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The Oligomeric Subunit *c* Rotor in the F_o Sector of ATP Synthase: Unresolved Questions in Our Understanding of Function

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We have proposed a model for the oligomeric *c*-rotor of the F_o sector of ATP synthase and its interaction with subunit *a* during H^+-transport driven rotation. The model is based upon the solution structure of monomeric subunit *c*, determined by NMR, and an extensive series of cross-linking distance constraints between *c* subunits and between subunits *c* and *a*. To explain the complete set of cross-linking data, we have suggested that the second transmembrane helix rotates during its interaction with subunit *a* in the course of the H^+-translocation cycle. The H^+-transport coupled rotation of this helix is proposed to drive the stepwise movement of the *c*-oligomeric rotor. The model is testable and provides a useful framework for addressing questions raised by other experiments.

KEY WORDS: F_oF_1-ATP synthase; F_o rotary motor; subunit *c*; subunit *a*; transmembrane helices; NMR; cross linking.

INTRODUCTION

In F_oF_1-ATP synthases, H^+-transport coupled rotation of an oligomeric rotor of *c* subunits is proposed to drive ATP synthesis via the coupled rotation of subunit γ within the $\alpha_3\beta_3$ catalytic hexameric unit of F_1 (Engelbrecht and Junge, 1997; Elston *et al.*, 1998; Noji *et al.*, 1997; Sambongi *et al.*, 1999; Pänke *et al.*, 2000). Based upon the NMR structure of purified, monomeric subunit *c* (Girvin *et al.*, 1998), and an extensive set of distance constraints derived by cross linking *in situ* (Jones *et al.*, 1998; Jiang and Fillingame, 1998), we have proposed a structural model for the oligomeric organization of subunit *c* (Dmitriev *et al.*, 1999) and its interaction with a functionally important transmembrane helix (TMH) of subunit *a* (Jiang and Fillingame, 1998). The credibility of the model is strengthened by its prediction of a closeness of residues that are thought to functionally interact during proton transport. In order to explain the complete set of cross-linking constraints between subunits *a* and *c*, we have also predicted that the *a*–*c* helical interactions change during H^+-transport driven rotation of the *c* oligomer (Jiang and Fillingame, 1998; Fillingame *et al.*, 2000). In one form of the model, a conformational change and approximately 180° rotation of TMH-2 within one subunit *c* is coupled to the 30° stepwise movement of the *c*-oligomeric rotor relative to the stationary TMH-4 of subunit *a*, in analogy to the mechanically coupled rotation of two meshed gears of unequal size (Rastogi and Girvin, 1999; Fillingame *et al.*, 2000). In presenting and discussing the model, we have emphasized a series of functional correlations with the predicted structure. However, several uncertainties and discrepant experimental observations remain to be clarified. It is these key unanswered questions that we will focus upon in this essay.

DISCUSSION

NMR Structure of Subunit *c* and Experimental Basis of Oligomeric Model

The solution structure of monomeric subunit *c*, a protein of 79 residues, was determined in a monophasic chloroform–methanol–H_20 mixture at pH 5 (Girvin *et al.*, 1998). The protein folds in a hairpinlike structure of two extended α-helices with the conserved

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Arg41–Gln42–Pro43 sequence at the apex of the hairpin. Residues 24 and 28 in TMH-1 lie close to Asp61 in TMH-2. These residue–residue interactions had been predicted from a variety of functional studies of the complete F_oF_1 complex. In one such study, it was shown that it was possible to switch the position of the essential, proton-binding aspartyl from position 61 in helix 2 to position 24 in helix 1 and maintain function (Miller *et al.*, 1990). One of the surprises of the NMR structure was that the side chains of Ala24 and Asp61 packed on different sides of the molecule. This led to the prediction that the subunits might pack in a "front-to-back" manner in the functional oligomer, such that the essential carboxyl group would occupy the same structural position whether linked to residue 61 at the front of one subunit or residue 24 at the back of the adjacently packed subunit. The general prediction of a front-to-back packing was confirmed by an extensive cross-linking study between Cys genetically introduced into the protein (Jones *et al.*, 1998).

The stoichiometry of *c* subunits in *E. coli* F_o was initially estimated by radiolabeling and yielded a value with an experimental uncertainty in the range of 9 to 12 (Foster and Fillingame, 1982). Subsequently, genetically fused proteins were generated by introduction of a loop between the C-terminus of the first subunit and the N-terminus of the next subunit (Jones and Fillingame, 1998). The genetically fused c_2 dimers and c_3 trimers were both functional, which suggested that the functional unit was a multiple of 2×3. Further, on introduction of appropriate Cys into the fused dimers and trimers, cross-linked products that maximized at the position of c_{12} were observed. Based upon these experiments, the stoichiometry of *c* subunits in F_o was concluded to be 12 (Jones and Fillingame, 1998). Based upon 21 intersubunit distance constraints, derived from cross linking, a model for the c_{12} oligomer was constructed by use of molecular mechanics and energy minimization (Dmitriev *et al.*, 1999). The key features of the model are a front-to-back packing of the flattened faces of 12-subunit *c* in a hollow cylindrical structure with TMH-1 and TMH-2 forming the inner and outer rings, respectively. The Asp61 side chain lies at the center of the four tightly packed TMH's of two interacting subunits, in a position where it would be shielded from the phospholipid phase of the membrane, where it is also closely juxtaposed to the side chains of residues 24 and 28 at the back face of the next subunit.

Observations Not Accounted for By the Simple Oligomeric Model

Cysteine in consecutive strings of residues at the N- and C- termini can be cross linked to form *c–c* dimers, which suggests considerable structural flexibility in these regions, both of which are projected to extend into the polar head group region of the lipid bilayer (Fillingame *et al.*, 2000; Jones *et al.*, 2000b). A number of cysteines introduced near the center of TMH-2 (*i.e.*, in the region of residues 58–66) can also be cross linked to form *c–c* homodimers. The cross linking of these residues cannot be explained by the cylindrical model described above with TMH-2 packing to form the outer ring. Cysteine introduced at some of these positions were also shown to form *a–c* heterodimers, via a second cysteine introduced into TMH-4 of subunit *a*, which led to the suggestion that *c*TMH-2 might turn when packed at subunit *a* interface (Jiang and Fillingame, 1998).

Correlations with the Crystal Structure of a Mitochondrial F_1-c_{10} Subcomplex

Stock *et al.* (1999) have noted the obvious correlation between the electron density of the *c* oligomer in a crystallized F_1–c_{10} subcomplex from yeast mitochondria and the NMR structure and oligomeric model of *E. coli* subunit *c*. Although the exact position of individual side chains is uncertain in the 3.9 Å resolution structure, the extended α helices and hairpin turn seen in the NMR model of *E. coli* subunit *c* closely overlay the published electron density. As in the *E. coli* oligomeric model, the shorter, somewhat kinked, TMH-2 was concluded to pack at the outer circumference of the oligomeric ring. On the other hand, the long, extended α-helices seen in the Stock *et al*. structure do not correlate with the suggested folding of *P. modestum* subunit *c* (Dimroth *et al.*, 1999, 2000), which was based upon determination of its secondary structure in SDS micelles (Matthey *et al.*, 1999). The major difference between the Stock *et al.* structure and the *E. coli* oligomeric model is the presence of 10 versus 12 subunit *c* in the oligomeric ring, an issue which will be addressed in the next section.

Hermolin *et al.* (1999) have proposed a model for the packing of *E. coli* subunit ε between polar loops of subunit *c*, a model based again upon NMR structures and cross-linking distance constraints. An antiparallel loop of β sheet of subunit ε is proposed to insert between the polar loops of adjacent subunit *c* in the oligomer. The proposed structure, and the residues subject to cross linking, are not easily reconciled with the proposed docking of subunit ε with the *c* oligomer in the coordinates 1qo1 deposited by Stock *et al.* (1999). Further refinements of the crystal structure will hopefully clarify the seriousness of the discrepancy.

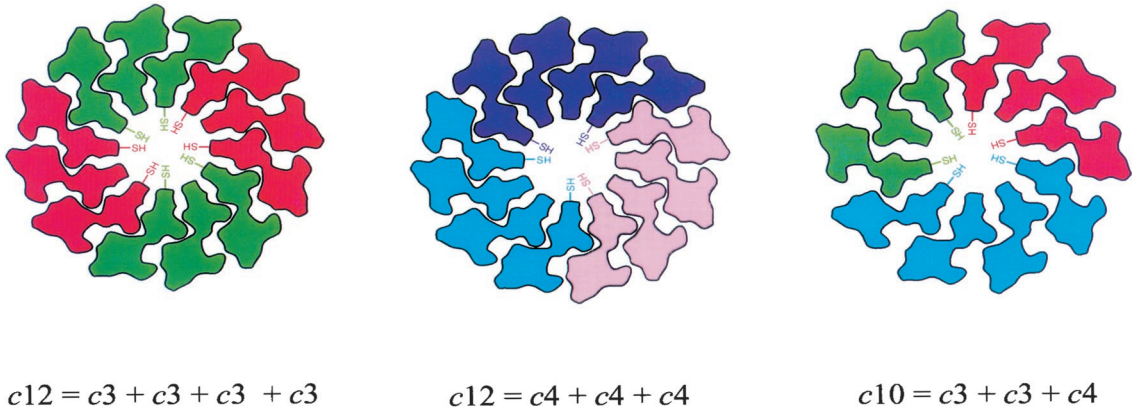

Fig. 1. Possible oligomeric rings formed by the mixing of genetically fused c_3 and c_4 subunits. Each fused subunit has an Ile30→Cys substitution in the first and last unit for cross linking to the neighboring fused subunit. The approximate position of the Cys residues in a cross section of the oligomeric ring model is shown. TMH-1 lies on the inside and TMH-2 lies on the outside of the ring.

The Preferred Stoichiometry of Subunits in the *c* Oligomer of *E. coli* Is Ten

The report of Stock *et al.* (1999) led us to reexamine the issue of subunit *c* stoichiometry, specifically to test whether c_{10} oligomers formed naturally in the membrane. The basic idea behind the experiment is illustrated schematically in Fig. 1. Mixtures of genetically fused, cysteine-substituted trimers (c_3) and tetramers (c_4) of subunit *c* were coexpressed and the *c* subunits cross linked in the plasma membrane (Jiang *et al.*, submitted). Prominent products corresponding to oligomers of c_7 and c_{10} were observed in the membrane and purified F_oF_1 complex, indicating that c_{10} oligomers were a natural product. Lesser amounts of larger oligomers corresponding to c_{12}, c_{15}, and c_{16} were also observed in the membrane fraction of cells expressing c_3 and c_4, but these did not copurify with the functional F_oF_1 complex and were concluded to be aberrant products of assembly in the membrane. We have concluded that the function measured previously on expressing c_3, and in this most recent study on expressing c_4, was due to incorporation of three c_3 or two c_4 fused subunits into an F_oF_1 complex with partial activity. However, the formation of predominantly c_7 dimers and c_{10} trimers, when c_3 and c_4 were coexpressed, indicates that the preferred stoichiometry in the F_oF_1 complex is clearly

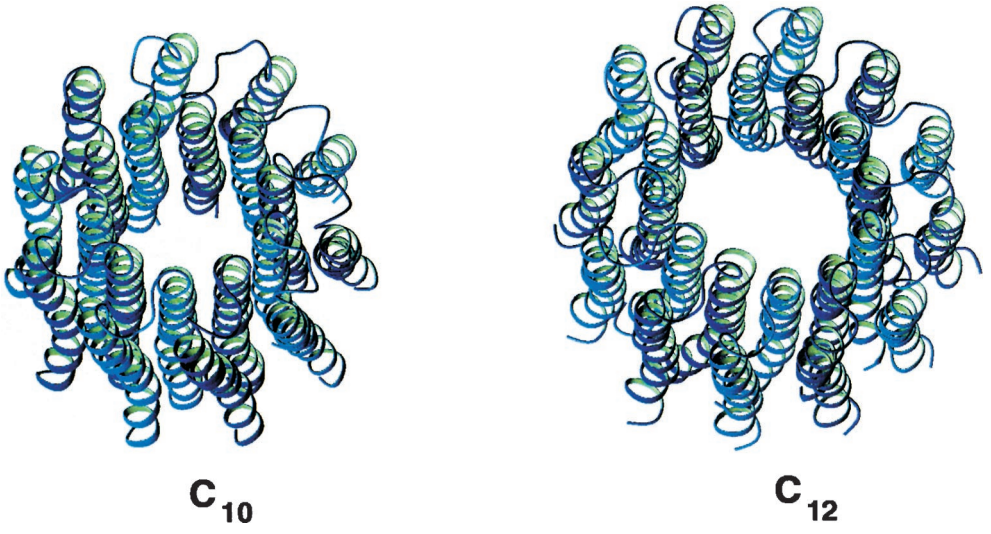

Fig. 2. Comparison of c_{10} and c_{12} oligomeric rings predicted using the cross-linking distance constraints of Dmitriev *et al.* (1999). The oligomeric cylinders are viewed from polar loop end of the subunit *c* hairpin.

10. We have examined the effect of a c_{10} versus c_{12} stoichiometry on the packing of subunits in an oligomeric ring by the same procedure described in Dmitriev *et al.* (1999). Predictably, the decameric ring is smaller, the maximal diameter for c_{10} ring being 51–55 versus 56–60 Å for the c_{12} ring (Fig. 2). The position of key residues, which are predicted to interact, is minimally affected, as illustrated in Fig. 3. For example, the distance between the Asp61 carboxylate carbon and Ala24 β-carbon is 4.23 ± .05 Å in the c_{12} model and 4.43 ± .06 Å in the c_{10} model. In conclusion, the change in stoichiometry of subunits in the ring has little affect on the predicted juxtaposition of interacting residues.

Is the Asp61 Proton/Cation-Binding Site Located between *c* Subunits?

The structural model discussed above suggests that the H^+-binding Asp61 carboxylate side chain is positioned between subunits in the *c* oligomeric ring. Others have suggested that the carboxylate might be positioned at the outer surface of the cylinder where it would more readily be exposed to the lipid soluble inhibitor dicyclohexylcarbodiimide (DCCD) (Engelbrecht and Junge, 1997; Elston *et al.*, 1998). In the *P. modestum* enzyme, residues Glu65, Ser66, and Gln32 (which correspond to residues Asp61, Ala62, and Ile28 in the *E. coli* enzyme) have been implicated in Na^+ binding (Kaim *et al.*, 1997). A similar cation-binding pocket seems to exist in a variant of the *E. coli* enzyme with substitutions of Asp61→Glu and Ala62→Ser (Zhang and Fillingame, 1995). The proton-translocating activity of this enzyme is inhibited by Li^+. The three aforementioned residues would fall of the front and back face of interacting subunits in the oligomeric model. On the other hand, Matthey *et al.* (1997) have suggested that monomeric subunit *c* is capable of binding Na^+ based upon Na^+ inhibition of reaction of DCCD with dodecyl sulfate-solubilized *P. modestum* subunit *c*, which is assumed to be in a monomeric state. If the conclusion that a single subunit *c* can bind Na^+ proves correct, where one alternative explanation is that multimeric species of subunit *c* persist in dodecyl sulfate, the binding could be accommodated by a swiveling of *c*TMH-2 relative to *c*TMH-1 by approximately 70° in the native oligomer relative to the NMR model. Since we do now know that the monomeric structure of subunit *c* does change dramatically on ionization of Asp61 (Rastogi and Girvin, 1999), such structural rearrangements in intrasubunit packing of helices seem more plausible, particularly if these rearrangements were stabilized by new intersubunit contacts.

Cross-Linking Pattern of *c*TMH-2 with *a*TMH-4 Is Not Easily Accommodated by NMR Structure of Subunit *c*

In the study of Jiang and Fillingame (1998), cross linking was observed between Cys introduced at a single face of the putative *a*TMH-4 to Cys at both the "front" and "back" faces of *c*TMH-2, as defined in the NMR model. To interpret the observed pattern of cross linking with the information from the NMR model, two possibilities were suggested: (1) that at some point in cycle of the enzyme, TMH-4 of subunit *a* would insert between *c* subunits of the oligomeric ring and thus be capable of reacting with both the front and back faces; and (2) that *c*TMH-2 would

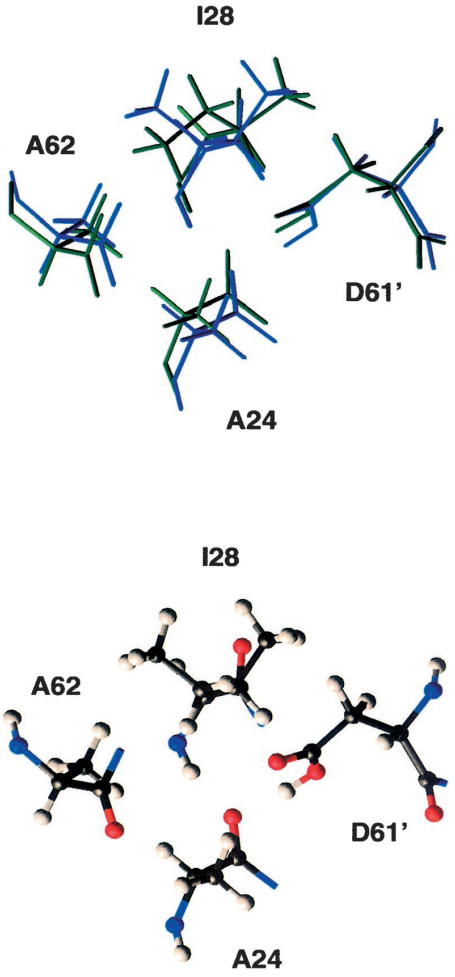

Fig. 3. Comparison of the relative positions of Asp61 side chain at the front face of one subunit *c* to the Ala24, Ile28, and Ala62 side chains at the back face of an interacting subunit *c* in c_{10} and c_{12} oligomeric rings. (A) Overlay of interacting subunits in c_{10} (black) and c_{12} (grey) oligomeric ring. (B) Positions of the interacting residues in the c_{10} ring.

rotate as it passes subunit *a*, *e.g.*, in response to protonation/deprotonation (Fig. 4). Such an event would also permit the predicted functional interaction between the conserved Arg210 residue of subunit *a* and the Asp61 carboxyl group, an event predicted to promote proton/cation release (Valiyaveetil and Fillingame, 1997; Elston *et al.*, 1998). The second suggested mechanism, *i.e.*, rotation of *c*TMH-2, would also provide an explanation for low yield *c* –*c* dimer formation seen between singly substituted Cys in TMH-2 of subunit *c* (Jones *et al.*, 1998). For example, as depicted in Fig. 4, dimer formation between Cys66 in *c* subunits 1 and 2 might take place during the proposed

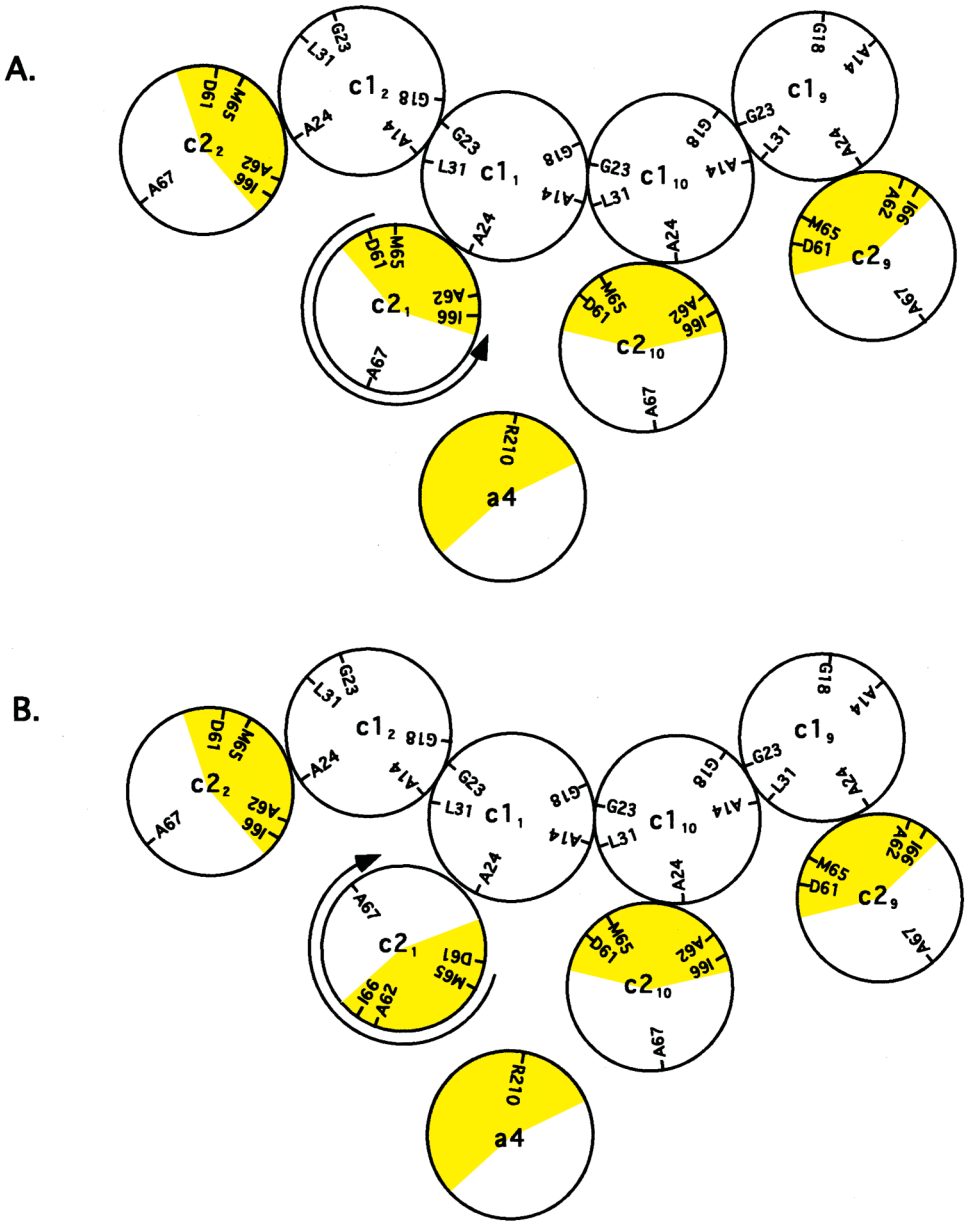

Fig. 4. Model for the suggested rotation of TMH-2 of subunit *c* during deprotonation of Asp61 via interaction with Arg210 on TMH-4 of subunit *a* (modified from Fillingame *et al.*, 2000). The surfaces of the helices subject to *a*–*c* cross-link formation is indicated by the grey shading. (A) Position of helices when Asp61 of *c* subunit No. 1 is protonated. Rotation of the helix in the direction indicated is proposed to occur as Asp61 deprotonates and forms a salt bridge with *a*Arg210. (B) Position of helices with Asp61 deprotonated. On reprotonation the TMH-2 of *c* subunit No.1 will rotate back to the original position. One of the helical rotations (the second in the model shown) is proposed to be coupled with the movement of the oligomeric rotor in the counterclockwise direction by 36° when the rotor has 10 *c* subunits. The first helical rotation would not be accompanied by rotor movement.

rotation from position A to B, as *c*TMH-2 swiveled from one side to the other of its junction with *c*TMH-1. With the publication of a new NMR structure of subunit *c* at pH 8 (Rastogi and Girvin, 1999), where *c*TMH-2 was shown to rotate by approximately 140° relative to the pH 5 structure, the suggested helix-rotation explanation is clearly plausible.

Does *c*TMH-2 Turn during the Protonation/Deprotonation Step While Interacting with Subunit *a*?

From the titration studies of Assadi-Porter and Fillingame (1995) we know that the major structural changes occurring between pH 5 and 8 in monomeric subunit *c* are due to ionization of Asp61 and not to changes in the ionization state of other carboxyl groups in protein. Based upon large chemical shift changes in the vicinity of Pro43, a major structural rearrangement of the loop region was predicted with the change in pH from 5 to 8 and that change has now been described in detail by Rastogi and Girvin (1999). Rastogi and Girvin (1999) and Fillingame *et al.* (2000) have both suggested that a protonation/deprotonation coupled turning of helix 2 with respect to helix 1 could be mechanically coupled to the rotation of the *c* oligomeric ring. Although we find the hypothesis attractive, we presently have no direct evidence to support such movements in the functional F_oF_1 complex. One could argue that the structural changes seen with the monomeric protein in solution might be muted by subunit–subunit interactions within the oligomer and not occur in the functional complex. Two discrepant observations require further consideration. In the rotary motor hypothesis for ATP synthesis, H^+-transport coupled rotation of the *c*-oligomeric rotor is proposed to be coupled to rotation of the γ subunit via a fixed linkage between subunits γ and ε and a set of *c* subunits in the rotor. The idea of a fixed linkage is now supported by cross linking and other indirect experiments (Schulenberg *et al.*, 1999; Jones *et al.*, 2000a). The predicted fixed linkage between a given set of subunit *c* and $\gamma\varepsilon$ is not easily reconciled with a major structural change in the loop region of the protein during each protonation/deprotonation event. Such a conformational change would be expected to transiently disrupt the fixed *c*-$\gamma\varepsilon$ complex. Conceivably, structural interactions between loop regions of subunit *c* and $\gamma\varepsilon$ at the bottom surface of the F_1 molecule could suppress the conformational change in the loop, but not prohibit rotation of *c*TMH-2 as it interacts with subunit *a*. A second puzzle, not resolved by the model, is the function of mutants in which the essential Asp is positioned at residue 24 rather than at residue 61. The turning of *c*TMH-2 would do not be expected to bring Asp24 in *c*TMH-1 to the predicted proximity with Arg210 in *a*TMH-4. However, the turning of the helix might still disrupt the H^+ binding site enough to promote proton release to the outlet channel. One could dismiss rationalization of the Asp24 mutants as being unimportant, because of the generally poor function of the Asp24Gly61 mutant (Miller *et al.*, 1990), but some Asp24-substituted mutants with secondary, optimizing mutations grow nearly as well as wild type (Fraga *et al.*, 1994). The mechanism by which these mutants function warrants further study in the context of current structural proposals for the rotary motor.

CONCLUSIONS

The rotary model discussed here provides an explanation for structural and other experimental observations made with *E. coli* F_o and suggests a testable mechanism by which H^+ transport drives rotary catalysis. Several apparently discrepant experimental observations are addressed here. These and other questions will be addressed in new experiments designed to test the validity of the model.

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