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Temperature-Sensitive Mutations at the Carboxy Terminus of the *a* Subunit of the *Escherichia coli* F_1F_0 ATP Synthase

STEVEN B. VIK,* DAISY LEE, AND PAMELA A. MARSHALL

Department of Biological Sciences, Southern Methodist University, Dallas, Texas 75275-0376

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Mutations were constructed in the *a* subunit of the F_1F_0 ATP synthase from *Escherichia coli*. Truncated forms of this subunit showed a temperature sensitivity phenotype. We conclude that the carboxy terminus of the *a* subunit is not involved directly with proton translocation but that it has an important structural role.

The F_1F_0 ATP synthase (EC 3.6.1.3) from *Escherichia coli* is a multisubunit, membrane-bound, proton-translocating ATPase, similar to enzymes found in the energy-transducing membranes of chloroplasts and mitochondria (for reviews, see references 10 and 21). Catalytic and transport functions are embodied in the two separable components of the enzyme, F_1 and F_0. F_1 is the site of ATP synthesis and hydrolysis and is composed of five subunits, and F_0 is a membrane-bound complex composed of three subunits, *a*, *b*, and *c*. When membranes are stripped of F_1, F_0 renders them permeable to protons (11).

All of the F_0 subunits are necessary for a functional, proton-translocating complex. The importance of the *a* subunit has been indicated by a series of studies of randomly generated (2, 20) and site-directed (3, 4, 12, 16, 17, 22, 23) mutations, many of which affect function at the level of proton movement through F_0. Most of these mutations lie in the region of highest amino acid sequence conservation, in the carboxy-terminal one-third of the polypeptide. Recent analysis of nonsense mutants (8) has confirmed the importance of the region between residues 252 and 271, the carboxy terminus.

The mechanism of proton translocation through F_0 and its linkage to net ATP synthesis is unknown. Recent work (7) has indicated that several residues at the carboxy terminus of the *a* subunit are not important for function. The work presented here describes three mutagenic approaches for defining further the role of the carboxy-terminal segment of the *a* subunit in the structure and function of the ATP synthase.

Materials and bacterial strains. Materials were obtained as described previously (23). Strain XL1-Blue (Stratagene) was used routinely for subcloning and mutagenesis. Strain RH305 (13) was used to characterize mutations in *uncB*. This strain is complemented by plasmids containing the wild-type *uncB* gene, but the nature of the *uncB205* mutation is unknown. Cultures were grown as previously described (23). Fractionation of cells and isolation of membranes and stripped membranes were carried out as described previously (3, 23).

Construction of plasmids. Plasmid pSBV10 was constructed from a derivative of pBDC6 (2) in which the unique *Nhe*I site was eliminated by digestion with *Nhe*I and *Acc*I, S1 nuclease treatment, and religation. Modified pBDC6 (Δ*Nhe*I) was digested with *Pvu*I and *Ava*I, generating three fragments of 3.78 kb, 0.16 kb, and 16 bp. The largest fragment was ligated with annealed synthetic oligodeoxynucleotides 1, 2, and 3, as shown in Fig. 1.

Construction of mutations. The *uncB* mutations analyzed in this study were constructed with the restriction sites introduced into *uncB* in pSBV10, as shown in Fig. 1. The mutations were analyzed phenotypically by transforming cells of strain RH305 (*uncB205 recA56*), which are complemented by plasmids bearing the wild-type *uncB* gene.

Amino acid sequence analysis. A comparison of the deduced amino acid sequences of eight bacterial *a* subunits revealed several conserved features at the carboxy terminus (Fig. 2). First, within a largely nonpolar region (residues 241 to 267), two hydrogen-bonding residues, Gln-252 and Tyr-263, are strictly conserved. Second, the extreme carboxy terminus is characterized by the presence of histidine, glutamic acid, and aspartic acid. In light of the possible role of such residues in proton translocation, a mutagenic analysis of some of these residues was undertaken.

Saturation mutagenesis of the last three residues at the carboxy terminus. To determine the possible significance of the last three residues at the carboxy terminus of the *E. coli* *a* subunit (Glu-269, Glu-270, and His-271), we carried out saturation mutagenesis as indicated in Fig. 1. More than 500 transformants of RH305 were analyzed for growth on minimal medium supplemented with the nonfermentable carbon

TABLE 1. Properties of Tyr-263 mutants[a]

Mutation at residue 263	Growth yield (%)[b]	Growth on succinate[c]	Membrane-bound ATPase activity (%)[d]	Sensitivity to DCCD (%)[e]	Proton translocation (%)[f]
Y (wild type)	100	+	79	77	100
Y → F	99	+	79	74	98
Y → A	100	+	75	59	98
Y → E	92	+	76	77	67
Y → G	91	+	77	76	71
Y → K	94	+	77	62	55
Y → Q	97	+	ND	ND	85
Y → stop	65	−	64	50	<10
None (no plasmid)	65	−	68	5	<10

[a] Values are the averages of at least two measurements, which did not differ by more than 5%. ND, not determined.
[b] Growth yields are expressed as percentages of the wild-type level.
[c] Growth was judged by the appearance of colonies on minimal medium-succinate plates at 37°C after 48 h. +, growth; −, no growth.
[d] ATPase activity found in the membrane fraction is expressed as a percentage of the total activity (membrane and supernatant fractions).
[e] Percentage of the membrane-bound ATPase activity that was inhibited by treatment with 50 μM DCCD at 37°C for 15 min.
[f] Extent of ATP-driven ACMA fluorescence quenching by everted membranes, expressed as a percentage of the wild-type level.

* Corresponding author.

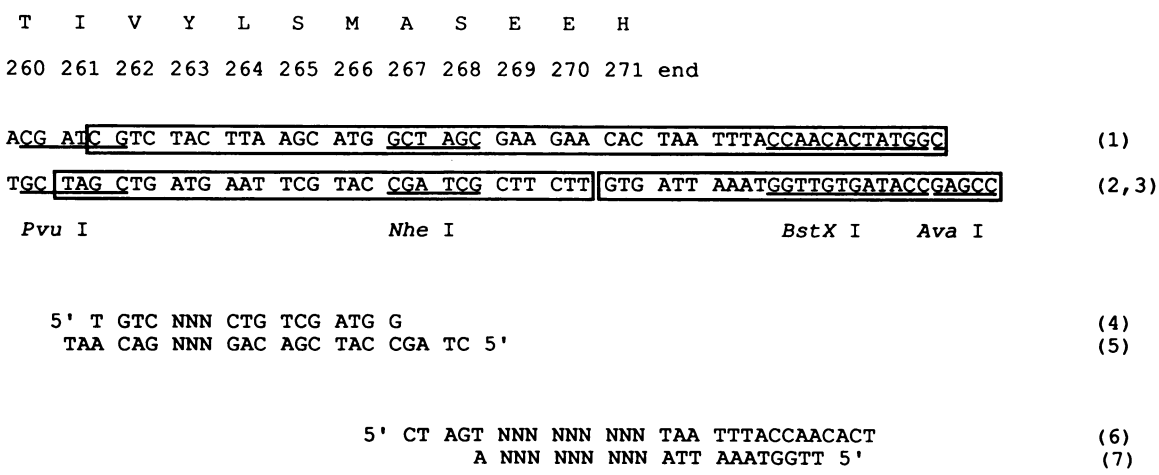

FIG. 1. Representation of the carboxy terminus of the *a* subunit. Oligonucleotides 1, 2, and 3 (indicated by boxes) were annealed and ligated to ends of pSBV10 generated by *Pvu*I-*Ava*I double digestion. Mutations at Tyr-263 were produced by oligonucleotides 4 and 5 with *Pvu*I and *Nhe*I. Mutations at Glu-269, Glu-270, and His-271 were produced by oligonucleotides 6 and 7 with *Nhe*I and *Bst*XI. Deletions in *uncB* were constructed by digestion of pSBV10 with *Bst*XI and treatment of the digest with BAL-31 nuclease for 20 min at 37°C. A nonsense linker (New England BioLabs), CTAGCTAGCTAG, was added to the digested pSBV10 before ligation. Restriction sites are underlined.

source succinate, but none were unable to grow, indicating that the ability of the ATP synthase to function was not compromised. DNA sequence analysis confirmed that the mutagenesis was successful (data not shown). It was concluded that the negatively charged carboxy terminus does not play an essential role in the structure or function of the ATP synthase or in the targeting of the protein to the inner membrane (24, 26).

Mutagenesis of Tyr-263. Tyr-263 is a residue strictly conserved among all known *a*-subunit homologs and, as was shown in *E. coli*, probably lies near the periplasm (15). Mutagenesis produced 17 different amino acid substitutions and 2 nonsense mutations (TAA and TGA). Aspartate and asparagine substitutions were not obtained. Growth yields of strain RH305 harboring plasmids containing each of the missense mutations were measured; all strains grew to within 90% of the wild-type levels. Furthermore, each strain was able to grow on minimal medium supplemented with succinate.

Several of the mutations were selected for further study (Table 1). Amino acid substitutions at residue 263 did not affect the binding of F_1 to F_0 or the sensitivity of membrane-bound ATPase activity to *N,N'*-dicyclohexyl carbodiimide (DCCD). Proton translocation was more sensitive to the nature of the substitution. Membranes containing the nonpolar Y-263→F-263 or Y-263→A-263 substitution showed wild-type levels of ATP-driven proton translocation, while polar residues were much less effective. These results suggested that the hydroxyl group of the conserved tyrosyl residue is not an essential feature and is therefore not likely to be involved in proton translocation.

Deletion mutants generated by BAL-31. The previous experiments failed to identify any critical residues at the carboxy-terminal region of the *a* subunit, but analysis of the Y-263 nonsense mutation indicated that a slightly truncated *a* subunit is not completely functional. To investigate this point further, we constructed a series of deletions (Fig. 1). Twelve such mutations corresponding to *a* subunits as small as 244 residues were analyzed (Table 2).

Growth yields of strain RH305 harboring these constructs are presented in Table 2. The truncated *a* subunits fell clearly into two groups: those larger than 266 residues produced growth yields of >95%, while those smaller than 263 residues produced growth yields of <80%. ATP-driven proton translocation, as measured by the 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence-quenching assay, was also normal for membranes with the longer *a* subunits (>266 residues), while the shorter ones were considerably less effective. Measurements of ATP hydrolysis activity (Table 3) indicated that membranes from strains with the shorter *a* subunits (<263 residues) contained slightly reduced levels of F_1 and that the sensitivity of membrane-bound ATPase activity to DCCD was also somewhat decreased.

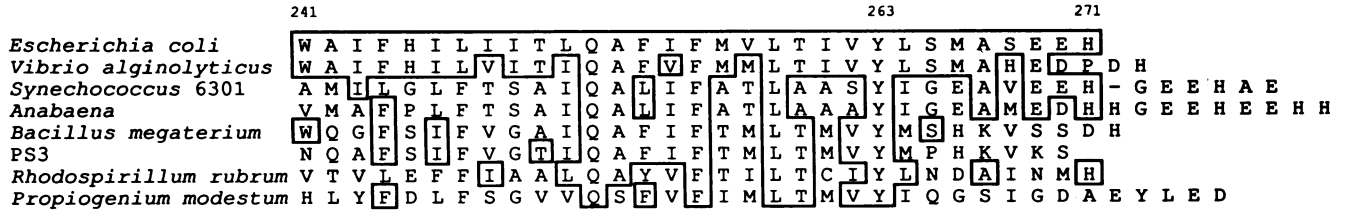

FIG. 2. Amino acid sequence comparison of bacterial *a* subunits. The carboxy-terminal 31 residues of the *E. coli a* subunit (25) are shown along with the corresponding regions of proteins from *Vibrio alginolyticus* (14), *Synechococcus* sp. strain 6301 (5), *Anabaena* sp. (18), *Bacillus megaterium* (1), PS3 (19), *Rhodospirillum rubrum* (9), and *Propiogenium modestum* (6). Residues identical to those in the *E. coli* protein are shown in boxes.

TABLE 2. Growth yields of strains bearing truncated *a* subunits

Mutation	Amino acid sequence[a]	Growth yield (%)[b]	Growth on succinate[c]
Wild type	WAIFHILIITLQAFIFMVLTIVYLSMASEEH	100	+
268	WAIFHILIITLQAFIFMVLTIVYLSMAS	97	+
267	WAIFHILIITLQAFIFMVLTIVYLSMA	97	+
261LAS	WAIFHILIITLQAFIFMVLTIVYLSMASEEHLAS	97	+
270PS	WAIFHILIITLQAFIFMVLTIVYLSMASEEPS	99	+
269LAS	WAIFHILIITLQAFIFMVLTIVYLSMASELAS	95	+
267X	WAIFHILIITLQAFIFMVLTIVYLSMALGPGHCGA	97	+
262LAS	WAIFHILIITLQAFIFMVLTIVLAS	77	±
261AS	WAIFHILIITLQAFIFMVLTIAS	68	±
256LAS	WAIFHILIITLQAFIFLAS	65	−
251LAS	WAIFHILIITLLAS	67	−
251AS	WAIFHILIITLAS	66	−
244	WAIF	66	−
None (no plasmid)		65	−

[a] Beginning at residue 241. Additional residues not found in the wild type are indicated in boldface type.
[b] Growth yields are expressed as percentages of the wild-type level.
[c] Growth was judged by the appearance of colonies on minimal medium-succinate plates after 48 h at 37°C. +, growth; −, no growth; ±, marginal growth.

Biochemical analysis of temperature-dependent mutants. Careful observation of the growth of strains containing the truncated *a* subunits on minimal medium-succinate plates revealed that while growth was not observed at 37°C in 48 h, slow growth was apparent at room temperature after 48 to 72 h. These results were also observed in strains containing the Y-263 nonsense mutation. In an attempt to correlate this enhanced growth with a biochemical property of the ATP synthase, we prepared membranes from cells grown at both 25 and 37°C. A pronounced increase in ATP-driven proton translocation was observed in membranes containing *a* subunits truncated at about residue 262 when cells were grown at 25°C versus 37°C. In Fig. 3, a comparison of ATP-driven fluorescence quenching by membranes isolated from several mutant strains grown at both 37 and 25°C is shown. Membranes bearing the Y-263 nonsense mutation (designated 262) showed the greatest stimulation of proton translocation at the lower temperature. Membranes in which the *a* subunit contained three additional residues, leucine, alanine, and serine, after the first 262 wild-type residues (designated 262LAS) also showed some stimulation when cells were grown at 25°C.

To assess passive proton permeability, we stripped membranes of F_1 and measured NADH-driven fluorescence quenching (Fig. 4). In Fig. 4A, stripped membranes prepared

TABLE 3. ATPase properties of membranes with selected truncated *a* subunits

Construct	Amino acid sequence[a]	F_1 binding (%)[b]	DCCD sensitivity (%)[c]
Wild type	TLQAFIFMVLTIVYLSMASEEH	89	74
268	TLQAFIFMVLTIVYLSMAS	91	75
267	TLQAFIFMVLTIVYLSMA	86	68
262LAS	TLQAFIFMVLTIVLAS	76	49
261AS	TLQAFIFMVLTIAS	70	43
251LAS	TLLAS	80	21
No plasmid		68	5

[a] Starting at residue 250. See Table 2, footnote *a*, for an explanation of boldface type.
[b] ATPase activity found in the membrane fraction is expressed as a percentage of the total activity (membrane and supernatant fractions).
[c] Percentage of the membrane-bound ATPase activity that was inhibited by treatment with 50 μM DCCD at 37°C for 15 min.

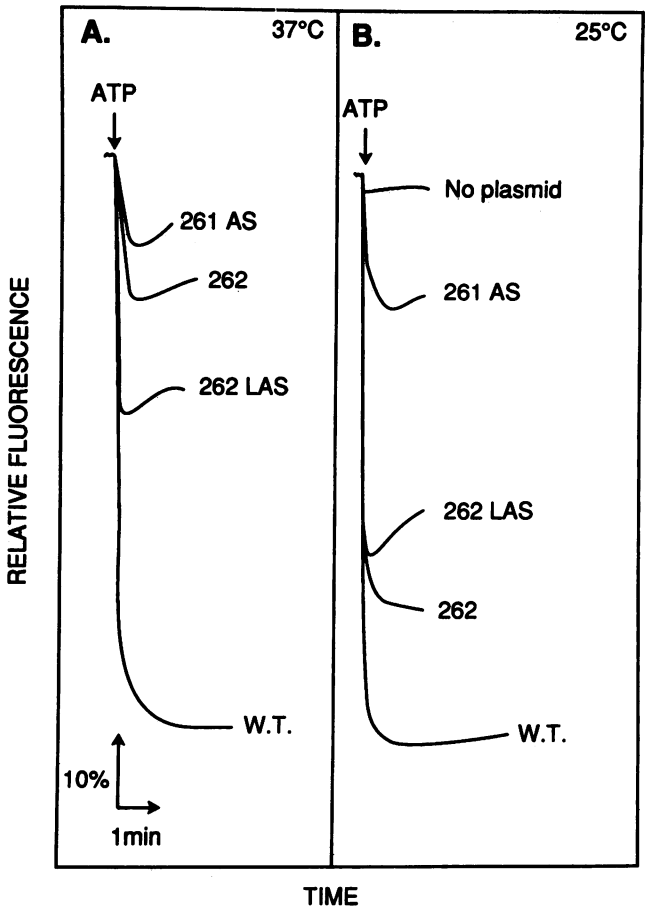

FIG. 3. ATP-driven fluorescence quenching by membranes containing truncated *a* subunits. (A) Membranes prepared from strain RH305 grown at 37°C and bearing plasmids coding for the wild-type *a* subunit or for *a* subunits truncated in the vicinity of residue 262. (B) Membranes from the same cells grown at 25°C. In each case, 2 μM carbonyl cyanide *m*-chlorophenylhydrazone abolished fluorescence quenching. W.T., wild type.

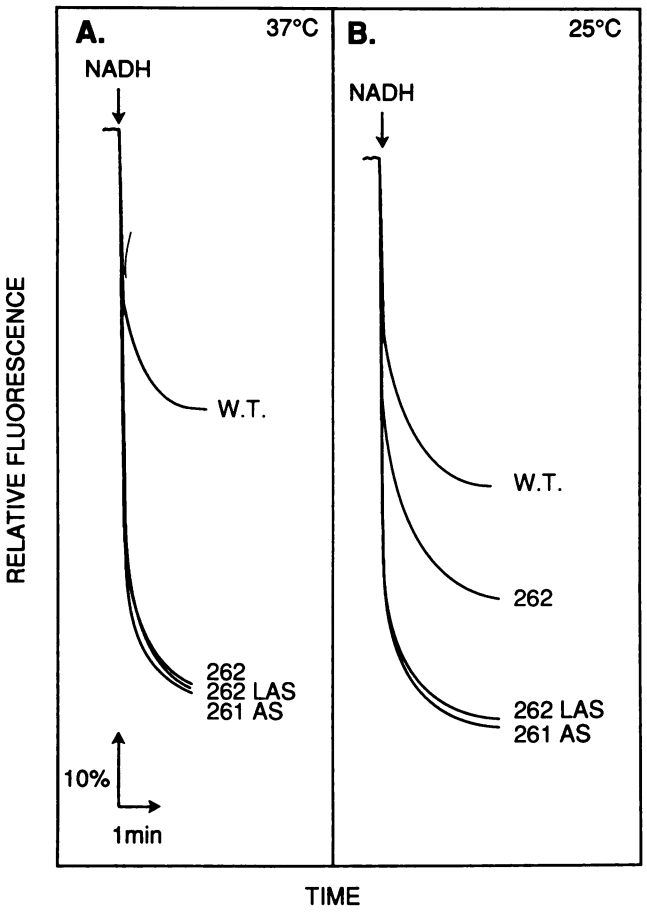

FIG. 4. NADH-driven fluorescence quenching by stripped membranes containing truncated *a* subunits. (A) Membranes from strain RH305 grown at 37°C and bearing plasmids coding for the wild-type *a* subunit or for forms truncated in the vicinity of residue 262. Before the assay, the membranes were stripped of F_1 by overnight treatment with 1 mM Tris (pH 8.0)–0.5 mM EDTA at 3°C. (B) Stripped membranes from the same cells grown at 25°C. W.T., wild type.

from cells grown at 37°C were assayed for proton leakiness by measuring NADH-driven fluorescence quenching. The wild-type membranes had diminished quenching because of proton leakage through F_0. All of the mutations (262, 262LAS, and 261AS) permitted similar rates of NADH-driven fluorescence quenching, which were higher than the rate in the wild type, indicative of reduced proton permeability. In Fig. 4B, results for stripped membranes prepared from cells grown at 25°C are shown. Stripped membranes containing the Y-263 nonsense mutation (designated 262) from cells grown at 25°C showed increased proton permeability, as evidenced by the reduced rate of NADH-driven fluorescence quenching. These results suggest that the defect due to the truncation of the *a* subunit in cells grown at 37°C lies at the level of proton movement through F_0.

This temperature dependence supports the interpretation that the role of residues 263 to 266 of the *a* subunit is to interact with and stabilize some portion of the proton channel. Apparently, the truncated forms of the *a* subunit have a near-native conformation when grown at 25°C. At the higher growth temperature of 37°C, a less active conformation of the truncated forms of the *a* subunit seems to be favored. This high-temperature effect could be due to an altered folding pattern or conceivably to partial proteolytic degradation of the carboxy terminus.

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