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Mutagenic Analysis of the *a* Subunit of F_1F_0 ATP Synthase in *Escherichia coli*: Gln-252 through Tyr-263

PHILLIP E. HARTZOG AND BRIAN D. CAIN*

Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida 32610

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The *a* subunit of F_1F_0 ATP synthase contains a highly conserved region near its carboxyl terminus which is thought to be important in proton translocation. Cassette site-directed mutagenesis was used to study the roles of four conserved amino acids Gln-252, Phe-256, Leu-259, and Tyr-263. Substitution of basic amino acids at each of these four sites resulted in marked decreases in enzyme function. Cells carrying *a* subunit mutations Gln-252→Lys, Phe-256→Arg, Leu-259→Arg, and Tyr-263→Arg all displayed growth characteristics suggesting substantial loss of ATP synthase function. Studies of both ATP-driven proton pumping and proton permeability of stripped membranes indicated that proton translocation through F_0 was affected by the mutations. Other mutations, such as the Phe-256→Asp mutation, also resulted in reduced enzyme activity. However, more conservative amino acid substitutions generated at these same four positions produced minimal losses of F_1F_0 ATP synthase. The effects of mutations and, hence, the relative importance of the amino acids for enzyme function appeared to decrease with proximity to the carboxyl terminus of the *a* subunit. The data are most consistent with the hypothesis that the region between Gln-252 and Tyr-263 of the *a* subunit has an important structural role in F_1F_0 ATP synthase.

F_1F_0 ATP synthase is a multimeric enzyme responsible for ATP synthesis in most organisms. The enzyme is composed of two subcomplexes referred to as F_1 and F_0 (8, 10, 29). F_1 contains the catalytic sites and consists of the α, β, γ, δ, and ε subunits. F_0 is intrinsic with respect to the membrane and facilitates proton translocation. F_0 consists of the *a*, *b*, and *c* subunits in a stoichiometry of 1:2:10 ± 1 (13), and all three subunits are apparently necessary for proton conductance (28). The amphipathic *b* subunit possesses a single membrane-spanning segment and a large hydrophilic domain which apparently interacts with F_1 (22). The *c* subunit is extremely hydrophobic and probably spans the membrane twice. Substantial biochemical and genetic evidence has linked the *c* subunit to proton translocation and the coupling proton translocation to catalytic activity (9).

The primary amino acid sequence of the carboxyl-terminal one-third of the *a* subunit bears striking homology with similar subunits from both the mitochondrial and chloroplast F_1F_0 ATP synthases (35). Recent mutant studies of the homologous region have suggested that the *a* subunit plays a central role in proton translocation through F_0. The first evidence came from the isolation of missense mutations which impaired proton translocation while apparently allowing assembly of an intact F_1F_0 ATP synthase complex (3). To investigate the specific amino acids which might participate in the translocation mechanism, a large number of missense mutations have been constructed by many investigators by using site-directed mutagenesis (4–7, 14, 19, 20, 32–34). By analyzing the phenotypes of multiple mutations at single positions, it has been possible to establish the relative importance of specific conserved amino acids for the function of the enzyme.

Although the topology of the *a* subunit of F_1F_0 ATP synthase is not known, the membrane-spanning segment proximal to the carboxyl terminus of the *a* subunit is thought to extend from about His-256 through Ser-268 (8). Evidence from localization of enzymatic activities of *a* subunit-alkaline phosphatase fusion products suggests the possibility of a shorter membrane-spanning segment between Phe-254 and Ser-268 (18). Regardless of the actual topology, the carboxyl-terminal membrane-spanning region of the *a* subunit is essential for enzyme function since removal of all or part of this segment results in apparent loss of F_1F_0 ATP synthase activity, such as occurs in strains carrying the Trp-235→End, Gln-252→End, and Tyr-263→End mutations (3, 6, 7, 34). Primary sequence comparisons between the *a* subunit present in the *Escherichia coli* enzyme and the corresponding subunits in other F_1F_0 ATP synthases have indicated that several amino acids in the carboxyl-terminal membrane span are among those strongly conserved in F_1F_0 ATP synthases.

Here we report the construction and biochemical analysis of mutations in several conserved positions in the carboxyl-terminal membrane-spanning segment. Novel mutations were generated in Gln-252, Phe-256, Leu-259, and Tyr-263. In each case, the least conservative mutations involving substitution of basic amino acids produced significantly reduced F_1F_0 ATP synthase function.

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MATERIALS AND METHODS

Materials. T-4 DNA ligase, T-4 polynucleotide kinase, proteinase K, and restriction endonucleases were supplied by Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), and New England BioLabs (Beverly, Mass.). The Taq-Traq sequencing system was the product of Promega (Madison, Wis.). Radionucleotides were purchased from Amersham Corp. (Arlington Heights, Ill.). Lysozyme, DNase, pyruvate kinase, and lactate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, Mo.). Difco Laboratories (Detroit, Mich.) was the source of bacterial growth media. All other reagents and chemicals were ob-

* Corresponding author.

TABLE 1. Strains and plasmids used

Strain or plasmid(s)	Genotype and description	Source or reference
1100	*bglR thi-1 rel-1* HfrPO1	15
MC4100	F^- *ΔlacU169 araD139 thiA rpsL relA*	17
PH105	*uncB2000* (a_{del}) in 1100 background	This work
RH305	*uncB205* ($a_{Val\text{-}239\rightarrow Ala,\ Pro\text{-}240\rightarrow Trp,\ Trp\text{-}241\rightarrow End}$) *recA56 srl*::Tn*10* in 1100 background	15
pBDC1	Ap^r *uncB* (*a*)	3
pBR325	Ap^r Cm^r Tet^r	NEB[a]
pMAK705	Cm^r *ori*(Ts)	12
pPH11	Cm^r *uncB2000* (a_{del})	This work
pPH12	Cm^r *uncB* (*a*)	This work
pPH15	Cm^r *uncB' uncE uncF uncH'* (a^- *c b* δ^-)	This work
pUNCB5.01–pUNCB5.04	Cm^r *uncB* (*a*) Phe-256 mutations	This work
pUNCB5.20–pUNCB5.16	Cm^r *uncB* (*a*) Phe-256 mutations	This work
pUNCB5.20–pUNCB5.23	Cm^r *uncB* (*a*) Leu-259 mutations	This work
pUNCB5.30–pUNCB5.36	Cm^r *uncB* (*a*) Tyr-263 mutations	This work

[a] New England BioLabs.

tained from Sigma or Fisher Scientific (Orlando, Fla.). Oligonucleotides were synthesized in the core facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida.

Organisms, media, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. Luria broth containing 0.2% (wt/vol) glucose was used as the primary medium. Minimal media employed for growth studies consisted of minimal A salts (23) supplemented with either glucose or succinate (0.2% [wt/vol]) as specified elsewhere in the text. Growth yields of strains were determined turbidimetrically in minimal A medium supplemented with 5 mM glucose. Chloramphenicol was added to media at concentrations of 20 μg/ml for liquid medium and 30 μg/ml for solid medium. Liquid cultures were aerated by continuous mixing on an orbital shaker or in a roller drum. Incubations were performed at 37°C unless otherwise indicated.

Recombinant DNA techniques. Large quantities of plasmid DNA were purified by using a CsCl step gradient (11), and smaller quantities were prepared by the rapid-screen method (2). Plasmid DNA for sequencing was prepared by a modification of the rapid-screen method. Cells were grown in 10 ml of Terrific Broth (1.2% bacto-tryptone [wt/vol], 2.4% bacto-yeast extract [wt/vol], 0.04% glycerol [vol/vol], 0.17 M KH_2PO_4, 0.072 M K_2HPO_4) overnight (31). Cells were lysed, and plasmid DNA was recovered essentially as described by Birnboim and Doly (2). The plasmid DNA was treated with 10 U of RNAce-It (Stratagene, La Jolla, Calif.) for 30 min and then treated with proteinase K overnight in the presence of 25 mM EDTA. The solution was extracted twice with phenol-chloroform (1:1) and twice with chloroform. The DNA was precipitated by addition of 0.5 volume of 7.5 M ammonium acetate and 2 volumes of ethanol and recovered by centrifugation at 12,000 × *g* for 20 min. The pellet was rinsed with 70% ethanol, dried under a vacuum, and suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA sequencing was done with the Taq-Traq sequencing system. Standard conditions were used for restriction endonuclease digestions, ligations, transformations, and agarose gel electrophoresis (27).

Sequencing of the *uncB* mutation in strain RH305. Genomic DNA was isolated by the method of Porter et al. (25) from strain RH305($a_{uncB205}$) and used for polymerase chain reaction amplification. The *uncB* gene was amplified by polymerase chain reaction with primers with the sequence GGT GCTGGTGGTTCAGATACTGGCAC and CCAGTTTGTTT CAGTTAAAACGTAGTAGTGTTGG under standard conditions (26). The nucleotide sequence was determined by using cycle sequencing in accordance with the protocol from the AmpliTaq Cycle Sequencing Kit (Perkin Elmer Cetus, Norwalk, Conn.). Sequence analysis revealed the RH305 mutation to be Val-239→Ala, Pro-240→Trp, and Trp-241→End (TAG stop codon). The results are consistent with the findings that the *uncB205* mutation mapped by recombination to the 3′ one-third of the *uncB* (*a*) gene and was suppressed by an amber tRNA suppressor mutation (2a).

Cassette mutagenesis of the *uncB* (*a*) gene. Plasmid pPH12 was constructed in multiple steps and consists of the following fragments from plasmids pBR325 and pBDC1 (*a*). The fragments from plasmid pBR325 were the 1.2-kb *Aat*II-*Bcl*I fragment, the 0.04-kb *Bam*HI-*Nar*I fragment, and the 2.3-kb *Nar*I-*Ase*I fragment. The 1.1-kb *Aat*II-*Ase*I fragment containing the *uncB* (*a*) gene came from plasmid pBDC1. The *Cla*I and *Hin*dIII restriction endonuclease sites in the promoter region of the *uncB* (*a*) gene were destroyed by digestion with both enzymes, followed by incubation with DNA polymerase I (large fragment) and ligation. The engineered *Hin*dIII and *Cla*I restriction sites were added by cassette mutagenesis, replacing the *Bcl*I-*Aha*II fragment from the *uncB* (*a*) gene (see Fig. 1A).

The *Bcl*I, *Hin*dIII, and *Cla*I restriction sites were used to construct site-directed mutations in the carboxyl-terminal region of *uncB* (*a*) by use of cassette mutagenesis (see Fig. 1B). Each mutation was confirmed by direct nucleotide sequence determination of the entire portion of the *a* subunit replaced by the mutagenic oligonucleotides.

Construction of strain PH105. The *uncB* (*a*) deletion strain PH105 was constructed via homologous recombination between the chromosome of strain 1100 and plasmid pPH15 essentially as described by Hamilton et al. (12). Plasmid pPH15 (a^- *c b* δ^-) was constructed by ligation of the 2.2-kb *Hin*dIII-*Cla*I fragment from the *unc* operon, minus the 617-bp *Bam*HI fragment from *uncB* (*a*), into plasmid pMAK705 digested with *Hin*dIII and *Cla*I. The *uncB* (*a*) deletion was confirmed by Southern analysis and by complementation (data not shown).

Cell fractionation. Preparation of subcellular fractions was done as described previously (16). *E. coli* cells were inocu-

lated into Luria broth containing 0.2% (wt/vol) glucose (500 ml) and grown to a density of approximately 150 Klett units. The cells were harvested by centrifugation (5,000 × *g*, 5 min), washed with TM buffer (50 mM Tris-HCl, 20 mM Mg_2SO_4, pH 7.5) and resuspended in 7 ml of TM buffer containing DNase I (10 μg/ml). Cells were disrupted at 14,000 lb/in^2 in a French pressure cell. Debris and unbroken cells were removed by two successive centrifugations (8,000 × *g* for 5 min each time). The particulate membrane fraction and soluble cytoplasmic fraction were separated by high-speed centrifugation (150,000 × *g*, 1.5 h). Membranes used for proton conduction studies were washed a second time with TM buffer. Membranes used for F_1 stripping experiments were washed with SB buffer (1 mM Tris-HCl, 0.5 mM EDTA, 2.5 mM 2-mercaptoethanol, 10% [vol/vol] glycerol, pH 8.0). The membranes were resuspended in SB buffer and gently agitated overnight. Stripped membranes were recovered by centrifugation (150,000 × *g*, 1 h) and ten resuspended in TM buffer. Resuspended membranes used in fluorescence assays were centrifuged (12,000 × *g*, 5 min) immediately prior to use to remove any remaining cell debris. All cell fractionation steps were performed at 4°C.

Assays. Protein concentrations were measured by using the modified Lowry procedure of Markwell et al. (21). Membrane energization (750 μg of protein, 3 ml of buffer, morpholinepropanesulfonic acid [MOPS], 10 mM $MgCl_2$, pH 7.3) was assayed by fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) (1). ATP hydrolysis was measured by a coupled assay to regenerate ATP in the system. Membranes (15 or 30 μg of protein) were added to the assay buffer (2 mM $MgCl_2$, 25 mM KCl, 5 mM KCN, 25 mM Tris-HCl, 5 U of pyruvate kinase per ml, 5 U of lactate dehydrogenase per ml, 2.5 mM phosphoenol pyruvate, 0.5 mM NADH, pH 8.0) and monitored by A_{350} for loss of NADH upon addition of ATP-$MgCl_2$ (2 mM). Treatment of fractionated membranes with 50 μM *N*,*N*′-dicyclohexylcarbodiimide (DCCD) for either 15 min at 37°C or 1 h at room temperature was used for inhibition of F_1F_0 activity. All assays utilized membrane samples prepared within 24 h of cell disruption.

RESULTS

Generation of *uncB* (*a*) mutations. The carboxyl-terminal one-third of the *a* subunit contains several amino acids which have apparently been conserved throughout nature. We constructed site-directed mutations in four of the conserved amino acids, Gln-252, Phe-256, Leu-259, and Tyr-263, by using a novel cassette site-directed mutagenesis vector, pPH12. The *uncB* (*a*) gene carried on plasmid pPH12 contains silent mutations which introduced unique *Hin*dIII and *Cla*I restriction sites. Together with a *Bcl*I site present in the native *uncB* (*a*) gene, these restriction endonuclease sites flanked the codons targeted for mutagenesis (Fig. 1).

The mutations generated by mutagenesis of plasmid pPH12 with each of the cassette oligonucleotides are listed in Table 2. Twenty-two mutations in the *uncB* (*a*) gene were recovered at the four sites. Four missense mutations altered Gln-252, seven amino acid substitutions affected Phe-256, three missense mutations appeared at Leu-259, and seven substitutions were found at Tyr-263. In addition, mutagenesis of Tyr-263 produced a mutant plasmid that contained a nonsense mutation at position Leu-259 due to fortuitous deletion of a thymidine nucleotide in the Leu-259 codon.

Effects of *uncB* (*a*) mutations on cell growth. The efficiency of F_1F_0 ATP synthase in *E. coli* was examined by correlation

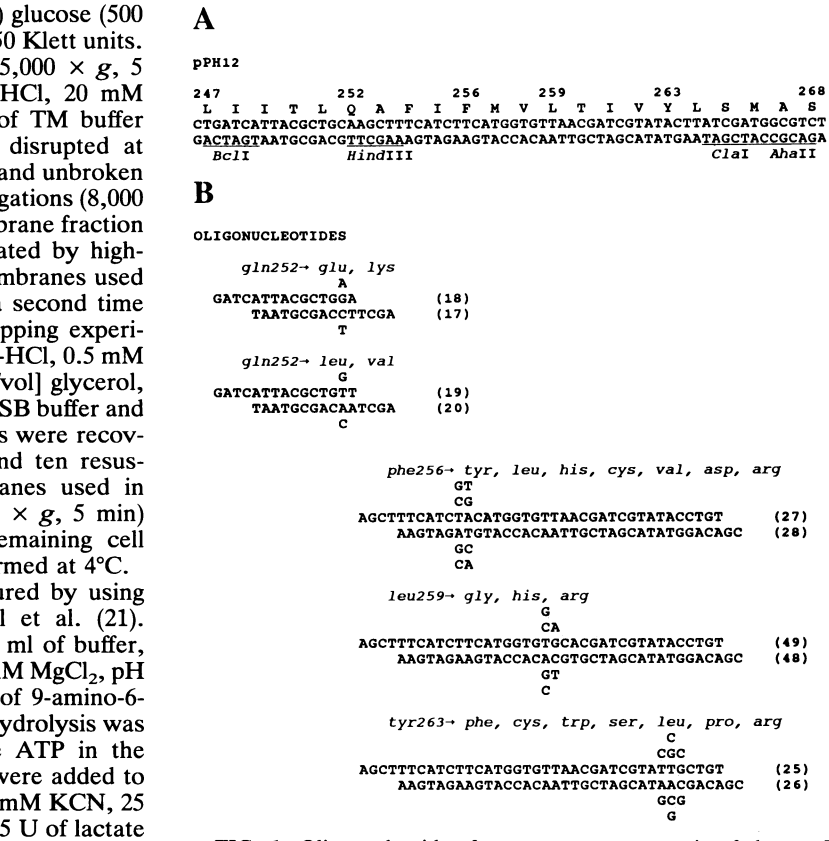

FIG. 1. Oligonucleotides for cassette mutagenesis of the *uncB* (*a*) gene. (A) Sequence of the *uncB* (*a*) gene replaced in plasmid pPH12 to obtain the silent changes for the *Hin*dIII and *Cla*I restriction sites. (B) Double-stranded oligonucleotides used for cassette mutagenesis at positions Gln-252, Phe-256, Leu-259, and Tyr-263.

to the growth yield of mutant strains in glucose-limited medium. Growth on succinate-based medium was also studied, since F_1F_0 ATP synthase is necessary for oxidative phosphorylation.

E. coli PH105 (a_{Del}), carrying an internal deletion of 617 bp of the *uncB* (*a*) gene, was constructed to serve as the host strain for growth studies (see Materials and Methods). Strain PH105 differed from the *uncB* (*a*) gene deletion strain BC2000 previously employed by us (3) in that PH105 (a_{del}) did not carry an altered *uncE* Shine-Dalgarno sequence that was present in the earlier construction (30). The mutant *uncB* (*a*) gene plasmids were transformed into strain PH105 (a_{del}) to study the capacity of the plasmids to complement the chromosomal deficiency.

Each strain, PH105 (a_{del}) harboring an *a* subunit plasmid, was streaked onto solid succinate minimal medium. Missense mutation plasmid pUNCB5.04 ($a_{Gln\text{-}252\rightarrow Lys}$), pUNCB5.16 ($a_{Phe\text{-}256\rightarrow Arg}$), and pUNCB5.22 ($a_{Leu\text{-}259\rightarrow Arg}$) and nonsense mutation plasmid pUNCB5.23 ($a_{Leu\text{-}259\rightarrow End}$) all failed to support growth on succinate (Table 2). Similarly, the strains carrying these plasmids attained cell densities in glucose-limited liquid medium comparable to that of strain PH105 with no plasmid. The results were consistent with loss of biologically significant levels of F_1F_0 ATP synthase.

Mutant plasmids, such as pUNCB5.02 ($a_{Gln\text{-}252\rightarrow Leu}$) and pUNCB5.03 ($a_{Gln\text{-}252\rightarrow Val}$), supported the development of

TABLE 2. Growth properties of mutations generated in the *uncB* (*a*) gene

Strain or plasmid	Mutation	Oligonucleotides[a]	Mutant codons	Growth on succinate[b]		Yield in limited glucose[c]
				37°C	42°C	
PH105	Deletion			−	−	64
pPH12	Wild type			+++	+++	98
pUNCB5.01	Gln-252→Glu	18-17	GAA	+++	+++	94
pUNCB5.04	Glu-252→Lys	18-17	AAA	−	−	70
pUNCB5.02	Glu-252→Leu	19-20	TTA	+	−	77
pUNCB5.03	Glu-252→Val	19-20	GTA	+	−	74
pUNCB5.10	Phe-256→Tyr	27-28	TAC	+++	+++	102
pUNCB5.11	Phe-256→Leu	27-28	CTC	+++	+++	101
pUNCB5.12	Phe-256→His	27-28	CAC	+++	+	98
pUNCB5.13	Phe-256→Cys	27-28	TGC	+++	+++	96
pUNCB5.14	Phe-256→Val	27-28	GTC	+++	+++	81
pUNCB5.15	Phe-256→Asp	27-28	GAC	+	+	87
pUNCB5.16	Phe-256→Arg	27-28	CGC	−	−	69
pUNCB5.20	Leu-259→Gly	49-48	GGC	+++	−	89
pUNCB5.21	Leu-259→His	49-48	CAC	+++	+	87
pUNCB5.22	Leu-259→Arg	49-48	CGC	−	−	70
pUNCB5.23	Leu-259→End	49-48	TAA	−	−	69
pUNCB5.30	Tyr-263→Phe	25-26	TTC	+++	+++	95
pUNCB5.31	Tyr-263→Cys	25-26	TGC	+++	+	93
pUNCB5.32	Tyr-263→Trp	25-26	TGG	+++	+++	92
pUNCB5.33	Tyr-263→Ser	25-26	TCC	+++	+	89
pUNCB5.34	Tyr-263→Leu	25-26	CTG	+++	+++	89
pUNCB5.35	Tyr-263→Pro	25-26	CCC	+++	+	92
pUNCB5.36	Tyr-263→Arg	25-26	CGG	+	−	79

[a] Oligonucleotides used for mutagenesis as shown in Fig. 1.
[b] Colony size after 48 h of incubation at the indicated temperatures on solid succinate (0.2% [wt/vol])–minimal medium A. Growth was scored as normal colonies comparable to the wild-type control strain (+++), smaller-than-normal colonies (+), or no visible colonies (−).
[c] Growth yield in 5 mM glucose–minimal medium A monitored turbidometrically with a Klett-Summerson colorimeter.

very small colonies on succinate medium and intermediate densities in the glucose-limited medium, suggesting reduced F_1F_0 ATP synthase function. Interestingly, plasmid pUNCB5.36 ($a_{Tyr\text{-}263\rightarrow Arg}$) also supported limited growth. This was the only plasmid in the present collection that encodes a mutation that substitutes a positively charged amino acid which allowed detectable enzyme function in vivo. Plasmid pUNCB5.35 ($a_{Tyr\text{-}263\rightarrow Pro}$) promoted growth at a level marginally less than that of the wild type. Apparently, Tyr-263 accommodates most mutations with little loss of enzyme activity.

Vik et al. (34) had previously reported that some missense mutations of Tyr-263 displayed temperature sensitivity. As shown in Table 2, incubation at 42°C resulted in reduced growth phenotypes not only for Tyr-263 mutations but for mutations in the other three positions as well. The most dramatic example of this temperature sensitivity was plasmid pUNCB5.20 ($a_{Leu\text{-}259\rightarrow Gly}$), which supported wild-type-like growth at 37°C but was unable to promote visible growth at 42°C.

In summary, the growth studies indicate that substitutions in Gln-252 exhibited the most pronounced effects on cell growth. The severity of growth inhibition seemed to diminish as the amino acid positions neared the carboxyl terminus of the *a* subunit. The latter point has been demonstrated by the finding that missense mutations affecting Tyr-263 were not sufficient for abolition of F_1F_0 ATP synthase function (Table 2; 6, 34).

Comparison of *a* subunit-defective strains. Initial studies using strain PH105 (a_{del}) carrying plasmid pPH12 (*a*) revealed a relatively low abundance of the F_1 subcomplex as assayed by ATPase activity and ATP-driven fluorescence quenching in inverted membrane vesicles (data not shown). Therefore, the *a* subunit mutation plasmids were transformed into strain RH305 ($a_{Val\text{-}239\rightarrow Ala,\ Pro\text{-}240\rightarrow Trp,\ Trp\text{-}241\rightarrow End}$), which has been used extensively in previous studies (3–5, 32–34). Strain RH305 ($a_{Val\text{-}239\rightarrow Ala,\ Pro\text{-}240\rightarrow Trp,\ Trp\text{-}241\rightarrow End}$) exhibits normal levels of F_1, which permitted analysis of the *a* subunit mutations for ATP hydrolysis and ATP-driven proton-pumping activities. Importantly, the growth characteristics on succinate-based medium of the recombinant plasmids complementing strain RH305 ($a_{Val\text{-}239\rightarrow Ala,\ Pro\text{-}240\rightarrow Trp,\ Trp\text{-}241\rightarrow End}$) were essentially the same as those reported for strain PH105 (a_{del}).

ATP synthase proton pumping. ATP-driven acidification of inverted membrane vesicles can be used as a measure of F_1F_0 ATP synthase to carry out proton translocation coupled to ATP hydrolysis. The acidification of the membrane vesicles derived from strain RH305 ($a_{Val\text{-}239\rightarrow Ala,\ Pro\text{-}240\rightarrow Trp,\ Trp\text{-}241\rightarrow End}$) harboring the *a* subunit mutation plasmids was monitored by observing the ATP-dependent quenching of the fluorescent dye ACMA. Electron transport chain-mediated NADH-dependent quenching was also determined for each preparation to ensure vesicle integrity. Membrane vesicles were prepared from each strain with an *a* subunit mutation plasmid which apparently resulted in a defect in F_1F_0 ATP synthase.

Membranes derived from cells with plasmids pUNCB5.04 ($a_{Gln\text{-}252\rightarrow Lys}$), pUNCB5.16 ($a_{Phe\text{-}256\rightarrow Arg}$), and pUNCB5.23 ($a_{Leu\text{-}259\rightarrow Arg}$) exhibited no significant quenching of ACMA

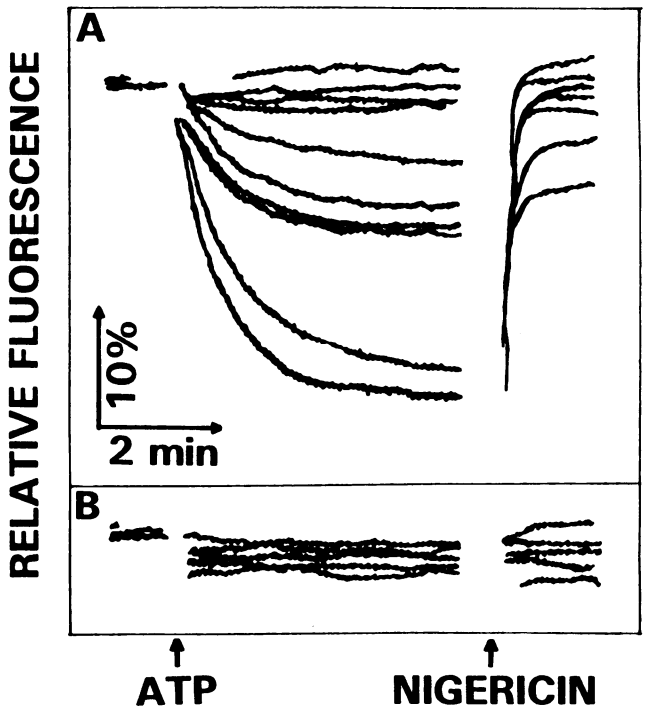

FIG. 2. ATP-driven ACMA fluorescence quenching in membranes derived from *a* subunit mutant strains. Assays were performed as described in Materials and Methods. The times of addition of ATP and nigericin are marked. (A) ATP-driven membrane energization. The traces represent the following mutant strains in descending order: RH305 ($a_{\text{Val-239}\rightarrow\text{Ala, Pro-240}\rightarrow\text{Trp, Trp-241}\rightarrow\text{End}}$), Phe-256→Arg, Leu-259→Arg, Gln-252→Lys, Tyr-263→Arg, Gln-252→Val, Phe-256→Asp, Gln-252→Leu, pPH12 (*a*), and Gln-252→Glu. (B) Membrane energization following treatment of membranes with 50 μM DCCD. The traces represent the following mutant strains in descending order: Gln-252→Val, Gln-252→Leu, Tyr-263→Arg, pPH12 (*a*), Gln-252→Glu, and Phe-256→Asp.

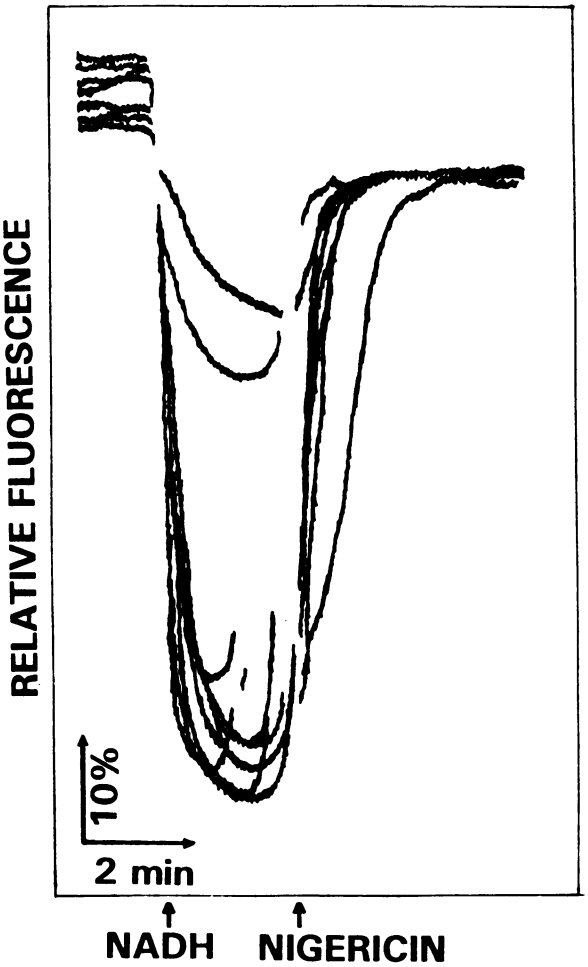

FIG. 3. Proton impermeability of stripped membranes from *a* subunit mutant strains. Assays were performed as described in Materials and Methods. The times of addition of NADH and nigericin are marked. The traces represent the following mutant strains in descending order: Gln-252→Glu, pPH12 (*a*), Gln-252→Leu, Phe-256→Asp, Tyr-263→Arg, Leu-259→Arg, Gln-252→Val, RH305 ($a_{\text{Val-239}\rightarrow\text{Ala, Pro-240}\rightarrow\text{Trp, Trp-241}\rightarrow\text{End}}$), Phe-256→Arg, and Gln-252→Lys.

fluorescence, suggesting apparent loss of proton-pumping activity (Fig. 2A). Vesicle acidification in the range of 25 to 50% of the fluorescence quenching seen in the positive control membranes from strain RH305 ($a_{\text{Val-239}\rightarrow\text{Ala, Pro-240}\rightarrow\text{Trp, Trp-241}\rightarrow\text{End}}$) with plasmid pPH12 (*a*) was observed in membranes from cells carrying plasmids pUNCB5.36 ($a_{\text{Tyr-263}\rightarrow\text{Arg}}$), pUNCB5.03 ($a_{\text{Gln-252}\rightarrow\text{Val}}$), pUNCB5.15 ($a_{\text{Phe-256}\rightarrow\text{Asp}}$), and pUNCB5.02 ($a_{\text{Gln-252}\rightarrow\text{Leu}}$). As expected, studies of membranes from cells carrying plasmid pUNCB5.01 ($a_{\text{Gln-252}\rightarrow\text{Glu}}$) resulted in fluorescence quenching similar to that of membranes from strain RH305 with plasmid pPH12 (*a*).

All membrane preparations which had detectable ATP-dependent acidification were studied with the proton translocation inhibitor DCCD. In every instance, treatment with DCCD reduced fluorescence quenching below detectable levels (Fig. 2B).

F_0-mediated passive proton permeability. To determine whether a mutation specifically affected F_0-mediated proton translocation, the permeability of membrane vesicles stripped of F_1 was examined (Fig. 3). Stripping of F_1 from membranes derived from wild-type cells caused F_0 to function as a passive proton conductor, allowing the collapse of an imposed electrochemical gradient. A mutation affecting F_0-mediated proton translocation typically reduces the capacity of F_0 to collapse proton gradients.

An electrochemical gradient was imposed by addition of NADH as a substrate for NADH dehydrogenase-driven vesicle acidification. The stripped membranes from cells harboring plasmid pPH12 (*a*) displayed little ACMA fluorescence quenching upon addition of NADH, demonstrating F_0 proton conductance. Similarly, analysis of stripped membranes from cells carrying plasmid pUNCB5.01 ($a_{\text{Gln-252}\rightarrow\text{Glu}}$) revealed abundant proton permeability. However, stripped membranes from cells with reduced-growth phenotypes resulted in significant ACMA quenching, indicating impairment of F_0-mediated proton conductance.

In summary, the studies of proton translocation were consistent with the notion that the mutational effects on cell growth are attributable to a reduction in ATP synthase activity due to a reduction in F_1F_0 proton translocation.

ATP hydrolysis activity. Mutations in the *a* subunit have been shown to affect F_1F_0 ATP synthase either by perturbing

TABLE 3. Properties of *uncB* (*a*) subunit mutations at Gln-252

Plasmid	Plasmid mutation	Avg ± SD ATPase sp act[a]	
		Total	DCCD sensitive[b]
pPH12	None (wild type)	0.800 ± 0.075	0.387 ± 0.052
pUNCB5.01	Gln-252→Glu	0.649 ± 0.034	0.305 ± 0.006
pUNCB5.02	Gln-252→Leu	0.768 ± 0.023	0.295 ± 0.013
pUNCB5.03	Glu-252→Val	0.726 ± 0.064	0.212 ± 0.004
pUNCB5.04	Glu-252→Lys	0.758 ± 0.085	0.000 ± 0.000
pPH11	Deletion	0.603 ± 0.033	0.062 ± 0.015

[a] Specific activity is expressed as micromoles of ATP hydrolyzed per milligram of protein per minute at pH 8.0. The values are averages of two assays done at two different protein concentrations.

[b] DCCD-sensitive activity is the amount of specific activity lost after treatment with 50 μM DCCD for 1 h at room temperature.

F_0 assembly via a structural defect or through alteration of an amino acid important in proton translocation. Membrane-associated, DCCD-sensitive F_1 ATPase activity was assayed as a measure of the structural integrity of the F_1F_0 ATP synthase complex.

Substitutions at position Gln-252 were analyzed to determine the activity of coupled F_1F_0 ATP synthase. The amount of DCCD-sensitive ATP hydrolysis activity (Table 3) mirrored the growth characteristics observed for the substitutions at Gln-252 (Table 2). Membranes derived from strain RH305 carrying plasmids pUNCB5.01 ($a_{Gln\text{-}252\rightarrow Glu}$), pUNCB5.02 ($a_{Gln\text{-}252\rightarrow Leu}$), and pUNCB5.03 ($a_{Gln\text{-}252\rightarrow Val}$) possessed significant levels of DCCD-sensitive ATP hydrolysis activity, indicating specific F_1 association with F_0. Study of membranes from strain RH305 with plasmid pUNCB5.04 ($a_{Gln\text{-}252\rightarrow Lys}$) revealed virtually no DCCD-sensitive F_1 ATPase activity, suggesting an interruption of productive coupling in F_1F_0 ATP synthase.

DISCUSSION

This report describes the mutagenesis of four of the strongly conserved amino acids in the carboxyl-terminal membrane-spanning segment of the *a* subunit. Gln-252 and Tyr-263 are conserved in the primary amino acid sequences of all reported F_1F_0 ATP synthases, while Phe-256 and Leu-259 are present in most of these enzymes (5). We report the first missense mutations in this region of the *a* subunit which appear to be sufficient to abolish F_1F_0 ATP synthase activity. All of the missense mutations previously reported in this area of the *a* subunit allowed detectable levels of enzyme function (6, 34).

Generally, as the carboxyl terminus is approached in the last membrane-spanning segment of the *a* subunit, mutations appear to have progressively less profound effects. In the case of the final conserved position in the *a* subunit Tyr-263, missense mutations which specify the substitutions of radically different amino acids, such as proline and arginine, do not abolish F_1F_0 ATP synthase activity (Table 2; 34). However, mutations resulting in the replacement of Gln-252, Phe-256, and Leu-259 with basic amino acids are sufficient for apparent loss of enzyme function. In each of these mutant strains, proton translocation through F_0 is decreased beyond detectable levels. It seems likely that introduction of charged amino acids into a membrane-spanning region may result in a conformational change sufficient to perturb proton translocation. The mutations Gln-252→Lys, Phe-256→Asp, Phe-256→Arg, Leu-259→Arg, and Tyr-263→Arg support this hypothesis.

However, changes at Gln-252 indicate a role beyond mere stabilization of a membrane span. In the case of Gln-252→Glu, a change to a charged amino acid, no significant effect was observed. In contrast, two noncharged substitutions, Gln-252→Leu and Gln-252→Val, substantially reduced activity. These data suggest that while Gln-252 is probably not directly involved in proton conductance, this position plays a very important role in the formation of a functional F_0.

Many of the amino acid changes in the carboxyl-terminal region of the *a* subunit allow appreciable F_1F_0 ATP synthase activity. However, the fact that many of these mutations display temperature sensitivity also supports the hypothesis that the carboxyl-terminal membrane-spanning segment plays a structural role in F_0 (Table 2).

Since our original report that missense mutations in the *a* subunit were sufficient for virtual abrogation of F_0-mediated proton translocation (3), at least 88 different missense mutations at 30 separate sites in the *a* subunit have been constructed by a number of independent investigators (3–7, 14, 17, 19, 20, 24, 32–34; Table 2). With the present work, all of the obviously conserved positions, as well as the charged amino acids which are predicted to lie within the membrane bilayer, have been studied. Although some minor controversies have appeared in the reported phenotypes, the combined data provide a substantial basis for considering the role of the *a* subunit in proton translocation.

The mutagenesis approach has established that Arg-210 is apparently essential for ATP synthase proton translocation (5, 6, 19). Several other amino acids, including Glu-219 and His-245, are probably intimately involved in the proton translocation machinery, since conservative substitutions confer dramatic effects on F_1F_0 ATP synthase (4, 6, 20). At the next level, only substitution of substantially dissimilar amino acids, such as the Gln-252→Lys, Gln-252→Val, and Gln-252→Leu mutations, resulted in substantial losses of enzyme function (Table 2 and Fig. 2). Many mutations had little or no effect on F_1F_0 ATP synthase. The present mutational map of the *a* subunit, coupled with the evidence from the *c* subunit, suggests that relatively few amino acids are directly involved in the proton translocation mechanism of F_0. Therefore, in our view, the ordered water channel recently considered by Fillingame (8) appears to be the most attractive mechanism for proton translocation through F_0. Few specific residues are absolutely required for formation of such a channel, but many substitutions might be expected to affect ion movement to various degrees. Unfortunately, until a high-resolution structural model of F_0 is obtained, the mechanism of proton conduction will remain open to conjecture.

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