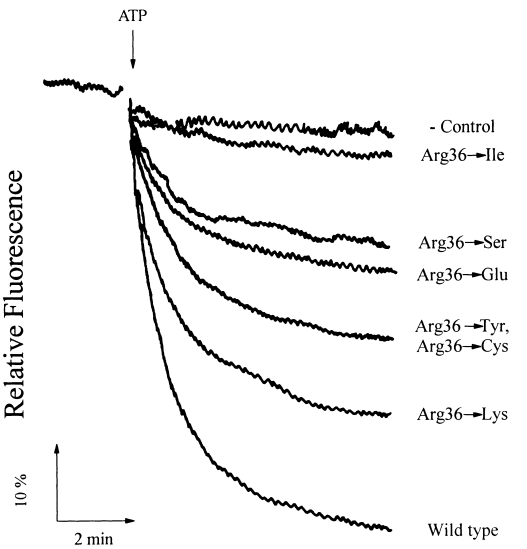

Fig. 3. ATP-driven energization of membrane vesicles prepared from *uncF*(*b*) gene mutants. 250 μg of membrane protein was suspended in assay buffer (50 mM MOPS, 10 mM $MgCl_2$, pH 7.3). ACMA was added to a final concentration of 1 μM, and fluorescence was recorded with an excitation at 410 nm and an emission at 490 nm. ATP was added as indicated to a final concentration of 0.4 mM. The *b* subunit substitutions are labeled next to each trace.

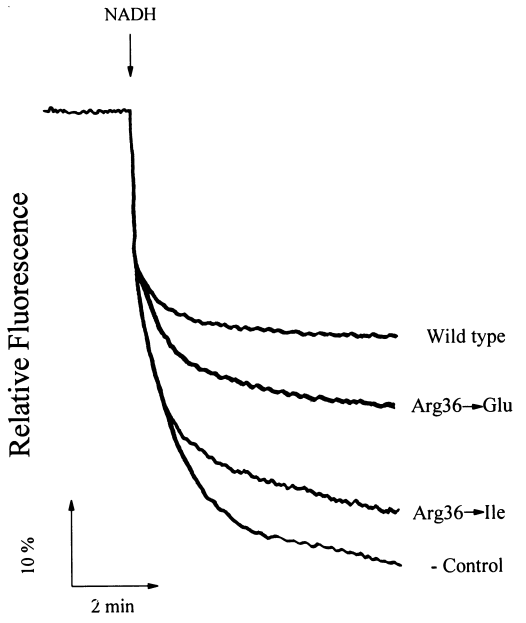

Fig. 4. Proton permeability of stripped membrane vesicles prepared from *uncF*(*b*) gene mutants. Membrane vesicles were prepared, and F_1 was stripped by gentle agitation in a low ionic strength buffer (1 mM Tris-HCl, 0.5 mM EDTA, 2.5 mM 2-mercaptoethanol, 10% (v/v) glycerol, pH 8.0). ACMA fluorescence was recorded after the addition of NADH to a final concentration of 0.5 mM. The *b* subunit substitutions are labeled next to each trace.

activity in membrane vesicles prepared from the Arg-36 mutants was used as an initial indication of coupled activity. Acidification of inverted membrane vesicles was monitored by fluorescence of ACMA. The level of NADH-driven fluorescence quenching of ACMA was strong and comparable in all preparations tested, indicating that the membranes were intact closed vesicles (data not shown). Membranes derived from strains TC212($b_{Arg-36\rightarrow Lys}$), TC216($b_{Arg-36\rightarrow Ser}$), TC217($b_{Arg-36\rightarrow Cys}$) and TC218($b_{Arg-36\rightarrow Tyr}$) had reduced ATP-driven proton pumping activity, reflecting the reduced amounts of assembled enzymes present (Fig. 3). Although the change in fluorescence in this assay is not strictly linear with respect to activity, it can be inferred that the intact F_1F_0 ATP synthase complex containing the altered *b* subunits was fully competent for coupled activity.

In contrast, ATP-driven proton pumping activity was nearly undetectable, despite the fact that levels of protein and F_1 activity comparable to the other strains were observed in membranes prepared from strain TC211($b_{Arg-36\rightarrow Ile}$) (Fig. 3). Membranes from strain TC211($b_{Arg-36\rightarrow Ile}$) that were stripped of F_1 were somewhat leaky when tested for NADH-driven quenching of ACMA fluorescence (Fig. 4). A much more interesting behavior was observed with membranes from strain TC215($b_{Arg-36\rightarrow Glu}$). These membranes had substantial amounts of ATP-driven proton pumping activity (Fig. 3). Since proton pumping is the physiological role of F_1F_0 ATP synthase under anaerobic conditions, strain TC215($b_{Arg-36\rightarrow Glu}$) was tested for growth on glucose in an anaerobic environment. The mutant grew well at 37°C on glucose minimal medium under anaerobic conditions yielding colony sizes comparable to the positive control strain KM200(*b*) (data not shown). One implication of the data was that the proton conduction mechanism remained intact and functional in F_1F_0 ATP synthase containing the $b_{Arg-36\rightarrow Glu}$ subunit. This was examined by measuring passive proton translocation through F_0 in stripped membranes prepared from strain TC215($b_{Arg-36\rightarrow Glu}$). As predicted, the stripped membranes proved to be leaky when a proton gradient was imposed by addition of NADH (Fig. 4). Apparently, the $b_{Arg-36\rightarrow Glu}$ mutation had little effect on proton translocation through the F_0 sector.

The coupling efficiency of the $b_{Arg-36\rightarrow Glu}$ subunit containing F_1F_0 ATP synthase was determined directly (Table 2). The amount of enzyme was adjusted by measuring the level of α subunit present in the membrane. Interestingly, the membranes from the negative control strain KM322 which lacks the *b* subunit [20] had a small amount of α subunits associated but had an extremely high apparent turnover for hydrolysis. Importantly, the KM322(negative control) membranes had very little ATP synthesis activity ($<5\%$ of KM200(*b*)). The defective transport function of TC211($b_{Arg-36\rightarrow Ile}$) was made apparent by its inability to synthesize ATP and a synthesis/hydrolysis ratio equal to the negative control. Clearly, the catalytic function of KM322(negative control) and TC211($b_{Arg-36\rightarrow Ile}$) are fully uncoupled from transport. The

Table 2
Alterations in F_1 activity associated with b_{Arg-36} substitutions

Strain	Mutation	% F_1[a]	ATP hydrolysis[b]	Turnover[d]	ATP synthesis[c]	Turnover[d]	Synthesis/hydrolysis[e]
KM200	Wild-type	2.05	1.55	482	0.219	68.1	0.141
KM322	Control	0.25	0.306	780	0.002	3.4	0.0065
TC211	$b_{Arg-36\rightarrow Ile}$	0.76	0.624	523	0.004	2.4	0.0065
TC215	$b_{Arg-36\rightarrow Glu}$	1.10	0.619	358	0.012	6.9	0.0193

[a]Percent of membrane protein represented by F_1 was determined by a quantitative immunoblot as described previously [35].
[b]ATP hydrolysis rates (μmol/min/mg) were measured at 30°C in a buffer containing 25 mM HEPES-KOH (pH 7.5), 200 mM KCl, 8 mM $MgSO_4$ (3.1 mM free Mg^{2+}), 10 mM glucose, 1 mM phosphoenolpyruvate, 5 mM ATP, 5 μM carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP), 50 μg/ml pyruvate kinase, and the reactions were started with the addition of 0.10 to 0.15 mg/ml *E. coli* membranes. Time points were taken every 2 min up to 8 min and stopped by the addition of 5% SDS. P_i was determined by the method of Taussky and Shorr [28].
[c]ATP synthesis rates (μmol/min/mg) were measured at 30°C with vigorous shaking in a buffer containing 25 mM HEPES-KOH, 200 mM KCl, 5 mM $MgSO_4$ (3.0 mM free Mg^{2+}), 10 mM glucose, 1 mM ADP, 10 mM [^{32}P]P_i, 50 units/ml hexokinase, and 0.12 mg/ml *E. coli* membranes at pH 7.5. The reactions were started with the addition of 2 mM NADH, time points were taken every 2 min up to 8 min, and stopped with ice cold 10 mM H_2SO_4. P_i was precipitated by the method of Sugino and Miyoshi [36] and the radioactivity remaining in the supernatant was determined by Cherenkov counting. Control experiments to determine $\Delta\mu H^+$-independent incorporation of [^{32}P]P_i into the nonprecipitable material was determined in the same conditions but with the presence of 5 μM CCCP and subtracted from the rates obtained in the absence of CCCP.
[d]Turnover (s^{-1}) was calculated using MW of F_1 (382 146 Da).
[e]Ratio was calculated by the turnover number for ATP synthesis divided by turnover number for ATP hydrolysis.

F_1F_0 ATP synthase in strain TC215($b_{Arg-36\rightarrow Glu}$) had a slightly reduced hydrolytic rate and a greater than sevenfold reduction in coupling efficiency relative to the positive control enzyme from KM200(*b*) (Table 2). It is apparent that the coupled ATP synthesis function is more defective than ATP-driven proton pumping. We note that the activity in the $b_{Arg-36\rightarrow Glu}$ enzyme was sensitive to both the F_1 inhibitor aurovertin D and the F_0 inhibitor dicyclohexylcarbodiimide, indicating that the hydrolytic function remained in communication with the transport mechanism. It should be noted that the absolute values reported for hydrolysis differ from those reported in Table 2 because these experiments were performed under different assay conditions chosen to maximize coupling.

4. Discussion

Analysis of primary protein sequence homology suggests that b_{Arg-36} is the most extensively conserved amino acid among all bacterial F_1F_0 ATP synthase *b* subunits. We have constructed a collection of mutations replacing b_{Arg-36} to investigate its functional role in the enzyme. The mutant strains have growth characteristics which indicate deficiencies in oxidative phosphorylation to varying degrees. Substitution of b_{Arg-36} with Lys, Ser, Cys and Tyr resulted in moderate reductions in the steady-state level of F_1F_0 ATP synthase in the membrane. In each case, the intact enzyme with these altered *b* subunits appeared to be functional. The $b_{Arg-36\rightarrow Ile}$ substitution also resulted in a moderate reduction in the level of F_1F_0 ATP synthase complexes, but the enzyme seemed to be virtually inactive. Apparently, replacing b_{Arg-36} with a hydrophobic group is incompatible with F_1F_0 ATP synthase activity. This suggests that while b_{Arg-36} is not essential for F_1F_0 ATP synthase, the amino acid in this position exerts significant influence on the function of the enzyme.

A more interesting result is that the $b_{Arg-36\rightarrow Glu}$ mutation appears to be a representative of the small group of mutations affecting the F_0 subunits which uncouple proton translocation from ATP synthesis in F_1. This is the first mutation of this type reported in the *uncF*(*b*) gene. The other substitutions alter the *c* and *a* subunits which, unlike the *b* subunit, are thought to participate in proton conduction [24,32–34]. Several observations are consistent with respect to these other missense mutations which yield the uncoupling phenotypes. First, like b_{Arg-36}, all substitutions occur at a strongly conserved position in F_1F_0 ATP synthase. Second, other amino acid substitutions at the same positions do not necessarily cause uncoupling of the enzyme. Third, with the exception of the $c_{Arg-41\rightarrow Lys}$ mutation [33], the uncoupling mutations are substitutions which result in a more acidic subunit. The effects of the amino acid substitutions in the polar loop of the *c* subunit on coupling can be rationalized by the observation of direct interaction between one of the uncoupling mutation sites c_{Gln-42} and the ε subunit [8], which, along with the γ subunit, form the rotating stalk of F_1F_0 ATP synthase. The linkage between the rotary stalk and the proton translocation mechanism has apparently been disrupted. Since neither the *a* subunit nor the *b* subunit has been shown to interact with the rotating stalk subunits, another interpretation may be necessary. Perhaps, these substitutions in the F_0 subunits facilitate exit of the proton from the channel along alternate paths not obligately coupled to rotation.

In summary, we have identified the first substitutions in the *b* subunit that yield phenotypes normally associated with subunits of functional importance to proton translocation and coupling of proton translocation to catalysis. The substitutions in the *b* subunit may be exerting a subtle influence on the conformations of the *a* or *c* subunits resulting in uncoupling. Alternatively, the interaction between the *b* subunit and F_1 may be dynamic, and the assignment of a purely passive structural role for the *b* subunit in F_1F_0 ATP synthase may be premature.

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