JOURNAL OF BACTERIOLOGY, Jan. 1994, p. 540–542
0021-9193/94/$04.00+0

Vol. 176, No. 2

Mutations in the δ Subunit Influence the Assembly of F_1F_0 ATP Synthase in *Escherichia coli*

ABIGAIL E. STACK AND BRIAN D. CAIN*

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

Received 26 July 1993/Accepted 13 November 1993

Missense mutations affecting Asp-161 and Ser-163 in the δ subunit of F_1F_0 ATP synthase have been generated. Although most substitutions allowed substantial enzyme function, the $\delta_{Asp\text{-}161\rightarrow Pro}$ substitution resulted in a loss of enzyme activity. The loss of activity was attributable to a structural failure altering assembly of the enzyme complex.

In *Escherichia coli* F_1F_0 ATP synthase, the δ subunit is required for the binding of F_1 to F_0 (1, 8, 9, 14, 16, 17). A narrow stalk connecting the two sectors is thought to consist of the F_0 *b* subunits and possibly F_1 subunits, including the δ subunit (5). The δ subunit appears to influence the functional state of F_0 during the assembly of the enzyme complex. Brusilow and coworkers (1, 16) have suggested that the α and δ subunits act not upon assembly of F_0 but upon activation of proton translocation.

Bacterial δ subunits have primary sequence homology to the δ subunits of chloroplasts and OSCP subunits of mitochondrial F_1F_0 ATP synthases (7, 9). The sequence identity implied that these amino acids might be important for folding the δ subunit and assembly of the enzyme complex. Therefore, the two amino acids with polar side groups which are present in all δ-like subunits, Asp-161 and Ser-163, were selected for study (Fig. 1). Previously, Jounouchi et al. (9) reported that single structurally conservative substitutions at both positions have little effect on in vivo F_1F_0 ATP synthase function. Mutations altering the Asp-161 and Ser-163 codons in bacteriophage M13KM01 (*acb*δ) were generated by oligonucleotide-directed mutagenesis (12) (Table 1). Each mutation was moved into plasmid pAES9 (*acb*δαγβε), allowing expression of the recombinant δ subunit gene in concert with the other F_1F_0 ATP synthase subunit genes. The negative control plasmid pAES10 (*acb*δ_{del}αγβε) was constructed by a deletion terminating the δ subunit at Ala-51. Plasmids were transformed into *E. coli* 1100ΔBC, which contains no F_1F_0 ATP synthase genes (10). Therefore, F_1F_0 ATP synthase in each mutant strain was derived entirely from the plasmid.

Growth of mutants. Growth on succinate minimal medium and growth yield in limiting glucose medium were used as indicators of the efficiency of F_1F_0 ATP synthase in vivo (15). Only strain 1100ΔBC harboring plasmid pAES9.04 ($\delta_{Asp\text{-}161\rightarrow Pro}$) or pAES10 ($\delta_{del}$) failed to grow on succinate medium (Table 2). The remaining plasmids complemented strain 1100ΔBC, indicating high levels of enzyme function. However, some strains displayed slightly reduced growth yield in limiting glucose medium, suggesting minor deficiencies in F_1F_0 ATP synthase. The Asp-161 mutants were chosen for further characterization because of the breadth of phenotypes observed in the growth studies.

Effects on F_1 activity. Membrane vesicles were prepared as described previously (3), and ATP hydrolysis activity (6) and proton pumping activity (2) were determined. Although most $\delta_{Asp\text{-}161}$ substitutions had no significant effect on F_1 activity, dicyclohexylcarbodiimide (DCCD)-sensitive F_1 ATP hydrolysis activity was absent from the membranes prepared from cells carrying either plasmid pAES10 (δ_{del}) or plasmid pAES9.04 ($\delta_{Asp\text{-}161\rightarrow Pro}$) (Table 2). This indicated that the $\delta_{Asp\text{-}161\rightarrow Pro}$ substitution altered the subunit structure sufficiently to disrupt binding of F_1 to F_0. The reductions in growth yield in all strains correlated directly with studies of ATP-driven proton pumping activity (Fig. 2). Membranes prepared from strains 1100ΔBC/pAES10 (δ_{del}) and 1100ΔBC/pAES9.04 ($\delta_{Asp\text{-}161\rightarrow Pro}$) produced identical results, indicating no detectable ATP-driven proton pumping activity. Intermediate levels of activity were observed in membranes from the other $\delta_{Asp\text{-}161}$ mutants.

Effects on F_0. F_1 was removed from the membranes to study the influence which the altered δ subunits have on F_0 (3). Proton permeability was assayed by imposing a proton gradient. The F_1-depleted membranes prepared from most of the $\delta_{Asp\text{-}161}$ mutants displayed considerable permeability; however, strong 9-amino-6-chloro-2-methoxyacridine fluorescence quenching was seen in membranes prepared from strains 1100ΔBC/pAES10 (δ_{del}) and 1100ΔBC/pAES9.04 ($\delta_{Asp\text{-}161\rightarrow Pro}$), indicating that neither preparation was proton permeable (data not shown). Plausible explanations for this blockage included (i) a defect in assembly of the F_0, (ii) a failure to open the F_0 proton channel in the absence of the δ subunit, and (iii) an altered δ subunit impairing F_0 proton conduction.

If F_0 in the $\delta_{Asp\text{-}161\rightarrow Pro}$ and δ_{del} mutant membranes is assembled, then addition of F_1 purified from wild-type cells should result in reconstitution of the F_1F_0 ATP synthase complex. Membrane vesicles were prepared, partially purified

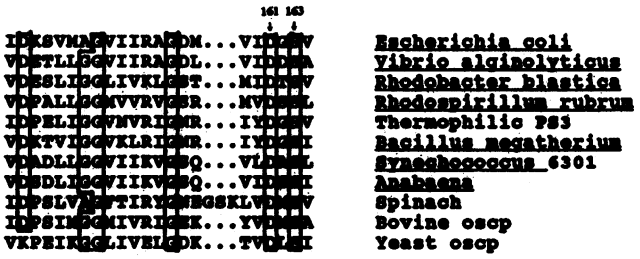

FIG. 1. Primary sequence homology of δ subunits. Boxes indicate sequence identity, and the positions of Asp-161 and Ser-163 are shown. Sequences were derived from GenBank sequences.

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, Box 100245 JHMHC, University of Florida, Gainesville, FL 32610. Phone: (904) 392-6473. Fax: (904) 392-2953.

TABLE 1. *E. coli* strains, plasmids, bacteriophage, and phagemids

Strain, bacteriophage, plasmid, or phagemid	Genotype or description	Source or reference
Strains		
1100	*bglR thi-1 rel-1* HfrP01	8
1100ΔBC	1100 deleted for *uncBEFHAGDC*	10
RH339	1100 *uncH239 recA56 srl*::Tn*10*	8
Plasmids		
pRPG51	Ap^r *uncFH*	8
pAES7	Ap^r *uncFH* Lys-131→Ala Arg-132→Ala Arg-135→Ala Lys-136→Ala Lys-138→Ala Lys-142→Ala	This study
pAES9	Cm^r *uncBEFHAGDC*	This study
pAES9.01	Cm^r Asp-161→Asn	This study
pAES9.02	Cm^r Asp-161→Ser	This study
pAES9.03	Cm^r Asp-161→His	This study
pAES9.04	Cm^r Asp-161→Pro	This study
pAES9.05	Cm^r Ser-163→Asp	This study
pAES9.06	Cm^r Ser-163→Asn	This study
pAES9.07	Cm^r Ser-163→Ala	This study
pAES9.08	Cm^r Ser-163→Thr	This study
pAES10	Cm^r *uncBEFH'AGDC*	This study
Bacteriophage M13KM01	M13mp19 *uncBEFHA'*	12
Phagemids		
pBS+	Ap^r *lacZ* f1(+) origin	12
pAES5	Ap^r *uncEFH*	This study
pAES6	Ap^r *uncEFH* Lys-131→Ala Arg-132→Ala Arg-135→Ala Lys-136→Ala Lys-138→Ala Lys-142→Ala	This study

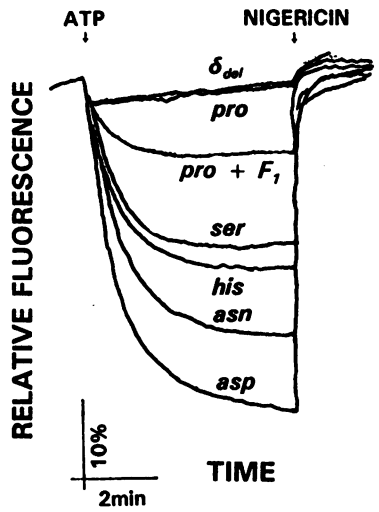

FIG. 2. ATP-driven proton pumping in membrane vesicles prepared from *uncH* (δ) gene mutants. Membrane vesicles were prepared (3), and the protein concentration was determined (11). The vesicles were suspended at a concentration of 150 μg/ml in buffer (50 mM 3-[*N*-morpholino]propanesulfonic acid [MOPS], 10 mM $MgCl_2$, pH 7.3) and 1 μM 9-amino-6-chloro-2-methoxyacridine. Arrows mark the addition of ATP (1 mM) and nigericin (0.5 μM). Traces are labeled according to the amino acid occupying position 161 of the δ subunit in the following strains: 1100ΔBC/pAES10 (δ_{del}), 1100ΔBC/pAES9.04 ($\delta_{Asp\text{-}161\rightarrow Pro}$), 1100ΔBC/pAES9.03 ($\delta_{Asp\text{-}161\rightarrow His}$), 1100ΔBC/pAES9.02 ($\delta_{Asp\text{-}161\rightarrow Ser}$), 1100ΔBC/pAES9.01 ($\delta_{Asp\text{-}161\rightarrow Asn}$), and 1100ΔBC/pAES9 ($\delta_{Asp\text{-}161}$). The trace labeled *pro* + F_1 represents membrane vesicles from 1100ΔBC/pAES9.04 ($\delta_{Asp\text{-}161\rightarrow Pro}$) reconstituted with partially purified F_1.

F_1 was added, and activity was determined by monitoring ATP-driven proton pumping. Addition of F_1 to membrane preparations from strains 1100ΔBC/pAES10 (δ_{del}) and 1100ΔBC/pAES9.04 ($\delta_{Asp\text{-}161\rightarrow Pro}$) resulted in reconstitution of the enzyme complex, indicating that at least some F_0 was present (Fig. 2). Either addition of the native F_1 catalyzed the rapid assembly of F_0 or, more likely, a few F_0 sectors were assembled in the absence of the δ subunit.

Immunoblot analyses using anti-*b*-subunit polyclonal antibody were performed to determine the amount of F_0 present in the membranes (Fig. 3) (4, 13). Membranes prepared from the two wild-type strains, 1100 and 1100ΔBC/pAES9, had virtually equivalent amounts of *b* subunit. When standardized to those in strain 1100, *b*-subunit levels were reduced 25 to 50% in membranes from strains 1100ΔBC/pAES9.01 ($\delta_{Asp\text{-}161\rightarrow Asn}$), 1100ΔBC/pAES9.03 ($\delta_{Asp\text{-}161\rightarrow His}$), and 1100ΔBC/pAES9.02 ($\delta_{Asp\text{-}161\rightarrow Ser}$). The amount of *b* subunit in the membranes

TABLE 2. Properties of mutations generated in the *uncH* (δ) gene

Strain or plasmid[a]	Mutation	Growth on succinate[b]	Growth yield[c] (%)	ATP hydrolysis sp act[d]	
				Total	DCCD sensitive
pAES9	Wild type	+	100	0.46 ± 0.14	0.22 ± 0.04
1100ΔBC	Deletion	−	35	0.20 ± 0.01	0.04 ± 0.01
pAES10	δ_{del}	−	ND[e]	0.26 ± 0.03	0.03 ± 0.01
pAES9.01	Asp-161→Asn	+	95	0.43 ± 0.12	0.21 ± 0.04
pAES9.02	Asp-161→Ser	+	81	0.44 ± 0.09	0.19 ± 0.02
pAES9.03	Asp-161→His	+	83	0.53 ± 0.09	0.17 ± 0.03
pAES9.04	Asp-161→Pro	−	49	0.24 ± 0.06	0.04 ± 0.02
pAES9.05	Ser-163→Asp	+	79	ND	ND
pAES9.06	Ser-163→Asn	+	79	ND	ND
pAES9.07	Ser-163→Ala	+	88	ND	ND
pAES9.08	Ser-163→Thr	+	83	ND	ND

[a] All plasmids were transformed into *E. coli* 1100ΔBC.
[b] Growth (+) or no growth (−) after 48 h of incubation at 37°C.
[c] Growth yield in 5 mM glucose-minimal medium monitored turbidimetrically.
[d] Reported as micromoles of ATP hydrolyzed per milligram of protein per minute at pH 8.0. DCCD-sensitive activity is the amount of specific activity lost after DCCD treatment.
[e] ND, not determined.

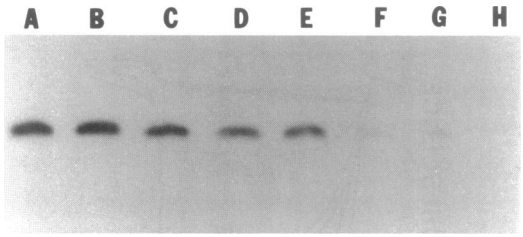

FIG. 3. Detection of *b* subunits in membranes prepared from *uncH* (δ) gene mutants. Membrane fractions were isolated from *E. coli* strains, and 15 μg of membrane protein was separated by electrophoresis in a 10% polyacrylamide–Tris–tricine–sodium dodecyl sulfate gel. Lane A, strain 1100; lane B, 1100ΔBC/pAES9 ($\delta_{Asp\text{-}161}$); lane C, 1100ΔBC/pAES9.01 ($\delta_{Asp\text{-}161\rightarrow Asn}$); lane D, 1100ΔBC/pAES9.02 ($\delta_{Asp\text{-}161\rightarrow Ser}$); lane E, 1100ΔBC/pAES9.03 ($\delta_{Asp\text{-}161\rightarrow His}$); lane F, 1100ΔBC/pAES9.03 ($\delta_{Asp\text{-}161\rightarrow Pro}$); lane G, 1100ΔBC/pAES10 (δ_{del}); lane H, strain 1100ΔBC.

prepared from strains 1100ΔBC/pAES10 (δ_{del}) and 1100ΔBC/pAES9.04 ($\delta_{Asp\text{-}161\rightarrow Pro}$) was 3% that of wild type. Importantly, the relative amounts of immunoreactive *b* subunit correlated with the levels of DCCD-sensitive enzyme activity, indicating that the altered δ subunits were affecting the amount of F_0 present in the membranes.

The level of F_0 was low in the mutants, as evidenced by both the amount of reconstituted F_1F_0 ATP synthase proton pumping activity and the amount of *b* subunit detected by immunoblot analysis. There are two possible interpretations for the reduced level of F_0. First, an absence of the δ subunit, and thus of F_1, renders the *b* subunit subject to proteolysis. This would interfere with the reconstitution and presumably the interaction required for activating the proton channel. Alternatively, incorporation of the *b* subunit into F_0 may be facilitated by the δ subunit.

The present work is in agreement with the conclusions of Jounouchi et al. (9) that $\delta_{Asp\text{-}161}$ and $\delta_{Ser\text{-}163}$ are tolerant to substitution with structurally similar amino acids. Only the $\delta_{Asp\text{-}161\rightarrow Pro}$ substitution was sufficient for loss of enzyme activity. Perhaps no specific amino acid in the δ subunit is essential for F_1F_0 ATP synthase function.

This work was supported by Public Health Service grant GM43495 (to B.D.C.) from the National Institutes of Health.

REFERENCES

1. **Angov, E., T. C. N. Ng, and W. S. A. Brusilow.** 1991. Effect of the δ subunit on assembly and proton permeability of the F_0 proton channel of *Escherichia coli* F_1F_0 ATPase. J. Bacteriol. **173:**407–411.
2. **Aris, J. P., D. J. Klionsky, and R. D. Simoni.** 1985. The F_0 subunits of the *Escherichia coli* F_1F_0-ATP synthase are sufficient to form a functional proton pore. J. Biol. Chem. **260:**11207–11215.
3. **Cain, B. D., and R. D. Simoni.** 1989. Proton translocation by the F_1F_0 ATPase of *Escherichia coli*: mutagenic analysis of the *a* subunit. J. Biol. Chem. **264:**3292–3300.
4. **Deckers-Hebestreit, G., R. D. Simoni, and K. Altendorf.** 1992. Influence of subunit-specific antibodies on the activity of the F_0 complex of the ATP synthase of *Escherichia coli*. J. Biol. Chem. **267:**12364–12369.
5. **Gogol, E. P., U. Lücken, and R. A. Capaldi.** 1987. The stalk connecting the F_1 and F_0 domains of ATP synthase visualized by electron microscopy of unstained specimens. FEBS Lett. **219:**274–278.
6. **Hartzog, P. E., and B. D. Cain.** 1993. Mutagenic analysis of the *a* subunit of F_1F_0 ATP synthase in *Escherichia coli*: Gln-252 through Tyr-263. J. Bacteriol. **175:**1337–1343.
7. **Hoesche, J. A., and R. Berzborn.** 1993. Primary structure, deduced from cDNA, secondary structure analysis, and conclusions concerning interaction surfaces, of the delta subunit of the photosynthetic ATP-synthase (E.C. 3.6.1.34) from millet (*Sorghum bicolor*) and maize (*Zea mays*). Biochim. Biophys. Acta **1142:**293–305.
8. **Humbert, R., W. S. A. Brusilow, R. P. Gunsalus, D. J. Klionsky, and R. D. Simoni.** 1983. *Escherichia coli* mutants defective in the *uncH* gene. J. Bacteriol. **153:**416–422.
9. **Jounouchi, M., M. Takayama, P. Chaiprasert, T. Noumi, Y. Moriyama, M. Maeda, and M. Futai.** 1992. *Escherichia coli* H^+-ATPase: role of the δ subunit in binding F_1 to the F_0 sector. Arch. Biochem. Biophys. **292:**376–381.
10. **Klionsky, D. J., W. S. A. Brusilow, and R. D. Simoni.** 1983. Assembly of a functional F_0 of the proton-translocating ATPase of *Escherichia coli*. J. Biol. Chem. **258:**10136–10143.
11. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:**206–210.
12. **McCormick, K. A., and B. D. Cain.** 1991. Targeted mutagenesis of the *b* subunit of F_1F_0 ATP synthase in *Escherichia coli*: Glu-77 through Gln-85. J. Bacteriol. **173:**7240–7248.
13. **McCormick, K. A., G. Deckers-Hebestreit, K. Altendorf, and B. D. Cain.** Characterization of mutations in the *b* subunit of F_1F_0 ATP synthase in *Escherichia coli*. J. Biol. Chem. **268:**24683–24691.
14. **Mendel-Hartvig, J., and R. A. Capaldi.** 1991. Structure-function relationships of the domains of the δ subunit in *Escherichia coli* adenosine triphosphatase. Biochim. Biophys. Acta **1060:**115–124.
15. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Monticello, R. A., E. Angov, and W. S. A. Brusilow.** 1992. Effects of inducing expression of cloned genes for the F_0 proton channel of the *Escherichia coli* F_1F_0 ATPase. J. Bacteriol. **174:**3370–3376.
17. **Sternweiss, P. C., and J. B. Smith.** 1977. Characterization of the purified membrane attachment (δ) subunit of the proton translocating adenosine triphosphatase from *Escherichia coli*. Biochemistry **16:**4020–4025.