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The essential carboxyl group in subunit c of the F_1F_0 ATP synthase can be moved and H^+-translocating function retained

(proteolipid/dicyclohexylcarbodiimide/mutants)

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ABSTRACT **The proteolipid subunit c of F_1F_0-type, H^+-transporting ATP synthases [ATP phosphohydrolase (H^+-transporting), EC 3.6.1.34] contains a conserved Asp/Glu residue that is thought to function in H^+ translocation. To test the importance of the position of this residue in the *Escherichia coli* enzyme, we used oligonucleotide-directed mutagenesis to move the carboxyl side chain from position 61 to position 58, 60, or 62. Mutant cells with these changes were incapable of growth via oxidative phosphorylation on succinate. An Asp-61 → Glu mutant grew on succinate but at 50% the efficiency of wild type. Hence, even minor changes in the position of the carboxyl group can significantly reduce function. In a second approach, slow-growing revertants to an Asp-61 → Gly mutant were isolated. In one such revertant, Ala-24 was changed to Asp, while the original Asp-61 → Gly mutation remained unchanged. The Asp-24–Gly-61 double mutant grew on succinate at 60% the efficiency of wild type. Hence the essential carboxyl group of subunit c can function when anchored at either position 24 or position 61, and this supports the idea that these residues may neighbor each other when subunit c is folded in the membrane. The rate of ATP-driven H^+ translocation by mutant membrane vesicles was estimated by the quenching of 9-amino-6-chloro-2-methoxyacridine fluorescence and corresponded to actual H^+ pumping rates <25% that of wild type.**

Membrane-associated, H^+-transporting ATP synthases [ATP phosphohydrolase (H^+-transporting), EC 3.6.1.34] catalyze the synthesis of ATP during oxidative phosphorylation (1). These enzymes are composed of two functionally distinct sectors termed F_1 and F_0. The F_1 moiety is easily released from the membrane surface and, when isolated, functions as an ATPase. The F_0 sector extends through the membrane and catalyzes H^+ transport across the membrane. In *Escherichia coli*, F_1 is composed of five types of subunits in an $\alpha_3\beta_3\gamma\delta\varepsilon$ stoichiometry; F_0 is composed of three types of subunits in an $a_1b_2c_{10}$ stoichiometry (2). Each subunit is encoded by a gene of the *unc* operon (3). Homologous subunits are found in mitochondria, chloroplasts, and other bacteria (1, 3).

Subunit c is a small, hydrophobic protein that is thought to fold in the membrane like a hairpin with two membrane-traversing α-helices (Fig. 1). It is thought to play a key role in F_0-mediated H^+ transport. H^+ translocation through F_0, and the ATPase activity of the coupled F_1F_0 complex, are specifically blocked by reaction of dicyclohexylcarbodiimide (DCCD) with Asp-61 of the *E. coli* subunit, or a Glu at the equivalent position in the "proteolipid" subunit of other species (4, 11). Reaction with one of the ≈10 subunits c in F_0 is sufficient to abolish activity (12). Mutation of Asp-61 to Gly or Asn also abolishes H^+ translocase activity (13–15). The rate of reaction of DCCD with Asp-61 is slowed in mutants with substitutions of Ala-24 → Ser (refs. 16 and 17; R.H.F., M.O., and D. Fraga, unpublished data) or Ile-28 → Thr or Val (18, 19). These results suggest that residues 24–28 may lie close to Asp-61 in the membrane and that DCCD may bind in this region of the first membrane-spanning segment before reacting with Asp-61 on the second membrane-spanning segment (20).

In the work reported here, we have characterized two variants of subunit c in which the essential carboxyl group has been repositioned in the center of the membrane. We conclude that the carboxyl group must be precisely positioned for optimal function, but that it can be anchored to either residue 61 of transmembrane segment 2 or to residue 24 of transmembrane segment 1.

METHODS

***E. coli* Strains.** Strain MJM125 (F^+, *asnB31*, *thi-1*) is the wild-type control strain used in these studies. Strains MJM381 (Asp-61 → Gly), MJM385 (Asp-61 → Glu), and MJM414 (Ala-24–Asp-61 → Asp-24–Gly-61) are isogenic with strain MJM125 and carry chromosomal mutations in the *uncE* gene for subunit c. These strains were constructed as described below and by Miller *et al.* (21). Strains LW180 [Δ*uncE334*-(Kan^R), *asnB31*, *thi-1*] (Kan^R, kanamycin resistant) and MJM62 [Δ*uncE334*(Kan^R), *ilv*::Tn*10*, *asnB31*, *thi-1*] are described by Miller *et al.* (21). Strain MM349 (*uncB402*), a derivative of strain RR1 that was made *recA56* (15), was constructed in this laboratory by Mary Mosher.

Oligonucleotide-Directed Mutagenesis. Oligonucleotide-directed mutagenesis was carried out with phage F2, a derivative of phage M13mp18 with the *uncE* gene cloned between the *Bam*HI and *Hin*cII sites of the multiple cloning region (21). Mutagenesis was carried out by the method of Kramer *et al.* (22) or by the method of Taylor *et al.* (23) with an Amersham kit. The desired mutation was confirmed, and unintended mutations were ruled out by sequencing the entire *uncE* gene as described (21).

Complementation Test for Function. The mutated *uncE* genes were cut out of the M13 replicative form with *Bam*HI and *Pst* I and ligated into the equivalent sites of plasmid pUC18 (24). Strain $MM994I^q$ [*uncE114* (Gln-42 → Glu)] (21) was transformed with the recombinant plasmids, and transformants were tested for growth on minimal agar containing 22 mM succinate and 30 μM isopropyl β-D-thiogalactopyranoside to induce the cloned *uncE* gene.

Revertants to the Asp-61 → Asn and Asp-61 → Gly Mutants. Strains MM441 and MM457 (25), which carry the Asp-61 → Asn and Asp-61 → Gly mutations in *uncE*, respectively, were grown overnight in LB medium and 0.1-ml aliquots were spread over minimal agar plates containing 22 mM succinate.

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Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; DCCD, dicyclohexylcarbodiimide.

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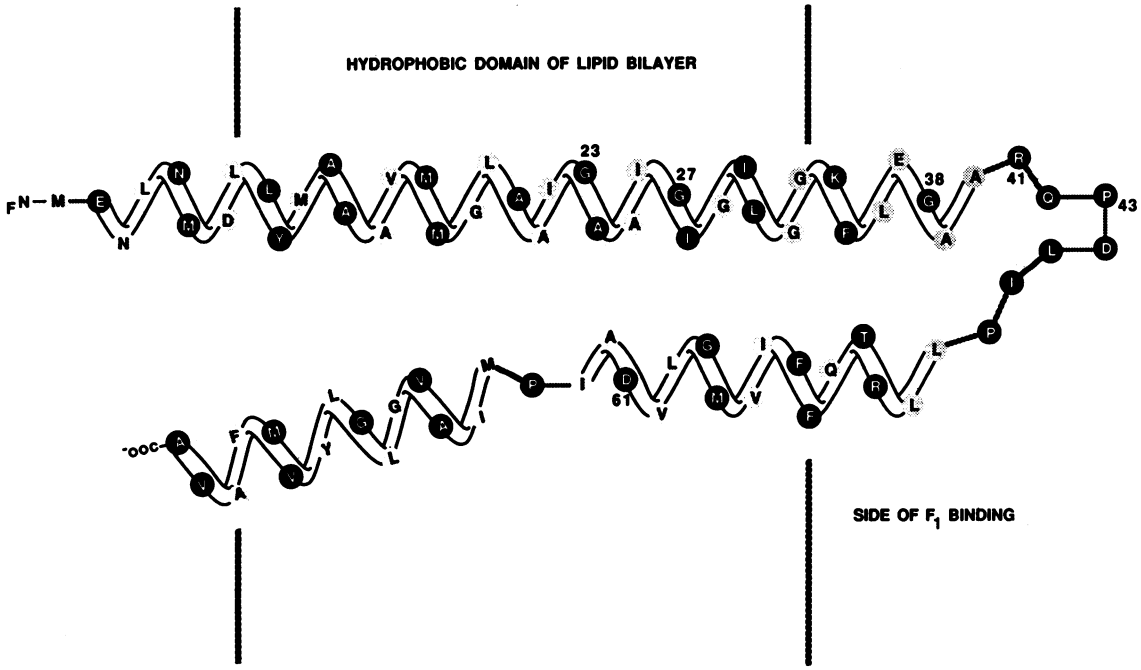

FIG. 1. Model for folding of subunit c in the membrane. The physical evidence supporting this model is discussed elsewhere (4–7). Solid circles are residues projecting toward the front of an α-helix and stippled circles are residues projecting toward the back. The most hydrophobic segments are centered in the lipid bilayer on the basis of an analysis by the method of Engelman *et al.* (8). The numbered residues are conserved in all species, except Asp-61, which is Glu in all other species (4). The kink at Pro-64 is hypothesized on the basis of the crystal structure of alamecithin (9) and is also favored by Fimmel *et al.* (10) based on studies of a mutant.

Revertant colonies were evident after 3–4 days at 37°C. Chromosomal DNA was extracted from the revertant cells by the method of Silhavy *et al.* (26), digested with *Hin*dIII, and ligated with *Hin*dIII-digested plasmid pBR322. Recombinant plasmids carrying the chromosomal *Hin*dIII fragment with the *uncBEFHA* genes were selected by genetic complementation of strain MM349 (*uncB402*)—i.e., transformants capable of growth on minimal agar plates containing 22 mM succinate and ampicillin (50 μg/ml) were selected. Plasmids with the expected DNA fragment were digested with *Pvu* II, and the blunt-ended *Pvu* II fragment containing the *uncE* gene was cloned into *Sma* I-digested M13mp18 replicative form. The DNA sequence of the sense strand was determined by use of a primer that hybridizes 48 nucleotides past the 3′ end of the *uncE* gene (nucleotides 2173–2189; ref. 21) and with a primer hybridizing at the 5′ end of the *uncE* gene (nucleotides 1886–1906).‡ The latter primer allowed sequencing past a *Pst* I site at position 1561 in the *uncB* gene.

Incorporation of Mutant Genes into the Chromosome. A *Pst* I/*Hpa* I fragment of the Ala-24–Gly-61 → Asp-24–Gly-61 revertant DNA—i.e., bases 1561–2162 including the *uncE* gene—was exchanged into an otherwise normal chromosomal *unc* operon. To do this, the *Pst* I/*Hpa* I fragment was first cloned into the equivalent *Pst* I and *Hpa* I sites of *uncBEFH* DNA (nucleotides 870–3216) in plasmid pDF163 (27). DNA containing the *uncE* gene was crossed into the chromosome of the *uncE* deletion strain LW180 [Δ*uncE334*-(Kan^R)] by a double recombination, and the new chromosomal *uncE* gene was transferred by P1 transduction to strain MJM62 as described (21) to generate strain MJM414. A similar procedure was used to transfer the Asp-61 → Glu mutation from the mutant phage F2 to the chromosome. In this case, the *Ava* I/*Hpa* I (nucleotides 1876–2162) fragments of *unc* DNA were exchanged with those in plasmid pDF163, and the mutant DNA was recombined into the chromosome and transduced into strain MJM62 to generate strain MJM385.

Other Procedures. Membranes were prepared and routine biochemical assays were performed as described (21).

RESULTS

Repositioning the Essential Carboxyl Group of Subunit c. We initially attempted to reposition the essential carboxyl group at residues near Asp-61 by oligonucleotide-directed mutagenesis. In a limited test, Asp or Glu was substituted at position 58, 60, or 62, and Gly or Ala was substituted for Asp at position 61 (Table 1). Each of the three double substitutions was tested for function by genetic complementation and proved completely negative in the test for growth on succinate. On the other hand, the Asp-61 → Glu substitution led to formation of a functional protein.

In a second type of approach, revertants of the Asp-61 → Gly and Asp-61 → Asn mutations were isolated on succinate minimal medium plates and screened for second-site suppressor mutations. The growth yield of the revertants on 0.02% glucose was first determined to distinguish cells that had likely reverted to wild type. All 24 revertants to the Asp-61 → Asn mutation gave growth yields near wild type. The DNA of one such revertant was sequenced and the expected reversion of Asn-61 back to Asp-61 (wild type) was found. In contrast, 15 of 19 revertants to the Asp-61 → Gly mutation gave a growth yield significantly less than wild type. DNA from two revertants giving a high growth yield was sequenced and the expected reversion to wild type was found. DNA from two revertants showing a lower growth yield was also sequenced. In both of the revertants giving a low growth yield, the original Asp-61 → Gly substitution was retained and a second substitution of Asp for Ala-24 was found.

Asp-61 → Glu and Ala-24–Asp-61 → Asp-24–Gly-61 Mutants Retain Partial Function. Prior to characterization, a small segment of mutant DNA that had been completely

‡The nucleotide numbering system is that described by Walker *et al.* (3).

Table 1. Mutants generated by oligonucleotide-directed mutagenesis

Amino acid change	Oligonucleotide	Activity
None (wild type)	5'-CGG GAT AGC ATC CAC CAG ACC CAT-3'	+
Asp-61 → Glu	5'- GG GAT AGC CTC AAC CAG ACC-3'	+
Gly-58–Asp-61 → Asp-58–Gly-61	5'-GAT AGC ACC CAC CAG ATC CAT-3'	−
Val-60–Asp-61 → Glu-60–Ala-61	5'-CGG GAT AGC AGC CTC CAG ACC-3'	−
Ala-62–Asp-61 → Asp-62–Ala-61	5'-CGG GAT ATC AGC CAC CAG ACC-3'	−

Oligonucleotides are complementary to the sense strand. Nucleotides that have been changed from wild type are underlined. Plasmids carrying the mutant genes were transformed into a *uncE114* (Gln-42 → Glu) mutant, and positive complementation was scored by growth on succinate minimal medium.

sequenced was exchanged into an otherwise normal *unc* operon in the chromosome. This was done to be certain that the properties observed resulted solely from the mutation that was detected by DNA sequencing, and not from possible changes that had not been detected. Growth of the chromosomal recombinant strains on succinate are compared to wild type in Fig. 2. The Glu-61 mutation reduced the growth rate and yield to ≈50% of wild type, whereas the Asp-24–Gly-61 double mutation resulted in a smaller impairment in growth rate and yield—i.e., to ≈60% of wild type. These results show that the mutant F_1F_0 complexes retain the capacity to synthesize ATP by oxidative phosphorylation, although at a rate lower than in wild type.

Properties of Membrane ATPase. Under normal ATPase assay conditions, the specific ATPase activity of mutant membranes was significantly lower than that of wild-type membranes (column 1, Table 2). The reduction in ATPase activity resulted from inhibition of F_1 activity on binding of F_1 to F_0. This is shown in column 2 of Table 2, where the total ATPase activity was measured after release of F_1 from the membrane by EDTA treatment (see ref. 25 for protocol). The relative stimulation of ATPase activity on release of F_1 was always greater for the Asp-24–Gly-61 mutant than for the Glu-61 mutant. The membrane ATPase activity of both mutants was reduced to ≈40% of wild type when assayed in the HMK assay buffer (column 3, Table 2). This buffer was used in the DCCD titration and 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence quenching experiments described below because the F_1 ATPase remains tightly bound (21, 28).

It was of interest to determine whether the membrane ATPase of the Asp-24–Gly-61 mutant was inhibited by DCCD since we knew that the Ala-24 → Ser mutation markedly reduced DCCD binding to subunit c (ref. 16; R.H.F., M.O., and D. Fraga, unpublished data). As shown in Fig. 3, the Asp-24–Gly-61 membrane ATPase was much less sensitive to inhibition by DCCD than the ATPase of wild-type membranes. The small amount of inhibition observed has to be attributed to reaction of DCCD with Asp-24 since the ATPase of the Ala-24–Gly-61 control membranes was completely resistant to DCCD. In addition, Fig. 3 shows that the Glu-61 membrane ATPase was considerably more sensitive to inhibition by DCCD than the wild-type membrane ATPase.

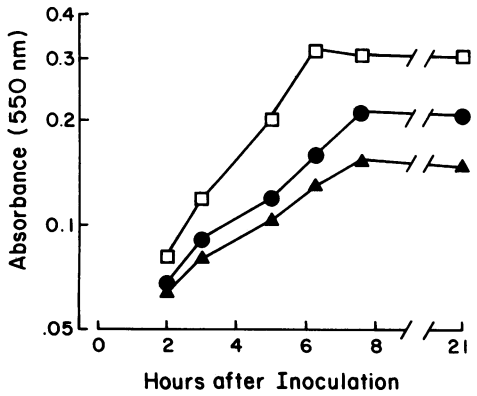

FIG. 2. Growth of Glu-61 mutant and Asp-24–Gly-61 double mutant by oxidative phosphorylation on a succinate carbon source. Cells were grown in minimal medium 63 containing 3.7 mM succinate and thiamine (5 μg/ml). Samples of the cultures were taken at the indicated intervals and the absorbance was read at 550 nm. □, Wild-type strain MJM125; ●, strain MJM414 (Asp-24–Gly-61); ▲, strain MJM385 (Glu-61).

ATPase-Coupled H^+ Translocation by Mutant Membranes. ATP-driven quenching of acridine dye fluorescence is a sensitive indicator of ATPase-coupled H^+ pumping to the interior of inside-out membrane vesicles. In several preliminary experiments with quinacrine, the ATP-driven quenching responses observed with mutant membrane vesicles was very small (<10% quenching), and we therefore changed to the use of a more sensitive indicator, ACMA. Typical ATP-driven quenching responses with ACMA are shown in Fig. 4. ATP hydrolysis by wild-type membrane vesicles resulted in 80–85% quenching of the ACMA fluorescence. The quenching response by mutant membrane vesicles was significantly reduced—i.e., on average 37% ± 4% (n = 8) quenching by the Asp-24–Gly-61 vesicles and 24% ± 3% (n = 5) quenching by the Glu-61 vesicles.

As a means of correlating the quenching responses with the actual rate of H^+ pumping, we chose to reduce the H^+ pumping rate of wild-type vesicles by slowing ATPase activity; we then correlated the reduction in ATP hydrolysis rate with the reduction in ACMA quenching response. The rate of ATP hydrolysis was reduced either by lowering the ATP substrate concentration or by inhibiting the enzyme with DCCD. The reduction in the ACMA quenching response proved to depend on the means that was used to reduce

Table 2. ATPase activity of mutants is inhibited when F_1 is membrane bound

Strain	ATPase activity of membrane, μmol·min^{-1}·mg^{-1}		
	Tris Mg assay buffer*		HMK assay buffer†
	F_1 bound	F_1 released	
MJM125 (wild type)	0.354	0.417	0.075
MJM385 (Glu-61)	0.258	0.456	0.031
MJM414 (Asp-24–Gly-61)	0.136	0.471	0.029

Values shown are from a single membrane preparation, but are representative of several independent preparations.

*Membranes were suspended at 10 mg of protein per ml in ice-cold TEDG buffer [1 mM Tris·HCl, pH 8.0/0.1 mM Na_2EDTA/1 mM dithiothreitol/10% (vol/vol) glycerol] and immediately diluted into 4 vol of TEDG buffer to release F_1 from the membrane, or into 4 vol of TMDG buffer [50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/1 mM dithiothreitol/10% (vol/vol) glycerol] where F_1 remains membrane bound (25). After 30 min at 30°C, 10-μl (20 μg) samples were diluted into 1.0 ml of Tris MgATPase assay buffer (50 mM Tris·H_2SO_4, pH 7.8/0.20 mM $MgSO_4$/0.4 mM [γ-^{32}P]ATP) and assayed for ATPase activity at 30°C.

†Membranes (125 μg of protein in 10 μl of TMDG buffer) were diluted into 1 ml of HMK buffer (final concentrations, 10 mM Hepes-KOH, pH 7.8/5 mM $MgCl_2$/300 mM KCl) and assayed for ATPase activity at 25°C using 1 mM [γ-^{32}P]ATP.

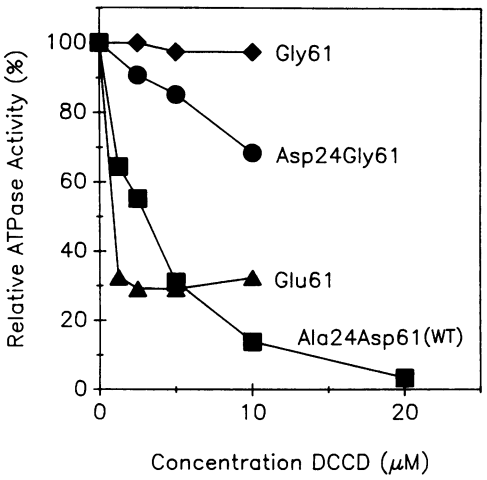

FIG. 3. Mutations change DCCD sensitivity of membrane ATPase. Membranes (125 μg of protein in 10 μl of TMDG buffer) (Table 2) were suspended in 0.9 ml of a 10/9 concentration of HMK assay buffer (see Table 2) at 25°C and treated with DCCD (added in 10 μl of ethanol) at the indicated concentrations for 20 min. The ATPase assay was initiated by addition of 100 μl of 10 mM [γ-^{32}P]ATP and the reaction was stopped after an additional 5 min. ■, Wild-type membranes; ●, Asp-24–Gly-61 mutant membranes; ▲, Glu-61 mutant membranes; ◆, Gly-61 mutant membranes. The specific ATPase activities of the membranes were, respectively, 0.078, 0.037, 0.028, and 0.032 μmol·min^{-1}·mg^{-1}.

ATPase activity, and two calibration curves were generated (Fig. 5). The 37% quenching response observed with Asp-24–Gly-61 vesicles hydrolyzing 1 mM ATP corresponded to that of wild-type vesicles hydrolyzing 8 μM ATP at 4% of the control rate or to DCCD-treated, wild-type vesicles hydrolyzing ATP at 24% of the control rate. The 24% quenching response by Glu-61 mutant vesicles corresponded to wild-type ATP hydrolysis rates of 2% and 18%, respectively.

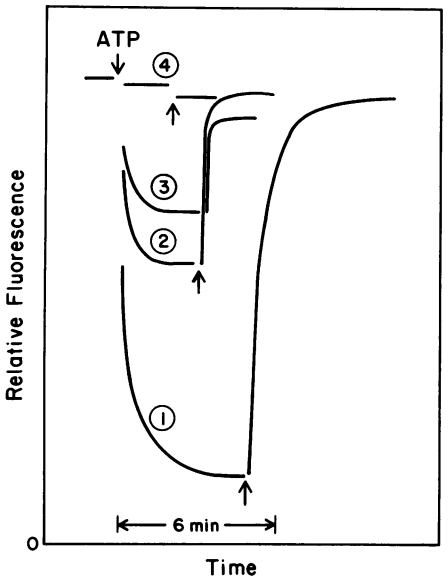

FIG. 4. ATP-driven quenching of ACMA fluorescence by wild-type and mutant membrane vesicles. The cuvette contained membrane vesicles (0.25 mg of protein in 20 μl of TMDG buffer) in 2 ml of HMK assay buffer (see Table 2). ACMA (100 μg/ml in ethanol) was added to 0.25 μg/ml and the fluorescence emission was recorded at 485 nm after excitation at 415 nm. At the downward arrow (↓) 20 μl of 100 mM ATP was added and the cuvette was mixed on a Vortex stirrer. At the upward arrow (↑), 10 μl of 1 mM carbonylcyanide *m*-chlorophenylhydrazone was added and the cuvette was mixed. Trace 1, wild-type membrane vesicles (strain MJM125); trace 2, Asp-24–Gly-61 mutant membrane vesicles (strain MJM414); trace 3, Glu-61 mutant membrane vesicles (strain MJM385); trace 4, Gly-61 mutant membrane vesicles (strain MJM381).

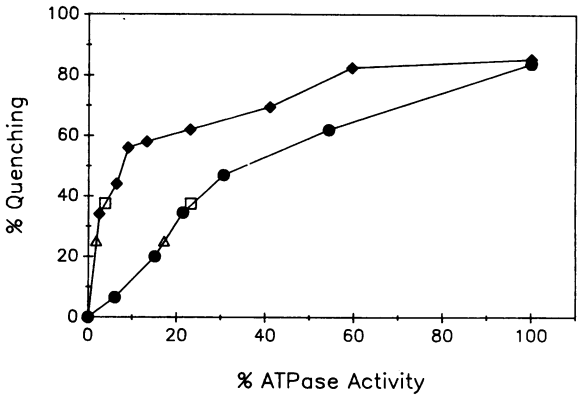

FIG. 5. Reduction of wild-type ACMA quenching response by reduction in ATPase activity and comparison with mutant quenching responses. DCCD curve (●), wild-type membrane vesicles at 0.5 mg/ml in HMK buffer were incubated at room temperature for 20 min with various concentrations of DCCD (0–15 μM). DCCD-treated samples were then diluted with 3 vol of HMK buffer and assayed for ATP-driven ACMA fluorescence quenching or ATPase activity on addition of ATP to 1 mM. [ATP] curve (◆), wild-type membrane vesicles at 0.125 mg/ml in HMK buffer were assayed for ATP-driven ACMA fluorescence quenching or ATPase activity after the addition of ATP to various final concentrations (5–1000 μM). For both curves, ATPase activity is plotted as a percentage of the activity measured at 1 mM ATP without DCCD treatment. The average quenching response of Asp-24–Gly-61 mutant membranes (□) and Glu-61 mutant membranes (△) is indicated on each calibration curve.

DISCUSSION

The most provocative finding of this study is that subunit c retains partial function when Asp is moved from position 61 to position 24. The simplest interpretation is that the protein does fold in the membrane like a hairpin, as depicted in Fig. 1, and that the Asp β-carboxyl group can assume the same relative position in the center of the membrane when anchored to either residue 24 of helix 1 or residue 61 of helix 2. The exact position of the carboxyl group appears to be critical to function since placement of a carboxyl group at position 58, 60, or 62 led to loss of function. The critical requirement for exact positioning of the carboxyl group is also suggested by the severely reduced function in the Asp-61 → Glu mutant.

In the limited screening carried out here, all revertants of the Asn-61 mutant gave growth yields close to wild type and were presumed to have reverted to the wild-type sequence. In contrast, most revertants of the Gly-61 mutant gave lower than wild-type growth yields, and these are likely to be second-site revertants. It seems possible that Asn at position 61 may sterically prevent insertion of an Asp at position 24 and thus prohibit formation of this type of second-site revertant. If this is the case, it would imply a tight packing of the protein in the pocket around residues 24 and 61. This may also be inferred by the conservation of other small residues in this region—i.e., the invariant Gly at positions 23 and 27, and invariant Gly or Ala at positions 25, 29, 58, and 62. A close association of residues 23–29 of helix 1 and residues 58–62 of helix 2 is also suggested by the mutation in residues 24 and 28 that decrease reactivity of Asp-61 with DCCD (ref. 18; R.H.F., M.O., and D. Fraga, unpublished data).

H^+ ATP synthase function is severely reduced in both the Asp-24–Gly-61 and Glu-61 mutants. This is indicated by both the slower growth of cells on succinate via oxidative phosphorylation (Fig. 2) and the reduced ATP-driven ACMA quenching response (Fig. 4). The efficiency of H^+ translo-

cation by the mutant F_1F_0 ATPases was calibrated by reducing ATPase activity in wild-type vesicles. The ACMA quenching response decreased more rapidly when ATPase activity was reduced by treatment with DCCD than when activity was reduced by decreasing the ATP substrate concentration (Fig. 5). Why do the two curves differ? One possible explanation is that DCCD preferentially reacts with F_1F_0 complexes in which ATPase and H^+ transport are properly coupled and that most of the DCCD-sensitive ATPase activity of the membrane is catalyzed by F_1F_0 complexes that are somehow defective in H^+ translocation. Alternatively, ATP hydrolysis at low substrate concentrations (<25 μM) may be preferentially coupled to H^+ transport. Because of the difference in the calibration curves, and the uncertainty of the explanation, our conclusions regarding the efficiency of ATP-driven H^+ translocation in the mutants are limited to ranges—i.e., 4–24% of wild type for the Asp-24–Gly-61 mutant and 2–18% for the Glu-61 mutant.§

The severity of the functional defects in the Asp-61 → Glu mutant are somewhat surprising, given that Glu is always found at this position in the protein of other species. The defect in function may be due to a stringent requirement for positioning of the residue 61 carboxyl group. Alternatively, the Glu for Asp substitution might significantly change the pK_a of the side-chain carboxyl. Such a change in pK_a could obviously reduce activity if protonation and deprotonation of the carboxyl was required during the catalytic cycle. The hyperreactivity of the Glu-61 carboxyl with DCCD may be related to these considerations. The additional methylene group in the Glu-61 side chain may place the carboxyl in a more exposed environment. Alternatively, the change in reactivity might relate to a change in the pK_a of the side-chain carboxyl group. Carbodiimides are now thought to react with the carboxylate anion in a general acid-catalyzed reaction (29). If the pK_a of the Glu-61 carboxyl group were lower than that of the wild-type Asp-61 carboxyl group, the reactivity would be expected to increase. In considering this explanation, note that for the amino acids, the pK_a of a Glu γ-carboxyl is higher (4.3), not lower, than that of an Asp β-carboxyl (3.9).

The finding that a functional subunit c is generated by substitution of Asp for Ala-24 in the Gly-61 mutant clearly indicates that the key functional element of this protein is a side-chain carboxyl group positioned at the center of the membrane. Although the carboxyl group can be anchored to either helix, it apparently needs to be precisely positioned in the center of the membrane as was discussed above. Previous studies show that Asn cannot substitute for Asp-61 (14, 15). This implies that either a carboxylate anion, or a group capable of undergoing a protonation–deprotonation cycle, may be required. Cox *et al.* (30) have suggested that the protonation and deprotonation of Asp-61 may be mediated by a proton wire consisting of hydrogen-bonding side chains on a transmembrane helix of subunit a. If a specific structural interaction between subunits a and c is required, the results reported here suggest that helix 1 and helix 2 of subunit c may interact as a unit during the proton transfer step. In this way, a structurally equivalent unit could be formed with the crucial carboxyl group anchored to either helix. The spans of residues 23–29 and 58–62 contain conserved Gly and Ala residues, and these segments may form the interacting structure. Further analysis of mutants in these regions may lead to a more extensive definition of these interactions.

§We have noted above that the ATP-driven quinacrine quenching response was even more reduced in the mutants. In the case of the Asp-24–Gly-61 mutant, we observed 6–7% quenching versus 80% quenching for wild type. When calibration curves similar to those shown in Fig. 5 were generated for quinacrine, the 7% quenching response was shown to correlate with ATPase activities of 3% and 21% when ATPase was reduced by decreasing ATP concentrations or by DCCD treatment, respectively. That is, there is a good correlation with the ACMA calibration curves.

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