COUPLING H⁺ TRANSPORT TO ROTARY CATALYSIS IN F-TYPE ATP SYNTHASES:
STRUCTURE AND ORGANIZATION OF THE TRANSMEMBRANE ROTARY MOTOR

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Summary

H⁺-transporting F₁F₀-type ATP synthases utilize a transmembrane H⁺ potential to drive ATP formation by a rotary catalytic mechanism. ATP is formed in alternating β subunits of the extramembranous F₁ sector of the enzyme, synthesis being driven by rotation of the γ subunit in the center of the F₁ molecule between the alternating catalytic sites. The H⁺ electrochemical potential is thought to drive γ subunit rotation by first coupling H⁺ transport to rotation of an oligomeric rotor of c subunits within the transmembrane F₀ sector. The γ subunit is forced to turn with the c₁₂ oligomeric rotor as a result of connections between subunit c and the γ and ε subunits of F₁. In this essay, we will review recent studies on the Escherichia coli F₀ sector. The monomeric structure of subunit c, determined by nuclear magnetic resonance (NMR), is discussed first and used as a basis for the rest of the review. A model for the structural organization of the c₁₂ oligomer in F₀, deduced from extensive cross-linking studies and by molecular modeling, is then described. The interactions between the a₁b₂ ‘stator’ subcomplex of F₀ and the c₁₂ oligomer are then considered. A functional interaction between transmembrane helix 4 of subunit a (aTMH-4) and transmembrane helix 2 of subunit c (cTMH-2) during the proton-release step from Asp61 on cTMH-2 is suggested. Current a–c cross-linking data can only be explained by helix–helix swiveling or rotation during the proton transfer steps. A model that mechanically links helix rotation within a single subunit c to the incremental 30° rotation of the c₁₂ oligomer is proposed. In the final section, the structural interactions between the surface residues of the c₁₂ oligomer and subunits ε and γ are considered. A molecular model for the binding of subunit ε between the exposed, polar surfaces of two subunits c in the oligomer is proposed on the basis of cross-linking data and the NMR structures of the individual subunits.

Key words: ATP synthase, proton transport, rotary motor, subunit c, nuclear magnetic resonance, F₀ structure, cross-linking, molecular modelling, Escherichia coli.

Introduction, perspective and overview

H⁺-transporting F₁F₀-type ATP synthases utilize a transmembrane H⁺ potential to drive ATP formation by a rotary catalytic mechanism. The simplest F₁F₀-type enzymes, as in the case of Escherichia coli, are composed of eight types of subunit with a biologically unique stoichiometry of α₃β₃γδε for the F₁ sector at the periphery of the membrane and a₁b₂c₁₂ for the transmembrane F₀ sector (Fillingame, 1997; Fillingame et al., 1998). From the atomic resolution X-ray structure of the α₃β₃γ portion of bovine F₁, the three α and three β subunits are known to pack alternately around a centrally located γ subunit, with the γ subunit interacting asymmetrically with the catalytic β subunits (Abrahams et al., 1994). Subunit γ was subsequently shown to rotate with respect to the three β subunits during catalysis (Duncan et al., 1995; Sabbert et al., 1996; Noji et al., 1997). Rotation of subunit γ is thought to change the binding affinities in alternating catalytic sites to promote tight substrate binding and product release during catalysis (Boyer, 1997). During ATP synthesis, the rotation of subunit γ must be driven by proton translocation through F₀.

The F₁ sector and proton-translocating F₀ sector are connected by a central stalk formed by the γ and ε subunits. The γ and ε subunits are both known to contact the c subunit at the surface of F₀ (Zhang and Fillingame, 1995b; Watts et al., 1995, 1996; Hermolin et al., 1999), and the 12 copies of the c subunit are thought to form an oligomeric ring that is proposed to rotate relative to a stationary a₁b₂ complex (Fig. 1). Protonation/deprotonation of Asp61 of subunit c at the center of the membrane is proposed to drive rotation of the c oligomer, with c-rotary movement being coupled to rotation of subunit γ by connections between the c, γ and ε subunits (Vik and Antonio, 1994; Vik et al., 1998; Junge et al., 1997; Engelbrecht and Junge, 1997; Elston et al., 1998). Low-resolution electron microscopic and atomic force microscopic images initially suggested a ring-like arrangement for the c oligomer, with subunits a and b lying at the periphery of the
Fig. 1. Rotary model for F₁F₀ modified from that presented by Elston et al. (1998). Rotation of the c₁₂ oligomer in the direction indicated is proposed to be driven by the binding of protons to Asp61 (white circle) via a periplasmic inlet channel at the bottom of the structure. The protonated binding site (black circle) then moves to the stator interface to the lipid phase of the membrane where, after 12 steps, it reaches an outlet channel with access to the cytoplasmic, F₁-binding side of the membrane. Arg210 on transmembrane helix 4 (TMH-4) of subunit a, indicated by (+), is proposed to promote proton release to the outlet channel. The γ and ε subunits are proposed to remain fixed to the top of one set of c subunits so that rotation of the rotor also drives rotation of subunit γ within the αβ₁β subunits of F₁. The b₂ and δ subunits of the stator hold the αβ₁β subunits in a fixed position as the γ subunit turns within to drive ATP synthesis. The actual size of subunits, relative to each other, varies from the proportions indicated here.

Subunit c is a hydrophobic protein of 79 residues that folds through the membrane in a hairpin-like structure with two membrane-traversing α-helices and a more polar loop region exposed to the F₁ binding side of the membrane (Fillingame, 1997). Asp61, centered in the second transmembrane helix, is known to protonate and deprotonate during H⁺ transport. The binding of the loop region to subunits γ and ε of F₁ is proposed to force rotation of subunit γ as proton transport drives rotation of the c₁₂ oligomer (Fig. 1). A high-resolution structure of monomeric subunit c has been solved by NMR, and the structure closely resembles the folding predicted for the native subunit in F₀ (Girvin et al., 1998). The protein folds as a helical hairpin in solution with residue–residue interactions as predicted from genetic analysis of the folded protein in vivo. These interactions include the close proximities of residues Ala24 and Ile28 in TMH-1 to Asp61 in TMH-2 (Miller et al., 1997). Asp61, centered in the second transmembrane helix, is known to protonate and deprotonate during H⁺ transport.

Extensive cross-linking studies with membranous native F₀ indicate an oligomeric ring of 10–12 subunits with TMH-1 inside and TMH-2 outside (Jones et al., 1998). The cross-links between genetically introduced cysteine residues were generated in part to test, and now do support, the NMR model. The stoichiometry of 12 c subunits per oligomeric ring is based upon cross-linking studies with genetically fused dimers and trimers of subunit c (Jones and Fillingame, 1998). A spacer loop was inserted between the C terminus of the first monomeric unit and the N terminus of the next on the basis of the precedent of V-ATPase subunit c, which is thought to have arisen by gene duplication (Nelson, 1992). The genetically fused c₂ dimers and c₃ trimers proved to be functional, suggesting that the stoichiometry was a multiple of 2 and 3. On introduction of cysteine residues into the N- and C-terminal helices of the dimers and trimers, and oxidation in the membrane, multimeric ladders extending to the equivalent of

Subunit b is estimated to extend 11 nm from the surface of the membrane (Rodgers and Capaldi, 1998). Subunit b is anchored in the membrane via a single transmembrane helix (TMH) at the N terminus, the structure of the segment having been determined recently using NMR (Dmitriev et al., 1999b). Subunit a is a highly hydrophobic protein which was recently shown to span the membrane with five transmembrane helices (Valiyaveetil and Fillingame, 1998; Long et al., 1998; Wada et al., 1999). It is presumed to be the major component of the alternate access channels shown in Fig. 1, but the placement of these channels in the protein is not yet easily reconciled with our current knowledge of the subunit. The interactions and role of aTMH-4, which contains the conserved and essential residue Arg210, will be considered in some detail below. In this essay, we will be emphasizing recent work that has helped to define details of the structural organization and interactions of subunits in F₀ and discuss this new information in the context of the rotary motor hypothesis.
Structure and organization of the rotary motif

were observed in both cases. Previous values for stoichiometry based upon quantification of biosynthetically incorporated radiolabel had ranged from 9 to 12 (Foster and Fillingame, 1982; von Meyenburg et al., 1982), i.e. a range within experimental error of the value of 12 derived from the cross-linking studies.

The structure of the oligomer has been modeled using molecular dynamics and energy minimization calculations from the solution structure of monomeric subunit c and 21 inter-subunit distance constraints derived from cross-linking of subunits in native F1F0 (Dmitriev et al., 1999a). In the oligomeric structure, the subunits pack to form a compact hollow cylinder with an outer diameter of 5.5–6.0 nm and an inner space with a minimal diameter of 1.1–1.2 nm (Fig. 2A,B). Phospholipids are presumed to pack in the inner space in the native membrane. The transmembrane helices pack in two concentric rings with TMH-1 on the inside and TMH-2 on the outside. The H+ -transporting Asp61 residue packs towards the center of the four transmembrane helices of two interacting subunits (Fig. 2C). The packing supports the suggestion that the proton-binding site is formed at the packed interface of two subunits, with Asp61 at the front face of one subunit interacting with Ala24, Ile28 and Ala62 at the back face of a second subunit. The packing can explain the interchange of the functional carboxyl group from position 61 to position 24 in the functional cA24D/D61G mutant (Miller et al., 1990), since the carboxyl would end up at approximately the same position between subunits whether attached to TMH-2 or TMH-1. The packing would also explain the slowed reaction of dicyclohexylcarbodiimide (DCCD) with Asp61 in the cA24S and cI28T DCCD-resistant mutants (Fillingame et al., 1991). In the Propionigenium modestum F1F0, which transports Na+, a Gln residue at the position of E. coli Ala28 and a Ser residue at the position of E. coli Ala62 provide additional liganding groups for Na+ binding to a Glu residue at the position of E. coli Asp61 (Kaim et al., 1997). The Na+-binding site would thus be expected to be formed by the front-to-back packing of two subunits. The positioning of the Asp61 carboxyl in the center of the interacting transmembrane helices, rather than at the periphery of the cylinder, suggests that the helices must rotate or swivel to open the proton-binding site to subunit a during proton transport.

An alternative model for a subunit c oligomer in which TMH-2 is packed in the inner ring and TMH-1 in the outer ring has been suggested by Groth and Walker (1997). However, as described by Dmitriev et al. (1999a), energy calculations from the cross-linking data strongly favor a structure with TMH-2 at the periphery of the ring and TMH-1 inside the ring. Groth et al. (1998) have constructed single Trp substitutions in residues 61–72 of helix 2 in an attempt to probe the arrangement of c subunits. Interpretation of the effects of most of the substitutions, where activities were severely compromised or abolished, is complicated and hence may not provide good evidence to distinguish between models. For example, some activity was observed with substitutions centered around Pro64, i.e. at residues 62, 63 and 65, which could reflect a required structural flexibility in this region related to proton binding and release from Asp61. The effects of substitutions at positions 69–72, which are expected to be located near the hydrocarbon–headgroup interface of the phospholipid bilayer, i.e. the usual position of Trp in membrane proteins, are quite compatible with the model presented by Dmitriev et al. (1999a), the expected positions of the bulky Trp side chains having been discussed more thoroughly by Dmitriev et al. (1999a). In addition, the Groth and Walker (1997) model is not easily reconciled with the extensive pattern of cross-linking observed between cysteine residues in TMH-2 of subunit c and cysteine residues in TMH-4 of subunit a, as discussed below.

Fig. 2. Structure of the oligomer deduced from energy calculations using distance constraints from cross-linking and the nuclear magnetic resonance model (Dmitriev et al., 1999a; reproduced with the permission of the publishers). (A) Top view from the F1 binding side and polar loop end of subunit c. Alternating c subunits are shown in different shades of blue. The positions of potential salt-bridging side chains are indicated, i.e. Lys34 and Arg50 of one subunit pack close to Glu37 and Asp44 of the neighboring subunit. (B) View from the side with the Asp61 residue indicated in yellow. The yellow atoms seen from the side are backbone atoms. (C) Cross section of the oligomer at the level of Asp61 with the carboxyl side chain (red and yellow atoms) packed between subunits.
Organization of subunits \(a\) and \(b\) relative to the \(c\) oligomer

Additional cross-linking analyses have established transmembrane interactions between subunits \(a\), \(b\) and \(c\) (Fig. 3). Cys-substituted subunit \(b\) can be cross-linked to form homodimers, preferentially with Cys substitutions at residues 2, 6, 10 and 14. The structure of the N-terminal region has been determined using NMR, and the structure of the dimer was modeled on the basis of these cross-links (Dmitriev et al., 1999b). The N-terminal segments of the two copies of subunit \(b\) in \(F_0\) are suggested to cross the membrane with the transmembrane helices in close association, with the direction of packing changing at a ‘hinge’ region near the cytoplasmic surface. A \(b-c\) dimer can be formed from \(b\)Cys2 to Cys74, Cys75 and Cys78 in the C-terminal region of subunit \(c\) (P. C. Jones, W. Jiang and R. H. Fillingame, unpublished results). The \(b-c\) dimer can be formed from bCys2, but not from cysteine residues lying deeper in the transmembrane regions of subunit \(b\) and \(c\) (i.e. not from bCys4, 5, 7 or 8 to cCys71 or from bCys7, 8, 11 or 12 to cCys67). The lack of cross-linking between these residue pairs may indicate that the transmembrane helices of subunits \(b\) move away from the \(c\)-oligomeric cylinder as they traverse the membrane at an angle relative to the more perpendicularly oriented \(c\)-oligomeric cylinder. Cys in TMH-4 of subunit \(a\) can be cross-linked to Cys in TMH-2 of subunit \(c\) over a span of 19 residues in each helix (Jiang and Fillingame, 1998), i.e. from residues 55 to 73 in \(c\)TMH-2 and from residues 207 to 225 in \(a\)TMH-4 (Fig. 3). The cross-linking pattern brings Asp61 of subunit \(c\) and Arg210 of subunit \(a\) close together in the transmembrane region, as would be predicted if they interact functionally. Cys residues at positions 227 and 228 in the loop region between \(a\)TMH-4 and \(a\)TMH-5 form strong cross-links with \(b\)Cys2 and cCys78 (Fig. 3; W. Jiang and R. Fillingame, unpublished results). The short loop between \(a\)TMH-4 and \(a\)TMH-5 is quite precisely placed at the periplasmic surface of the membrane by multiple Cys substitutions from residues 230 to 232, which preferentially react from the periplasmic surface (Fig. 3; for key results, see Valiyaveetil and Fillingame, 1998; Wada et al., 1999). On the basis of this and other information, the depth of placement of the helical hairpin of subunit \(c\) in the membrane phospholipid bilayer can be estimated fairly precisely (Fig. 4). The placement conforms well with predictions made from hydrophathy analyses.

The Cys residues in TMH-2 of subunit \(c\) which cross-link with TMH-4 of subunit \(a\) fall on both the ‘front’ and ‘back’ faces of subunit \(c\) in the NMR model (Jiang and Fillingame, 1998). Given the arrangement of the \(c_{12}\) oligomer discussed above, with the front face of one subunit packed against the back face of a second subunit, the cross-linking pattern is difficult to understand. Two explanations were suggested (Jiang and Fillingame, 1998). In the first, TMH-4 of subunit \(a\) was suggested to insert between \(c\) subunits of the oligomer in order for Arg210 to interact with the carboxyl of cAsp61 during its deprotonation (Fig. 5). In such a model, the \(c\) subunits would swivel about the centrally located TMH-1s to provide the gap for helix insertion, and the packing interactions between TMH-1 and TMH-2 within a single subunit \(c\) would remain unperturbed. In the second explanation, TMH-2 of the \(c\) subunit at the \(a-c\) stator interface was suggested to swivel relative to \(c\)TMH-1, not only to make the cross-linkable residues accessible to \(a\)TMH-4 but also to expose Asp61 at the periphery of the rotor. This idea, which is described in greater detail in Fig. 6, now seems plausible on the basis of a new NMR structure for subunit \(c\) performed at pH 8, where Asp61 should be fully ionized (Rastogi and Girvin, 1999). The previous structure was solved at pH 5, where Asp61 should be protonated. Ionization of the carboxyl group was known from previous studies to change the structure significantly (Assadi-Porter and Fillingame, 1995), particularly in the region of Asp61, in the polar loop and at the C- and N-terminal ends of the protein. In the new pH 8 structure, the protein folds over the N-terminal segment to Arg41, essentially as at pH 5, but the loop then unravels somewhat and twists such that TMH-2 reorients by 120° as it packs with TMH-1. It is not clear whether the twisting of the loop causes reorientation of the helices or vice versa. The reorientation of helices places Asp61 closer to the periphery of the ring, where both an interaction
entire rotor by 30° by a rolling of the position shown in Fig. 6B, and possibly force movement of the promote deprotonation of Asp61 as it moves past Arg210 to the 240° counterclockwise movement could simultaneously by a counterclockwise rotation of 240° as shown in Fig. 6A. The Fig. 6 could occur by a 120° clockwise movement or conversely 1998; see Fig. 6). The rotation of TMH-2 to the peripheral position shown in Fig. 6 could be reprotonated from the inlet channel at the carboxyl was shown to cross-link to the amine moiety of phosphatidylethanolamine by Lötscher et al. (1984). Cys residues introduced at positions 74, 75 and 78 in subunit b and to Cys227 and Cys228 in the periplasmic loop between aTMH-4 and aTMH-5 (P. C. Jones, W. Jiang and R. H. Fillingame, unpublished results). The side chain of Asp61 is shown in grey. The positions of all charged residues (red and green) and polar residues (yellow) are indicated. The approximate widths of the fatty acyl interior and polar head group regions of a dioleoylphosphatidylcholine lipid bilayer are indicated (from Wiener and White, 1992). Residues Met11–Leu31 and Ile55–Met75 are predicted to span the fatty acyl region of the lipid bilayer. TMH, transmembrane helix.

with Arg210 and the cross-linking between the c and α subunits described previously could take place (Jiang and Fillingame, 1998; see Fig. 6).

The rotation of TMH-2 to the peripheral position shown in Fig. 6 could occur by a 120° clockwise movement or conversely by a counterclockwise rotation of 240° as shown in Fig. 6A. The 240° counterclockwise movement could simultaneously promote deprotonation of Asp61 as it moves past Arg210 to the position shown in Fig. 6B, and possibly force movement of the entire rotor by 30° by a rolling of the α–c surfaces against each other. Reprotonation of Asp61 could occur by one of two means. A continued counterclockwise rotation of TMH-2 by 120° would complete a 360° rotation cycle for TMH-2 and return the unprotonated Asp61 to its original position, where it would be reprotonated from the inlet half-channel. Conversely, the ionized carboxylate could be reprotonated from the inlet channel at the position shown in Fig. 6B, and on reprotonation return to its original position by a clockwise rotation of 240°. The 30° movement of the rotor could also be driven at this step. The portion of subunit α making up the inlet channel remains to be defined. It could be formed, at least partially, by polar residues Asn214, Glu219, Asn238 and His245 on the periplasmic sides defined. It could be formed, at least partially, by polar residues Asn214, Glu219, Asn238 and His245 on the periplasmic sides and to Cys227 and Cys228 in the periplasmic loop between aTMH-4 and aTMH-5 (P. C. Jones, W. Jiang and R. H. Fillingame, unpublished results). The side chain of Asp61 is shown in grey. The positions of all charged residues (red and green) and polar residues (yellow) are indicated. The approximate widths of the fatty acyl interior and polar head group regions of a dioleoylphosphatidylcholine lipid bilayer are indicated (from Wiener and White, 1992). Residues Met11–Leu31 and Ile55–Met75 are predicted to span the fatty acyl region of the lipid bilayer. TMH, transmembrane helix.

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Fig. 5. Suggested cross-sectional arrangement of TMH-4 of subunit a with subunits of the c12 oligomer. The relative positions of α carbons of transmembrane helices are those indicated by the nuclear magnetic resonance model (Girvin et al., 1998) for intra-subunit contacts and by the oligomeric model (Dmitriev et al., 1999a) for contacts between subunits. An insertion of aTMH-4 between c subunits of the oligomer has been postulated to account for cross-linking of aN214C with cA62C or cM65C (residues highlighted in green) and for the proposed functional interaction between aArg210 (highlighted in blue) and cAsp61 (highlighted in red) (Jiang and Fillingame, 1998). Alternatively, these interactions could take place after rotation of cTMH-2 relative to aTMH-4, as illustrated in Fig. 6. TMH, transmembrane helix.
Fig. 6. Model for the suggested rotation of TMH-2 of subunit c during deprotonation of Asp61 via interaction with Arg210 on aTMH-4. The surfaces of helix–helix interaction demonstrated by cross-link analysis are indicated by the yellow shading. (A) Arrangement of helices prior to deprotonation of Asp61 as subunit c5 of the c12 oligomer approaches subunit a. In the step between this arrangement and that shown in arrangement B, cTMH-2 is proposed to rotate in the counterclockwise direction by 240° and, on passing Arg210 in aTMH-4, to release a proton to the cytoplasmic outlet channel during ATP synthesis. (B) Arrangement of helices after deprotonation of Asp61. Reprotonation from the periplasmic inlet channel and a subsequent conformational change allow clockwise rotation of cTMH-2 back to its original position relative to cTMH-1. Alternatively, the helix could return its original position by continued rotation in the counterclockwise direction by 120° and be reprotonated at that site (see text). (C) In the process of one of the helix rotations in either step A→B or step B→C, the ring of subunits c is proposed to be translocated to the right in a counterclockwise direction by 30° (see text).
Interaction of subunit $\varepsilon$ with the loop region of subunit $c$

The polar loop of subunit $c$ was shown to interact directly with the $\gamma$ and $\varepsilon$ subunits of $F_1$ by Cys–Cys cross-linking studies (Zhang and Fillingame, 1995b; Watts et al., 1995, 1996) in experiments designed to test interactions suggested by suppressor mutation analysis (Zhang et al., 1994). In the most recent analysis (Hermolin et al., 1999), an extensive series of cross-links was interpreted by using the solution NMR structures of subunits $c$ and $\varepsilon$ (Girvin et al., 1998; Wilkens et al., 1995). Cysteine residues in the continuous span of residues 26–33 of subunit $\varepsilon$ form a turn of antiparallel $\beta$-sheet extending from the bottom of the subunit as a well-defined lobe (Fig. 7A). The side chains of residues 42 and 44 project from opposite sides of the loop of subunit $c$, i.e. the ‘front’ and ‘back’ face, respectively (Fig. 7B), which indicates that the cross-linkable domain of subunit $\varepsilon$ must pack between the loops of adjacent subunits $c$ in the $c_{12}$ oligomer for cross-linking to occur from both faces. The interaction between subunits has been modeled on the basis of cross-linking distance constraints and places the residue 26–33 lobe of subunit $\varepsilon$ between loops of subunit $c$ in a well-packed structure (Fig. 7C). The modeling indicates that the cross-linking of subunit $\gamma$ with these same residues in subunit $c$ (Watts et al., 1995, 1996) must take place by interaction with a separate pair of $c$ subunits, perhaps the adjacent pair, in the oligomer. An interesting question posed by the changes in polar loop structure seen in the pH 8 NMR model of subunit $c$ is whether the binding interactions in the loop change during the deprotonation–protonation interactions of subunit $c$ with subunit $a$ (discussed in the previous section).

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References


complex (ECF₁Fₒ) extends through the stalk and contacts the c subunits of the Fₒ part. *FEBS Lett.* **368**, 235–238.


