Mutations in Single Hairpin Units of Genetically Fused Subunit c Provide Support for a Rotary Catalytic Mechanism in F\textsubscript{0}F\textsubscript{1} ATP Synthase*

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Previously, we constructed genetically fused dimers and trimers of subunit c of the Escherichia coli ATP synthase based upon the precedent of naturally occurring dimers in V-type H\textsuperscript{+}-transporting ATPases. The c\textsubscript{2} and c\textsubscript{3} oligomers have proven useful in testing hypotheses regarding the mechanism of energy coupling. In the first part of this paper, the uncoupling Q42E substitution has been introduced into the second loop of the c\textsubscript{2} dimer or the third loop of the c\textsubscript{3} trimer. Both mutant proteins proved to be as functional as the wild type c\textsubscript{2} dimer or wild type c\textsubscript{3} trimer. The results argue against an obligatory movement of the \(\varepsilon\) subunit between loops of monomeric subunit c in the c\textsubscript{12} oligomer during rotary catalysis. Rather, the results support the hypothesis that the c-\(\varepsilon\) connection remains fixed as the c-oligomer rotates. In the second section of this paper, we report on the effect of substitution of the proton translocating Asp\textsuperscript{61} in every second helical hairpin of the c\textsubscript{2} dimer, or in every third hairpin of the c\textsubscript{3} trimer. Based upon the precedent of V-type ATPases, where the c\textsubscript{2} dimer occurs naturally with a single proton translocating carboxyl in every second hairpin, these modified versions of the E. coli c\textsubscript{2} and c\textsubscript{3} fused proteins were predicted to have a functional H\textsuperscript{+}-transporting ATPase activity, with a reduced H\textsuperscript{+}/ATP stoichiometry, but to be inactive as ATP synthases. A variety of Asp\textsuperscript{61}-substituted proteins proved to lack either activity indicating that the switch in function in V-type ATPases is a consequence of more than a single substitution.

The mechanism by which H\textsuperscript{+} movement through the F\textsubscript{0} membrane sector drives rotation of the \(\gamma\) subunit during ATP synthesis remains to be established (10). The \(\gamma\) and \(\varepsilon\) subunits are known to interact with each other and appear to rotate as a fixed unit (10–13). Rotation of the \(\varepsilon\) subunit during ATP hydrolysis by F\textsubscript{1} ATPase has been directly demonstrated (14, 15). In addition, the \(\gamma\) and \(\varepsilon\) subunits are known to interact directly with the \(\gamma\) subunits of F\textsubscript{0} (16–18). Subunit c is known to fold in the membrane as a hairpin of two hydrophobic \(\alpha\)-helices connected by a polar loop on the F\textsubscript{0} binding side of the membrane (19). The conserved Asp\textsuperscript{61} carboxyl, centered in the second transmembrane helix, is known to catalyze proton transport via interaction with subunit \(\alpha\) (10, 19). In a recently determined NMR structure of monomeric subunit c, the folding and interaction of the two transmembrane helices takes place as is predicted from genetic and chemical studies of F\textsubscript{0} in situ (20). The 12 c subunits are now known to be arranged in an oligomeric ring, or cylinder, with subunits a and b of F\textsubscript{0} placed at the periphery of the ring (2, 10, 21–26). A recent 4-Å resolution x-ray diffraction model of a yeast mitochondrial F\textsubscript{1}-c\textsubscript{10} subcomplex supports the previously deduced oligomeric ring structure and the c-\(\varepsilon\) and c-\(\gamma\) connections between the surfaces of F\textsubscript{0} and F\textsubscript{1} (27). The sequential protonation-deprotonation of Asp\textsuperscript{61} at the a-\(\varepsilon\) interface was proposed to drive rotation of the c-oligomer in stepwise 30° increments (6, 28–30), and in so doing drive rotation of subunit \(\gamma\) within F\textsubscript{1}. Direct evidence for rotation of the c-oligomeric ring was recently presented (31). In an alternative hypothesis, the \(\gamma\) and \(\varepsilon\) subunits were proposed to move from one subunit c to the next as a consequence of sequential conformational changes at the c-\(\varepsilon\) interface brought about by proton binding and release through each subunit c (19, 32).

In a previous study, we constructed genetically fused functional dimers and trimers of subunit c based upon the precedent of the subunit c in vacuolar (V-type) ATPases. The functional fusion proteins provided a novel experimental system to test whether a functional polar loop was required in each subunit c of the oligomer, as would be expected if the \(\gamma\) subunits sequentially moved from one c subunit to the next in the process of rotation. The cQ42E polar loop substitution had been

H\textsuperscript{+}-transporting F\textsubscript{0}F\textsubscript{1} ATP synthases utilize the energy of a transmembrane electrochemical H\textsuperscript{+} gradient to catalyze formation of ATP. Closely related enzymes are found in the plasma membrane of eubacteria, the inner membrane of mitochondria, and the thylakoid membrane of chloroplasts (1). The enzyme is composed of distinct extramembranous and transmembrane sectors, termed F\textsubscript{1} and F\textsubscript{0}, respectively. Proton movement through F\textsubscript{0} is reversibly coupled to ATP synthesis or hydrolysis in catalytic sites of F\textsubscript{1}. Each sector of the enzyme is composed of multiple subunits with the simplest composition being \(\alpha_{\beta_{2}}\gamma\delta\varepsilon\) for F\textsubscript{1} and \(\alpha_{b_{2}}\varepsilon\gamma_{2}\delta\) for F\textsubscript{0} in the case of the Escherichia coli enzyme (2–4). Homologous subunits are found in mitochondria and chloroplasts. An atomic resolution x-ray structure of the \(\alpha_{\beta_{2}}\gamma\) portion of bovine F\textsubscript{1} shows the three \(\alpha\) and three \(\beta\) subunits alternating around a centrally located \(\gamma\) subunit, with the \(\gamma\) subunit interacting asymmetrically with the three \(\beta\) catalytic subunits (5). Subunit \(\gamma\) was subsequently shown to rotate with respect to the three \(\beta\) subunits during catalysis (6–8). Rotation of \(\gamma\) is thought to change the binding affinities in alternating catalytic sites to promote tight substrate binding and product release during catalysis (9). During ATP synthesis, the rotation of \(\gamma\) must be driven by proton translocation through F\textsubscript{0}.

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shown to “uncouple” H+ translocation from ATP synthesis or hydrolysis (33), a phenotype that could be suppressed by second site substitutions in Glu31 of subunit ε (34). In this study we show the Q42E substitution can be introduced into every second loop of the c2 dimer or every third loop of the c3 trimer without altering function. The result is clearly consistent with the suggestion that the c-ε interface remains fixed during rotary catalysis (6, 16, 28–30). The c subunits of V-ATPases are composed of four transmembrane helices and seem to have evolved by gene duplication of an F0 type progenitor gene (35). They have a single H+ transporting carboxylate in the fourth transmembrane helix or second helical hairpin. In this study, we have studied the effect of removal of one of the essential aspartates in the F-type c2 dimer or c3 trimer to test the hypothesis that the enzyme might still function but as a V-type, H+-pumping ATPase with a reduced H+/ATP stoichiometry (35). We were unable to replace any of the transmembrane Asp in a variety of substituted dimers or trimers without complete loss of function.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—The plasmids used in this study are derivatives of plasmid pDP163, which contains the wild type uncBEFH genes (bases 870–3216 coding subunits a, c, b, δ) cloned between the HindIII and SphI sites of plasmid pBR322 (36). The plasmids carrying the wild type c2 monomer (pNOC) and fused c2 dimer (pPJ2), c3 trimer (pPJ3), and c4 tetramer (pPJ4) were derived from plasmid pNOC, which has a C21S substitution in subunit b, a mutation which has no effect on function (4, 24). These plasmids and the derivatives described below were expressed in the recA chromosomal uncBEFH deletion strain J3001 (pyrE41, entA43, argR1, rpsL109, supF44, ΔuncBEFH, recA56, srl:Trp10) (4). (Complementation of the unc (succinate minus) phenotype was tested by transferring transformant, ampicillin-resistant colonies to minimal medium plates (38) containing 2 mM succinate, 2 mg/liter thiamine, 0.2 mM uracil, 0.2 mM l-arginine, 20 μM dihydriodiloxanobenzoin acid, and 100 mg/liter ampicillin.

Generation of cQ42E-substituted Plasmids—Plasmid pC1Q42E was generated by subcloning a PstI/BstEII fragment containing the cQ42E substitution from the whole unc operon plasmid pYZ186 (34) into the respective sites of plasmid pDF163 and the cloning was confirmed by sequencing. Plasmid pCQ2Q42E was subsequently used to generate plasmids pCQ2Q42E, pC3Q42, and pC4Q42E, carrying, respectively, genes for a subunit c2 dimer (c2), and c3 trimer (c3) and c4 tetramer (c4) harboring in each case a single substitution in the last polar loop. A single AvaI site is present in the plasmid pCQ2Q42E at bases 1976–1981 in the uncE gene in codons for amino acid residues 31 and 32 of subunit c2. In plasmid pPJ2 encoding the fused c2 dimer, the AvaI fragment cloned between two AvaI sites encodes the C-terminal segment from residues 31 and 32 of the first subunit, the linking peptide, and the N-terminal segment to residues 31 and 32 of the second subunit. This AvaI fragment was cloned into the AvaI site of the cut and alkali phosphate-treated plasmid pCQ2Q4E to generate plasmid pCQ2Q4E. Similarly, plasmids pPJ3 or pPJ4 were partially digested with AvaI and the appropriate large AvaI fragment purified from an agarose gel and cloned into the AvaI site of pCQ2Q4E to generate pCQ3Q4E and pC4Q4Q2E. All constructs were confirmed by restriction mapping and sequencing.

Generation of Plasmids with Single Substitutions for Asp→Glu—The c2 dimeric constructs with Asp→Glu substitutions in the first or N-terminal segment were constructed using the PCR2 strategy described in Jones and Fillingame (4) wherein a plasmid encoding the substitution (D61N, D61E, or D61S) was used to generate the PCR product B. The substitutions generated by this method were c2D61N, c2D61E, and c2D61S.3 Plasmids bearing the c2D61N, c2D61E, and c2D61S substitutions were generated by cloning the AvaI fragment from the aforementioned substituted c2 plasmids into an incomplete AvaI digest of plasmid pPJ2. Plasmids bearing the c3D61N, c3D61E, and c3D61S were generated by the previously cited PCR strategy (4) using plasmid encoding the D61N substitution to generate PCR product A. Wild type AvaI fragments from pPJ2 were cloned into partial AvaI digest of the c3D61N plasmid product to generate plasmids bearing c3D61N and c3D61S. Each of the constructs was confirmed by DNA sequencing.

**Biochemical Assays**—Membranes were prepared and stored in TMG buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM dithiothreitol, 10% (v/v) glycerol) at 20 mg/ml after passage of cells through a French press (33). ATPase activity and protein were determined as described (33). ATP-driven ACMA quenching and NADH-driven quinone acquenching assays were carried out in HMK assay buffer (10 mM HEPES-NaOH, pH 7.5, 5 mM MgCl2, 300 mM KCl) as described (33). Membranes were solubilized for SDS electrophoresis by dilution of samples into SDS sample buffer (6.25 mM Tris-HCI, pH 6.8, 2% SDS, 10% (v/v) glycerol, and 0.01% bromphenol blue) and incubation for 1 h at room temperature immediately before electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out using the Tris-Tricine buffer system of Schägger and von Jagow (39) with a 0.75-mm-thick 15% acrylamide slab gel (14.5% acrylamide, 0.45% bisacrylamide), and proteins transferred from the gel electrophoretically onto a polyvinylidene difluoride membrane (40). Immunostaining was carried out using the ECL system (Amersham Pharmacia Biotech). Rabbit antiserum to subunit c was pretreated as described (41) and diluted 1:10,000 prior to use.

**RESULTS**

Effect of the Q42E Substitution in the Last Loop of Subunits c2 and c3—A Q42E mutation was introduced into the last polar loop in the c2 dimer, c3 trimer, and c4 tetramer to generate subunits c2Q42E, c3Q42E, and c4Q42E, respectively. The modified c subunits were expressed from a plasmid that also encoded the α, β, and δ subunits of F0F1 in the recA, ΔuncBEFH chromosomal deletion strain J3001. Subunits α, β, γ, and ε are expressed from the chromosome of this strain. Growth of transformants was tested on succinate minimal medium, where growth depends upon a functional oxidative phosphorylation system. As described previously, the c2Q42E mutant was unable to grow on succinate. However, mutants expressing the c2Q42E- and c3Q42E-substituted proteins grew similarly to strains expressing the wild type version of the c2 dimer (c2Q42E) (Table I), strains expressing the c3 wild type) or c4Q42E protein did not grow on succinate minimal medium. Wild type and mutant proteins were expressed at roughly comparable levels, as indicated by the immunoblot shown in Fig. 1A. The anti-c serum used had previously been shown to be directed to residues in the polar loop (41), and the lighter band for c4Q42E membranes may reflect reduced antibody binding to the substituted polar loop. Although several of the bands run as doublets for reasons that we do not understand.

**Table I**

| Subunit version | Growth on succinate (mm) |
|-----------------|--------------------------|
| c2 (wild type)  | 2.0–3.0                  |
| c2 (Q42E)       | 0.8–1.0                  |
| c3 (Q42E)       | 1.2–1.6                  |
| c3 (Q42E)       | 0.8–1.0                  |
| c4 (Q42E)       | 0                        |

1. The unc DNA numbering system corresponds to that used by Walker et al. (37).
2. The abbreviations used are: PCR, polymerase chain reaction; ACMA, 9-amino-6-chloro-2-methoxyacridine; LDAO, laurendihydroxylamine oxide; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DCCD, dicetylhexylcarbodiimide.
3. In the text, the repeated residues are designated by *, **, and *** to indicate the second, third, or fourth hairpin repeat. For example, c2 dimers with an Asp→Glu substitution in the first or second hairpin are designated c2D61N or c2D61E, respectively.

*Subunit c derivatives or fusion proteins were expressed from a pNOC-like plasmid in ΔuncBEFH (a,b,β) chromosomal background strain J3001 (recA), except as noted.

*Colony size after 72 h of incubation at 37 °C.

Expressed in strain JWP109 (ΔuncBEFH, recA).
Mutation of Fused Subunit c in E. coli F₀F₁ ATP Synthase

Fig. 1. Anti-subunit c immunoblot of membranes from strains expressing wild type and mutant c₁, c₂, c₃, and c₄ subunits. 50 µg of membrane protein was applied in each lane. A, immunoblot showing wild type and Q42E-substituted subunits. B, immunoblot showing wild type and Asp⁶₁-substituted subunits.

stand, the immunoblots give no indication that the fused proteins are degraded to smaller units, and seem to rule out proteolysis as a possible explanation for the observed function.

The ATPase activity of membranes prepared from these cells is shown in Table II. The LDAO-activated ATPase activity is the best indicator of the total amount of F₁ bound to the membrane (42). In wild type membranes, LDAO results in a 3–4-fold activation of membrane-bound F₁ ATPase. The low LDAO-activated ATPase activity of the c₁, Q42E membranes may indicate significant loss of F₁ ATPase from the membrane during cell rupture and membrane preparation, although the amount of F₀ in these membranes could also be somewhat reduced based upon the immunoblotting (Fig. 1). The extent of loss of F₁ ATPase from the chromosomal Q42E mutant had previously been shown to be strain-dependent (36). The c₂ and c₃ membranes (wild type or Q42E) show similar extents of activation by LDAO. Less F₁ ATPase activity seems to be lost during preparation of the c₂ (Q42E) and c₃ (Q42E) membranes than from c₁, Q42E membranes. In the c₄ mutant membranes (wild type or Q42E), the membrane-bound ATPase is more extensively inhibited and, when treated with LDAO, activated approximately 8-fold.

The ATP-driven ACMA quenching response of these membrane vesicles is shown in Fig. 2. As reported previously, the c₁ (Q42E) mutant shows virtually no activity, whereas quenching activity comparable to that seen for the wild type dimer and trimer was observed for c₂(Q42E) and c₃(Q42E) membranes. As we have reported previously (4), the c₅ trimeric protein promotes better ATP-driven proton pumping, as measured by ACMA quenching, than does the c₂ dimer. This is clearly unusual since strains expressing the c₂ dimer grow better by oxidative phosphorylation on succinate minimal medium (Table I). ATP-driven ACMA quenching had not previously been tested with the membranes from the c₁ tetramer (4). As shown here (Fig. 2), mutant membranes from either the c₂(wild type) or c₃(Q42E) show a significant quenching response even though the activity is insufficient to support growth by oxidative phosphorylation (Table I).

The cQ42E mutant was described as being “uncoupled” because its plasma membrane vesicles, containing normal amounts of bound F₁, were permeable to protons (34). This was reflected by a reduction in the NADH-driven quinacrine quenching response, caused by partial collapse of the H⁺ electrochemical potential due to proton transport through F₀. Membrane vesicles of the c₁(Q42E) transformant strain exhibit this uncoupled phenotype as illustrated in Fig. 3. In compari-
son to c1 (wild type), the c1(Q42E) membrane vesicles exhibit a significantly reduced NADH-driven quinacrine quenching response, which on DCCD treatment to block proton permeation via F0 was restored to wild type levels. In contrast, this uncoupled phenotype was not apparent with the c2(Q42E) and c3(Q42E) mutant membranes.

The passive proton permeability of the mutant membrane vesicles was also examined after removal of F1 from the membrane (Fig. 4). The c1 membrane vesicles (wild type and Q42E) exhibited a high proton permeability, as indicated by the small NADH-driven quinacrine quenching response, which was greatly enhanced by DCCD treatment to block proton transport by F0. In contrast, stripping of F1 from the c2 membranes (wild type or Q42E) only marginally increased the proton permeability, suggesting perhaps that the c2 dimeric proton channel was less stable. The response of c3 membrane vesicles (wild type or Q42E) was close to that seen with c1 membranes. This more active passive transport capacity may relate to the greater ATP-driven H\(^+\) transport activity seen in c3 versus c2 membrane vesicles.

Effect of Substitution of Asp61 in Fused Dimers, Trimers, and Tetramers of Subunit c—Based upon the precedent of eucaryotic V-type ATPases, one might expect to be able to substitute one of the multiple Asp61 carboxylates of the E. coli fused subunit c and retain ATP-driven proton pumping activity with a reduced H\(^+\)/ATP ratio. We generated a variety of Asp61-substituted fused proteins to test this hypothesis (Table II). Each of the mutated fused proteins was expressed, as shown by immunoblotting (see Fig. 1B for examples). None of the transformant strains expressing these proteins proved capable of growth on succinate minimal medium, which indicated a loss of oxidative phosphorylation activity. The membrane ATPase activity of all of the Asp61-substituted strains was very low (Table II), and the very low activity can be attributed to inhibition of the F1 ATPase in the membrane-bound state. This is indicated by the 15–35-fold activation caused by LDAO treatment (Table II).

The Asp61 substitutions made all seem to totally abolish ATP-driven proton pumping as assayed in the ACMA quenching assay (Fig. 5). The following combinations were tested. In direct analogy to the vacuolar ATPase proteolipid, the Asp in the first hairpin of the dimer was replaced by Asn, Ser, and Gly (Fig. 5B). An Asp→Asn substitution was also tried in the second hairpin of the c2 dimer (Fig. 5B). Gly and Ser were substituted in the second (middle) hairpin of the c3 trimer and Asn substituted in the third hairpin (Fig. 5C). The final substitution attempted was Asp→Asn in the fourth hairpin of the c4 tetramer (Fig. 5D).

**DISCUSSION**

Substitution of Q42E into the last polar loop of the c2 dimer and c3 trimer of subunit c resulted in functional enzymes that permitted growth on succinate minimal medium. Relative to the wild type c2 and c3, the substitution of Q42E in every second or every third loop had little effect on in vitro function, i.e. the extent of ATP-driven quenching of ACMA fluorescence was equivalent on comparing wild type and Q42E c2 dimers or wild type and Q42E c3 trimers. Further, in contrast to the monomeric Q42E c1 mutant, membranes of the c2 and c3 Q42E mutants did not exhibit an obvious proton leak. The facile genetic complementation of the Q42E c1 mutant supports the idea that an F\(_0\)F\(_1\) with a mixture of wild type and Q42E subunits can be functional (33, 43). The results are most easily interpreted by a rotational model, where the c subunit remains fixed in linkage with a single c subunit of the the oligomeric ring, as suggested by others (16). Cross-linking analysis suggests that the Glu31 residue packs near the Glu42 residue at the interface of two subunit c, which are packed in a front-to-back manner, and subunit \(\epsilon\) is predicted to pack at neighboring dimeric c-interfaces (18). The observed uncoupling may require a substitution of Q42E in each subunit c to disrupt a specific, fixed c-\(\epsilon\) linkage.

Based upon accessibility of Cys in the cQ42C-substituted mutant, Watts and Capaldi (45) have suggested that four or five c subunits may be involved in F1 binding via the \(\epsilon\) and \(\gamma\) subunits. The results presented here indicate that portions of the \(\epsilon\) and \(\gamma\) subunits are able to pack at the interface of Q42E-substituted subunit c without disruption of function. The dimensions of subunit \(\epsilon\) indicate that it is likely to have contacts with at least two subunit c within the oligomeric ring. The finding that all second site suppressor mutations isolated in the cQ42E mutant mapped to eGlu31 (34) suggest a very specific interaction between these residues, and that this portion of subunit \(\epsilon\) is likely packed with wild type loops of the c2 and c3 substituted proteins. Ketchum and Nakamoto (44) have recently shown that second site suppressors to the \(\gamma\)E208K mutant map to residues in the loop region of subunit c that fall on both faces of subunit c in the NMR structure. These results are consistent with the region surrounding residue 208 of subunit \(\gamma\) also packing between c subunits, as is proposed for the Glu31 region of subunit c (18).

The results discussed above support a model where the \(\epsilon\) subunit remains fixed to a special pair, or pairs, of subunits as the c-oligomer rotates to drive the rotation of the \(\gamma\) subunit. The question had been debated, based upon the differing effects of Cys-Cys cross-linking with cQ42C mutants. Based upon the inhibitory effect of cross-linking between \(\epsilon\)E31C and cQ42C on ATP-driven quenching of ACMA fluorescence, Zhang and Fillame (17) had suggested that the \(\epsilon\) subunit might move from one subunit c to the next in response to conformational changes linked to the protonation and deprotonation of Asp61. We have subsequently examined \(\epsilon\)E31C/cQ42C membranes after cross-linking and conclude that at least a portion of the diminution of...
FIG. 4. Comparison of proton leakiness of Q42E stripped membranes using NADH-driven quinacrine quenching response. Stripped membrane were diluted to 0.3 mg/ml in HMK assay buffer (10 mM HEPES, pH 7.5, 5 mM MgCl₂, 300 mM KCl) containing 0.357 μg/ml quinacrine, and NADH was added to 50 μM. The reversal of quenching is due to consumption of NADH in the cuvette. DCCD-treated membranes (+DCCD) were diluted into HMK assay buffer and incubated with 20 μM DCCD for 15 min at room temperature prior to addition of NADH.

FIG. 5. Tests for ATP-driven quenching of ACMA fluorescence with Asp⁶¹-substituted c₁, c₂, c₃, and c₄ membranes. Membranes were diluted to 0.75 mg/ml in HMK assay buffer (10 mM HEPES, pH 7.5, 5 mM MgCl₂, 300 mM KCl) and ACMA added to 0.3 μg/ml. ATP was added to 0.94 mM and the uncoupler SF6847 added to 0.3 μM at the times indicated. Comparison of quenching response is as follows: A, quenching by c₁ monomer membranes; B, quenching by c₂ dimer membranes; C, quenching by c₃ trimer membranes; D, quenching by c₄ tetramer membranes.

the ATP-driven ACMA quenching response can be attributed to increased proton leakiness.⁴ In contrast, Watts et al. (16) noted minimal effects of cross-linking of the γY205C and c⁴Q42C subunits on membrane ATPase activity and suggested that these results were most consistent with a fixed γε-c rotor hypothesis. The γ-c cross-link did abolish ATP-driven ACMA quenching, but this was attributed to a proton leak induced by the cross-linking reaction (16). The situation has recently been clarified in the study of Schulenberg et al. (46), who introduced the c⁴Q42C mutation into second loop of a c₂ dimer. Near quantitative cross-linking of c₂Q42C to εE31C resulted in minimal disruption of ATP synthesis or ATP-driven ACMA quenching and strongly supports the fixed γε-c rotor hypothesis. Ironically, cross-linking of εQ42C to γY207C led to uncoupling, as was the case in cross-linking of γY205C to monomeric εQ42C (16).

What then is the molecular basis for an uncoupled phenotype, where ATP hydrolysis is uncoupled from proton translocation and the junction between F₀ and F₁ perturbed such that membrane is leaky to protons? Presumably, the γ subunit of F₁ is permitted to rotate to the top of the ring of c subunits while F₁ is still held in place. To explain the high proton permeability of whole membranes, the mutations at the γε-c interface must result in structural changes that lead to opening of proton channels through F₀, where the opening would normally only take place during coupled ATP hydrolysis or synthesis in F₁. The question of whether the oligomeric ring of subunit c is rotating during uncoupled proton transport remains to be answered. It is possible that the c subunits oscillate back and forth between exit and entrance half-channels on the two sides of the membrane to promote the bidirectional, passive H⁺ translocation. Such an oscillation might require a structural change in subunit α that alters the proximity of αArg₂₁₀α to cAsp⁶¹α, which is postulated to promote unidirectional rotor motion (30). In support of this idea, the αR₂₁₀A substitution itself leads to an uncoupled, H⁺-leaky phenotype (47). The passive H⁺ conductance of whole or stripped cAsp⁶¹α membranes actually exceeds that of wild type stripped membranes.

Eight different versions of c₂, c₃, and c₄ fusion proteins were created wherein one of the multiple Asp⁶¹ residues was replaced by Asn, Gly, or Ser. None of the strains expressing these subunits exhibited a detectable ATP-driven quenching response measured with inverted membrane vesicles. The lack of a quenching response may result at least partially from the severe inhibition of the membrane ATPase activity in each strain (Table II).⁵ The inhibition observed suggests that F₁ is bound to the membrane in a coupled fashion and that replacement of one Asp⁶¹ in a fused multimer of two, three, or four subunits is sufficient to abolish H⁺ translocation by F₀. The inhibitory effect of the single Asp⁶¹ substitution is consistent with the results from previous studies where reaction of DCCD with approximately one of the 12 c subunits in the oligomer was shown to abolish ATPase activity (49), and where reconstitution of F₁ with one cD61G subunit or two cD61N subunits per c-oligomer was shown to abolish passive H⁺ translocation activity (50). Does H⁺ movement require the interaction of two adjacent carboxylates in F-type ATPases? Based upon a new NMR structure of subunit c in its deprotonated form, Rastogi and Girvin (51) have recently suggested that there may be

⁴ J. Hermolin and R. H. Fillingame, unpublished results.

⁵ The severe inhibition of membrane-associated F₁ ATPase seen with the Asp⁶¹ substitutions in Table II proves to be unexpected, in that chromosomal cD61N and cD61G strains show high rates of ATPase activity even though proton translocation through F₀ is blocked (48), i.e., in the chromosomal strains, the F₁ appears to assemble with F₀ in an uncoupled fashion.
large movements around Asp at the subunit interface and proposed a possible interaction of protonated and deprotonated carboxylates during H+ transport. Assuming that the c-oligomer of V-type (vacuolar) ATPase rotates past a site of protonation-deprotonation within a subunit α equivalent, with the H+ translocating carboxylate present on every other hairpin unit, then the mechanism may differ substantially from that in F,F,F ATP synthases where rotation seems to be designed to occur in 30° increments. The conformational interactions and electrostatics taking place between V0 subunits of V-type ATPases may be designed to facilitate the movement of the rotor in 60° or 120° increments since these enzymes seem to function unidirectionally as ATPases (35, 52).

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