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Minireview

H^+ Transport and Coupling by the F_0 Sector of the ATP Synthase: Insights into the Molecular Mechanism of Function

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The F_0 sector of the ATP synthase complex facilitates proton translocation through the membrane, and via interaction with the F_1 sector, couples proton transport to ATP synthesis. The molecular mechanism of function is being probed by a combination of mutant analysis and structural biochemistry, and recent progress on the *Escherichia coli* F_0 sector is reviewed here. The *E. coli* F_0 is composed of three types of subunits (*a*, *b*, and *c*) and current information on their folding and organization in F_0 is reviewed. The structure of purified subunit *c* in chloroform–methanol–H_2O resembles that in native F_0, and progress in determining the structure by NMR methods is reviewed. Genetic experiments suggest that the two helices of subunit *c* must interact as a functional unit around an essential carboxyl group as protons are transported. In addition, a unique class of suppressor mutations identify a transmembrane helix of subunit *a* that is proposed to interact with the bihelical unit of subunit *c* during proton transport. The role of multiple units of subunit *c* in coupling proton translocation to ATP synthesis is considered. The special roles of Asp61 of subunit *c* and Arg210 of subunit *a* in proton translocation are also discussed.

KEY WORDS: F_1F_0ATP synthase; F_0 sector; subunit *c*; H^+ translocation; binding and coupling F_1; coupling via conformational changes.

BACKGROUND

In this essay I was asked to review recent work from my laboratory pertaining to the mechanism of proton flow from F_0 to F_1 and its coupling to ATP synthesis. In doing this, I will focus largely on information pertaining to the *Escherichia coli* F_0F_1 ATP synthase with only occasional reference to other systems. Many of the conclusions made here are more thoroughly documented elsewhere (Fillingame, 1990). In trying to highlight the key unanswered questions, I have taken some liberties in speculating on possible mechanisms or structural alternatives when I thought these speculations would stimulate thought and alternative hypotheses.

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The *E. coli* enzyme is a reversible, H^+-transporting ATP synthase. It differs from its mitochondrial and chloroplast counterparts in that the direction of function switches with physiological conditions, i.e., it functions as an ATP driven H^+ pump when cells are grown anaerobically. The F_1 portion of the enzyme is easily removed from the membrane, and in soluble form, functions as an ATPase. The F_0 portion of the enzyme spans the membrane lipid bilayer and, in the absence of F_1, promotes passive proton translocation across the membrane. The *E. coli* enzyme appears to have the simplest subunit composition of any known F_1F_0, with five types of subunits in F_1 and three types of subunits in F_0. A homologue of each subunit is found in both the mitochondrial and chloroplast enzymes (Fillingame, 1990). The stoichiometry of subunits in F_1 is $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ and in F_0 is $a_1b_2c_{10} \pm 1$ (Foster and Fillingame, 1982). Proton translocation by F_0, and coupled ATP synthesis/hydrolysis in F_1, is blocked by

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the specific, covalent reaction of dicyclohexylcarbodiimide (DCCD) with Asp61 of subunit *c*. Modification of one subunit *c* per F_0, i.e., one per 9–12 total subunit *c*, is sufficient to completely inhibit the ATPase activity of the complex (Hermolin and Fillingame, 1989).

For reasons that I have summarized elsewhere (Fillingame, 1990), proton translocation is thought to be coupled to ATP synthesis by an indirect mechanism, i.e., by long-range conformational changes transmitted from F_0 to F_1. The conformational change in F_1 is now thought to be required for the release of tightly bound ATP from its site of synthesis, and for the tight binding of ADP and P_i at a second catalytic site (Cross, 1981; Senior, 1988). Such sites, at least two and perhaps three, are thought to alternate, cooperatively, during catalysis by the enzyme. By this model, proton translocation through F_0 would have to be coupled, obligately, to a conformational change that was ultimately transmitted to the catalytic binding sites in F_1. According to this view, the proton pathway need never traverse the F_1 part of the enzyme.

STRUCTURE AND FUNCTION OF SUBUNITS IN F_0

The general transmembrane topography of subunits *b* and *c* is fairly well established, while that of subunit *a* is still debated. Subunit *b* (156 residues) is thought to be anchored to the membrane via a single membrane spanning α-helix at its N-terminus (Fillingame, 1990). The remainder of the subunit is polar, highly charged, and mostly α-helical. It is thought to extend from the F_1 binding side of the membrane, perhaps primarily as a dimer of two elongated α-helices in a four helix bundle (Dunn, 1992), i.e., perhaps as the stalk seen in some electron micrographs. The extramembrane domain is required for the high-affinity binding of F_1 to F_0 (Fillingame, 1990).

Subunit *c* (79 residues) is thought to fold like a hairpin with two membrane-traversing α-helices and a relatively polar loop region extending from the F_1 binding side of the membrane (Girvin *et al.*, 1989). Such an arrangement is supported by both chemical and genetic experiments, although these experiments have not yet defined the exact membrane-spanning sequences (Fillingame, 1990). Mutations in the polar loop can disrupt the binding of F_1 to F_0 (Miller *et al.*, 1989; Fraga and Fillingame, 1989), and indicate that this region, together with the extramembrane domain of subunit *b*, is critical in the binding of F_1. On the other hand, subunit *a* appears to play no role in F_1 binding (Paule and Fillingame, 1989). Feng and McCarty (1990) have reached identical conclusions regarding the role of these three subunits in binding chloroplast F_1 to F_0. The polar loop also appears to play a special role in the coupling of proton translocation to ATP synthesis. This was first deduced from the phenotype of an "uncoupled" Q42E mutant where F_1 was bound normally to F_0, but where ATP hydrolysis was uncoupled from proton translocation and protons were found to leak through the membrane at the F_0–F_1 interface (Mosher *et al.*, 1985). An identical phenotype has now been documented for an R41K mutant (in preparation). Other changes in Q42 and P43 lead to minor indications of uncoupling without total loss of function (Miller *et al.*, 1989; Fraga and Fillingame, 1989). The R41Q42P43 sequence is highly conserved among all species with no variation found in either the Arg or Pro. It is somewhat surprising then that Ala and Ser should substitute so efficiently for P43 (Miller *et al.*, 1989). In a more thorough survey of 38 substitutions in the region E37 to L45, only R41 was shown to be absolutely essential (Fraga and Fillingame, 1991). All other residues are tolerant to at least limited changes, although sufficiently drastic changes in any residue resulted in loss of function. The ensemble of conserved amino acids in the region may collectively maintain the essential features of structure and explain the tolerance of the region to single amino acid substitutions. An explanation for the critical role of R41 in function is still lacking. The function of subunit *c* in proton translocation will be considered more fully below.

Subunit *a* (271 residues) has been proposed to fold in the membrane with 4, 5, 6, 7, or 8 membrane-traversing α-helices (Fillingame, 1990; Cain and Simoni, 1989; Howitt *et al.*, 1990; Bjorbaek *et al.*, 1990; Lewis *et al.*, 1990). The first three transmembrane helices are fairly consistently predicted, and are generally consistent with information from alkaline phosphatase and β-galactosidase fusions (Lewis *et al.*, 1990; Lewis and Simoni, 1992). A fusion and deletion analysis convincingly indicates that the N-terminus is on the periplasmic side of the membrane (Lewis and Simoni, 1992). Further, a fusion protein of alkaline phosphatase with the C-terminal residue folds to maintain activity while still indicating a periplasmic location of the alkaline phosphatase reporter (Lewis *et al.*, 1990). It therefore seems likely that subunit *a* has an even number of membrane-spanning helices, most

likely either six or eight for the reasons cited (Fillingame, 1990; Lewis and Simoni, 1992). The function of subunit *a* in proton translocation will be considered below.

ORGANIZATION OF SUBUNITS IN THE F_0 COMPLEX

How are the subunits of F_0 organized? It is currently difficult to reconcile all available information. Some ideas for organization are based upon the structural organization of F_1, the extrinsic sector of the complex with which it must interact. The 50–60 kdal α and β subunits of F_1 are thought to alternate in a hexagonal ring, with the smaller γ, δ, and ε subunits occupying space inside or beneath this ring. The *b* subunit dimer, which is required for binding F_1 to F_0, may extend into the hollow cavity observed inside the $(\alpha\beta)_3$ subunit ring (Dunn, 1992). This would place both *b* subunits either to one asymmetric side or in the center of F_0. Cross-linking studies, where an a_1b_2 product was observed (Hermolin *et al.*, 1983; Aris and Simoni, 1983), make it likely that subunit *a* and subunit *b* lie close to each other in the complex. Where then do we place the 9–12 subunit *c*? An obvious possibility is that they lie in a ring around the a_1b_2 subcomplex. However, subunits *a* and *b* are both susceptible to major labeling by TID (3-[trifluoromethyl]-3-*m*[^{125}I]iodophenyldiazirine), presumably from the hydrophobic phase of the lipid bilayer (Hoppe and Sebald, 1986). In addition, subunit *c* seems to be somewhat protected from labeling by TID. A portion of the N-terminal transmembrane span (L4–L19) is labeled on one apparent face of an α-helix, but the remaining portion of the transmembrane span is not labeled (Hoppe and Sebald, 1986). Portions of the C-terminal helix are also labeled by TID, again with a periodicity suggesting exposure of one face of an α-helix (residues F53–M57, M65–F76). In short, one might expect that a larger surface of subunit *c* would be labeled. From considerations of mechanism, discussed below, one could speculate that the subunit *c* are arranged in three units of subunit *c* trimers, and that each trimer is permanently fixed beneath each $\alpha\beta$ pair.

STRUCTURE OF ISOLATED SUBUNIT *c*

Dr. Mark Girvin of our laboratory is attempting to determine the structure of isolated subunit *c*, as it occurs in chloroform–methanol–H_2O solvent, by multidemensional NMR methods (Girvin and Fillingame, 1991; manuscripts in preparation). We are interested in this structure because we now know that important features of the native protein are retained in this solvent. These features include the unique chemical reactivity of Asp61 with DCCD. Further, a hairpin-like folding is retained such that the I28T mutation makes the isolated protein resistant to reaction with DCCD, just as the mutation does in the native protein of F_0 (Fillingame *et al.*, 1991). Direct evidence for a hairpin-like folding stem from NOEs measured between residues on helix-1 and residues on helix-2 at both ends of the putative, membrane-spanning regions. This means that the protein must be folding such that the two helices come to within 5 Å of each other at each end.

Further information on the folding of subunit *c* was obtained by derivatizing Asp61 of the native protein in F_0 with a nitroxide analogue of DCCD (NCCD; *N*-[2,2,6,6-tetramethyipiperidyl-1-oxyl]-*N'*[cyclohexyl]-carbodiimide), and then, following purification of the protein, measuring resonance broadening of proton resonances close to the nitroxide radical. In theory, the resonance of protons within 10–11 Å of the nitroxide should be broadened beyond detection, whereas the resonances of protons in a range of 11–22 Å should be predictably broadened and the intensity partially reduced. Significantly, resonances on both helices were broadened by the NCCD derivatization (Fig. 1). As predicted, resonances of residues close to D61 were obliterated. Based upon distances calculated from the resonance broadening of protons more distant from D61, the piperidyl ring containing the nitroxide group was placed in a pocket between the side chains of M57 and V60. Another face of the binding pocket is provided by the side chain of L31 extending from the opposite transmembrane helix. Other affected resonances on transmembrane helix-1 include those of residues G23, A24, A25, K34, and F35.

Based upon the distances calculated from resonance broadening, a model was developed for this region of subunit *c* and the helical–helical interaction. An energy-minimized version of the modeled protein, lacking NCCD, shows the β-carboxyl of D61 lying between the side chains of A24 and I28. Significantly, the A24S, I28T, and I28V mutations reduce reactivity of DCCD with D61 of the native protein in F_0 (Fillingame *et al.*, 1991). The model could account for

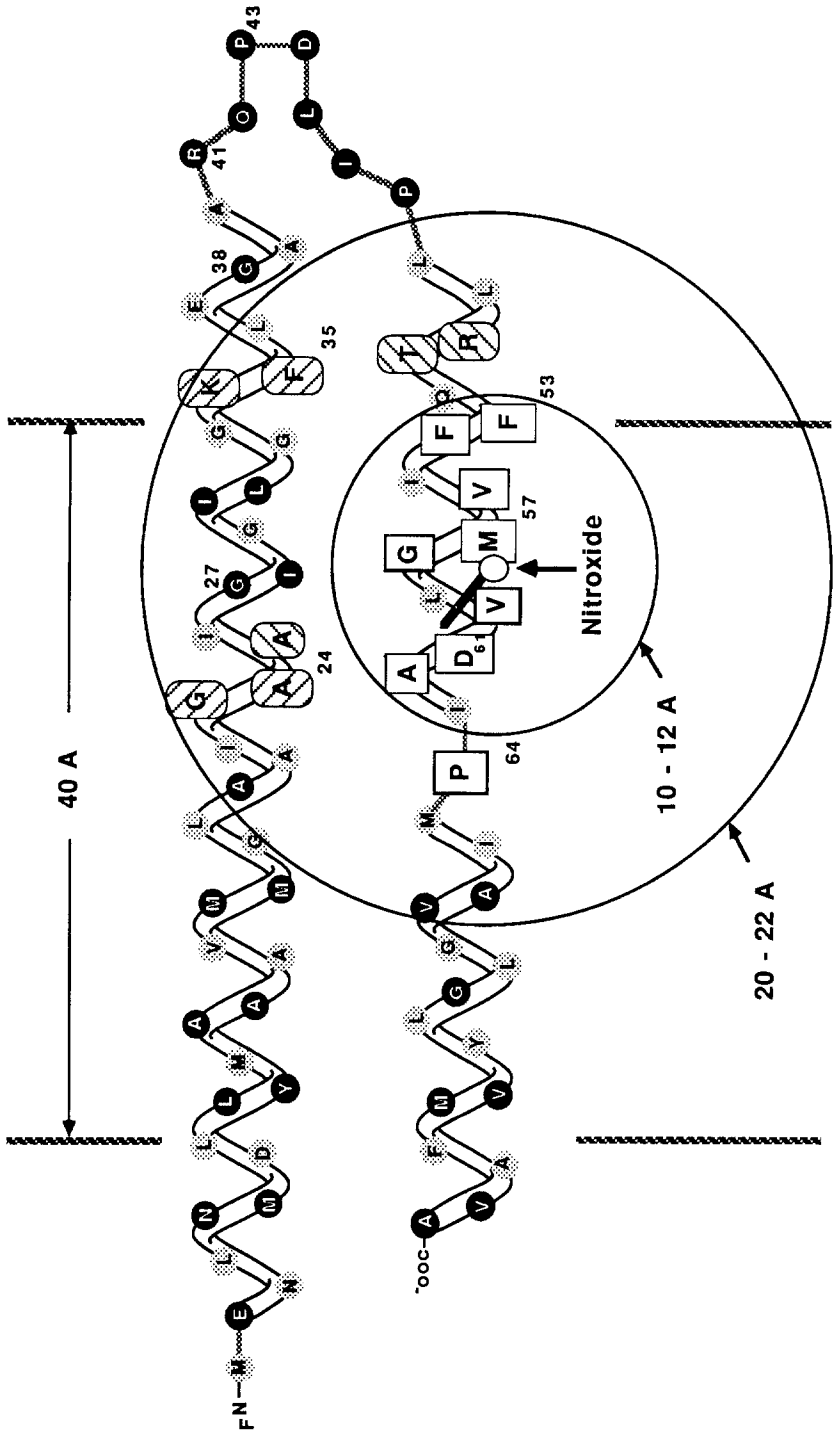

Figure 1 Location of NCCD nitroxide in hairpin folding model of subunit *c*. The model is a modification of that given in Miller *et al.*, (1990), where the predicted transmembrane domains are indicated by the 40 Å spans between vertical lines. The position of the piperidyl ring and nitroxide radical is indicated by the thick line and open circle between V60 and M57. Residues where proton resonance intensity is broadened to zero are highlighted by the open boxes. Residues where proton resonance intensity is broadened but still detectable are indicated by the hatched ovals. Sequence-specific assignments have not been made for other residues within the circles.

DCCD resistance if the hydroxyl groups of the A24S or I28T mutants were to repel binding of the second cyclohexyl ring between residues 24 and 28. However, it would not account for the resistance conferred by the V28 substitution. Perhaps the small cavity created by the I28V substitution permits greater access of H_2O to a region around D61. The stable N-acylurea adduct of DCCD and the D61 β-carboxyl is formed via an unstable (i.e., hydrolyzable) O-acylisourea intermediate. A greater competing reaction of H_2O with the O-acylisourea intermediate could explain the DCCD resistance of the I28V mutant.

HELICAL INTERACTIONS AND THE ESSENTIAL CARBOXYL GROUP DURING H^+ TRANSLOCATION

The β-carboxyl group of Asp61 in subunit *c* appears to play an essential catalytic role in proton translocation. The D61E mutation profoundly reduces activity, and indicates a remarkably specific chemical or structural requirement (Miller *et al.*, 1990). Mutation of the residue to Asn or Gly abolishes activity. The lack of any activity in the N61 mutant indicates a requirement for a side chain capable of more than hydrogen bonding, and suggests that a residue capable of protonation and deprotonation may be required. A reversible, ATP-driven H^+ pump requires at lease one H^+ or H_3O^+ binding site, the pK_a of which must change during the proton-translocation cycle (Fillingame, 1990). Irrefutable evidence that D61 is the proton-binding site in F_0, and that it undergoes a protonation–deprotonation cycle for each proton translocated, is still lacking. However, it remains the best candidate for this binding site.

Remarkably, the essential carboxyl group in subunit *c* can be moved from position 61 to position 24 with retention of activity (Miller *et al.*, 1990). This was discovered while analyzing slow growing revertants of the D61G mutant. The revertants functioned less well than wild type, and when sequenced, were shown to retain the original D61G mutation and acquire the secondary A24D mutation. ATP-driven proton translocation rates in D24G61 double mutant were reduced to < 20% of wild type (Miller *et al.*, 1990). A large reduction in rate of passive, F_0-mediated proton translocation correlates with the reduced ATPase-coupled transport (unpublished observations). In interpreting these observations, it is notable that the A24 methyl and D61 β-carboxyl lie close to each other (4–5 Å) in the independently derived structural model discussed above.

We have rationalized retention of function in the D24G61 double mutant by suggesting that the two transmembrane helices come together as a unit during the process of proton translocation. By this view, either helix could serve as a scaffold to anchor the essential carboxyl group at the same position near the middle of the membrane. The structural elements necessary for protonation–deprotonation, and the coupled, pK_a altering conformational change, would be provided by the unit of two interacting helices. It is noteworthy that residue 24 lies in a segment of highly conserved sequence on helix-1, i.e., between G23 and G29 where the consensus sequence is G-X-G-X-G-X-G. This is also the area of the protein where mutations confer DCCD resistance (Fillingame *et al.*, 1991). We have suggested that the Gly-rich sequence may provide hinge-like flexibility to the region and perhaps enable rotation of helical units as the protonated carboxyl group moves from a more polar, low pK_a, environment to a more hydrophobic, high pK_a, environment.

Interaction of Subunit *a* and Subunit *c* during H^+ Translocation

Nearly 100 mutants of subunit *a* have been generated, primarily using site-directed approaches. Much of the attention has focused on the C-terminal 80–85 residues since this is the most conserved section of the protein and it is also predicted to be largely transmembrane (see Fillingame, 1990 for complete references). Of the nearly 30 residues mutated in the whole protein, only R210 appears to be absolutely essential; here even the most conservative substitution of Lys abolishes proton translocating function (Cain and Simoni, 1989; Lightowlers *et al.*, 1987). A number of other conserved residues have been replaced (e.g., P190, E196, N214), often with suprisingly negligible effects on function. Other residues can be mutated, such that function is abolished (e.g., H245Y, H245L, E219L), but other substitutions give at least minor indication of residual function (e.g., H245E, E219H, and perhaps E219Q), and seem to rule out an absolute requirement for these residues. It seems possible that some of the polar residues, which appear important but not absolutely critical to function, may play key roles in hydrogen bonding. Such residues might participate on a direct pathway of proton conduction (proton wire), or less directly, e.g., in the binding of

water in a narrow channel. Mutations that partially disrupt function might do so by kinetically slowing the process of proton transfer from group to group in a proton wire, or by slightly diverting the pathway of proton flow, particularly if the hydrogen bonding function of the residue can be replaced by water. I have previously argued against the idea of a proton wire composed exclusively of side chains from a single α-helix (Fillingame, 1990). The idea of a channel that utilizes aqueous filled spaces to facilitate ion movement is certainly bolstered by the discovery of a Na^+-translocating F_1F_0 ATPase in *Propionigenium modestum*, an enzyme that can interchangeably pump Na^+ or H^+ (Laubinger and Dimroth, 1989). Many of the conserved residues found in the *E. coli* subunit *a* and subunit *c* are also found in the *P. modestum* enzyme. In addition to the role of water, a second key question concerns the special function of the R210 residue. One possibility is formation of a transient salt bridge with the carboxylate side chain of D61 in subunit *c*. In a relatively hydrophobic environment, such an interaction could serve to stabilize the carboxylate group in a low pK_a form, where it would be cyclically protonated during H^+ translocation-driven ATP synthesis (discussed further below).

Given that both subunits *a* and *c* are required for H^+ transiocation, the question then becomes, how do they interact during that process? We believe we have obtained new insights into that interaction from the analysis of optimizing, third-site mutations to the D24G61 double mutant. The double mutant grows more slowly than wild type on a succinate carbon source, and variants with optimizing mutations were selected as larger colonies (Fraga, 1990). Surprisingly, of the 18 optimizing mutations now characterized, 13 map to subunit *a*. Of the 13 mutations in subunit *a*, 10 are variants of wild type residues A217, I221, and L224. The periodicity of residues suggests that they may lie on a single face of an α-helix, and in at least some models for subunit *a* (Cain and Simoni, 1989; Fillingame, 1990) the same helix would include both E219 and R210. We suggest that during the process of proton translocation this single helix of subunit *a* must interact with the two helical unit of subunit *c* anchoring the essential carboxyl group (Fraga, D., and Fillingame, R., in preparation). It seems possible that such a helical–helical interaction could be facilitated by the docking of conserved aliphatic side chains from subunit *a* helix-5 (e.g., M215, L220, I221, L224, I225) in the groove of holes provided by the G-X-G-X-G-X-G motif in helix-1 of subunit *c* (Fillingame, 199l).

REQUIREMENTS IN COUPLING H^+ TRANSLOCATION TO ATP SYNTHESIS

The requirement that three H^+ be translocated per ATP synthesized stems largely from comparisons of steady-state phosphorylation potentials and proton electrochemical potentials in a number of systems (reviewed in Fillingame, 1980). If H^+ translocation and ATP synthesis are coupled via conformational changes transmitted from F_0 to F_1, then the simplest mechanism would require that all three H^+ be released simultaneously in an event initiating the conformational change. Coupling, then, would require multiple H^+ binding sites, and the conserved Asp61 of subunit *c* is the most obvious candidate. If three subunit *c* must be protonated per ATP synthesized, and three $\alpha\beta$ pairs alternate in F_1 during catalysis, then one could easily rationalize a stoichiomety of nine subunit *c* per F_0 if a subunit *c* trimer and $\alpha\beta$ pair acted as a unit. By this reasoning, the single subunit *a* per F_0 complex would most likely be involved catalytically in the sequential loading of the three subunit *c*. In such a model (Fillingame, 1990), three D61, low pK_a carboxylates would be sequentially protonated from the side of the membrane with a high local H^+ concentration. After each protonation, the protonated carboxyl could move away from the protonation site, conceivably to a region in the membrane of lower polarity, where the group would assume a higher pK_a. This could occur by movement away from the protonated R210 of subunit *a*. However, access of the high pK_a carboxyl to the opposite side of the membrane (low local H^+ concentration) would be prohibited until all three subunits *c* were protonated. The protonation and movement of the third subunit *c* carboxyl group to environment of high pK_a would then permit a concerted conformational change moving all three high pK_a carboxyls to the side of H^+ release, and this movement would be coupled to the conformational change transmitted to F_1. The structural mechanics of how the above might happen is of course not clear. The idea that the two helices of subunit *c* should work as a structural unit should be considered. In addition, the idea of interaction between the conserved Gly-rich segment of this unit and the aliphatic side chains in the region of A217–L224 of subunit *a* helix 5 seems promising. Our model building is consistent with the possibility that the subunit *c* helices could wrap around each other to form a fairly rigid superhilical structure. If this was the case, and if the helices did turn with respect to a third helix fixed vertically in the

membrane, this would lead to a vertical translocation of the subunit *c* unit and provide a possible means of transmitting a conformational change to F_1.

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