Insights into the Rotary Catalytic Mechanism of FₐFₐ ATP Synthase from the Cross-linking of Subunits b and c in the Escherichia coli Enzyme*

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Phil C. Jones†, Joe Hermolin, Weiping Jiang, and Robert H. Fillingame§

From the Department of Biomolecular Chemistry, University of Wisconsin Medical School, Madison, Wisconsin 53706

The transmembrane sector of the FₐFₐ rotary ATP synthase is proposed to organize with an oligomeric ring of c subunits, which function as a rotor, interacting with two b subunits at the periphery of the ring, the b subunits functioning as a stator. In this study, cysteines were introduced into the C-terminal region of subunit c and the N-terminal region of subunit b. Cys of N2C subunit b was cross-linked with Cys at positions 74, 75, and 78 of subunit c. In each case, a maximum of 50% of the b subunit could be cross-linked to subunit c, which suggests that either only one of the two b subunits lie adjacent to the c-ring or that both b subunits interact with a single subunit c. The results support a topological arrangement of these subunits, in which the respective N- and C-terminal ends of subunits b and c extend to the periplasmic surface of the membrane and cα-Asp-61 lies at the center of the membrane. The cross-linking of Cys between bN2C and cV78C was shown to inhibit ATP-driven proton pumping, as would be predicted from a rotary model for ATP synthase function, but unexpectedly, cross-linking did not lead to inhibition of ATPase activity. ATP hydrolysis and proton pumping are therefore uncoupled in the cross-linked enzyme. The c subunit lying adjacent to subunit b was shown to be mobile and to exchange with c subunits that initially occupied non-neighboring positions. The movement of exchange of subunits at the position adjacent to subunit b was blocked by dicyclohexylcarbodiimide. These experiments provide a biochemical verification that the oligomeric c-ring can move with respect to the b-stator and provide further support for a rotary catalytic mechanism in the ATP synthase.

H⁺-transporting FₐFₐ ATP synthases utilize the energy of a transmembrane electrochemical H⁺ gradient to drive 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 transmembranous sectors, termed F₁ and F₀, respectively. Proton movement through F₀ is reversibly coupled to ATP synthesis or hydrolysis in catalytic sites on F₁. Each sector of the enzyme is composed of multiple subunits, with the simplest composition being α₃β₃γδε for F₁ and α₁β₁c₁₂ for F₀ 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 α₂β₂γ protein of bovine F₁ shows the 3 α and 3 β subunits alternating around a centrally located γ subunit, with the γ subunit interacting asymmetrically with the 3 β catalytic subunits (5). Subunit γ was subsequently shown to rotate with respect to the 3 β subunits during catalysis (6–8). Rotation of γ is thought to change the binding affinities in alternating catalytic sites to promote substrate binding and product release during catalysis (9). The γ and ε subunits are known to interact with each other and probably rotate as a fixed unit (10–14). During ATP synthesis, the rotation of γ must be driven by proton flux through F₀, but the structure of F₀ and mechanism of coupling remain to be established (15).

The 12 c subunits of F₀ are now known to be arranged in an oligomeric ring with subunits a and b of F₀ positioned at the periphery of the ring (2, 16–21). Subunit c folds in the membrane as a hairpin of two hydrophobic α-helices connected by a polar loop on the F₁ binding side of the membrane (2, 22, 23). The conserved Asp-61 carbohydrate, centered in the second transmembrane helix, catalyzes proton transport via interaction with subunit a (15, 22, 24–29). The conserved polar loop region of subunit c interacts directly with the γ and ε subunits of F₁ (30–32), and proton flux-driven rotation of the c-ring is proposed to drive rotation of γ within F₁ (6, 29, 33–36). In such a model, subunits a and b pack to the side of the c-ring, where they are proposed to function as a stator to hold F₁ fixed to the membrane as the c₁₂ rotor drives rotation of subunit γ within the α₁β₁γε hexamer. Direct evidence for rotation of the c-oligomeric ring was recently presented (37, 38), although the interpretation has been challenged (39).

The arrangement of subunits described above is now supported by a 4 Å resolution x-ray diffraction density map of an F₁c₁₀ subcomplex purified from yeast mitochondria (40). The structure of monomeric subunit c determined in chloroform-methanol-water solvent by NMR (23) and the ring-like structure proposed from an extensive series of Cys-Cys cross-links (19, 21, 41) fit remarkably well with the density map. The monomeric structure of subunit ε (42, 43) and the proposed c-ε and c-γ connections between the surfaces of F₀ and F₁ (30–32) are also accommodated well by the map. The details of the interactions between the c-ring and subunits a and b, and the interactions between the stator subunits and the F₁ domain, remain to be elucidated.

Subunit a is thought to fold in the membrane with five

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† Present address: Medical Research Council, Dunn Human Nutritional Unit, Cambridge CB22QH, United Kingdom.

‡ Present correspondence should be addressed: Dept. of Biomedical Chemistry, 1300 University Ave., University of Wisconsin-Madison, Madison, WI 53706. Tel.: 608-262-1439; Fax: 608-262-5253; E-mail: rhfillin@facstaff.wisc.edu.

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transmembrane helices (TMHs)\(^1\) (44–46), the fourth of which is known to interact extensively with TMH-2 of subunit c (20). The interaction of the conserved Arg-210 residue in \(\alpha\)TMH-4 with cTMH-2 is thought to be critical during protonation-deprotonation of cAsp-61 (27–29). Cross-linking and modeling experiments do indicate that helix-1 of subunit c should be packed on the inside of the ring and helix-2 on the outside (19, 21), and this predicted packing is also suggested by the x-ray diffraction map (40). The placement of helix-2 at the periphery of the cylinder is consistent with the cross-linking of this helix, but not helix-1, to \(\alpha\)TMH-4 (20). Previous electron microscopic studies also indicate that subunit a, as well as subunit b, should lie at the periphery of the ring (16–18).

Subunit b is a amphipathic protein of 156 residues (47). The elongated, polar, and largely helical C-terminal domain is thought to be anchored to the lipid bilayer by a single N-terminal \(\alpha\)-helix, which should extend to the periplasmic surface of the bacterial inner membrane (47, 48). The cytoplasmic domain associates to form a dimer that is necessary for \(F_1\) binding (49–51). Interactions between the cytoplasmic domain of subunit b and subunit \(\delta\) of \(F_1\) have been demonstrated in solution (52), and interactions between subunit b and subunits \(\alpha\) and \(\delta\) at the top of the \(F_1\) molecule were recently demonstrated in \(F_1\)\(_b\) (53, 54). To reach the top of the \(F_1\) molecule, subunit b is estimated to extend 110 Å from the surface of the membrane (53).

Electron micrographs now indicate a second stalk at the periphery of the \(F_1\)\(_b\) molecule that is presumed to represent a dimer of b subunits extending from \(F_0\) to the top of \(F_1\) (55–58). The positioning of the TMHs of the two b subunits relative to the c-oligomeric ring has yet to be determined. As shown here, at least one of the TMHs must be spatially proximal to the ring, as a Cys sulphydryl substituted into the N-terminal segment is efficiently cross-linked to Cys in the C-terminal region of subunit c. We also report experiments, using subunit b-c cross-link formation, that support the idea of subunit c movement relative to a subunit b stator, as is predicted from rotary models for ATP synthase function.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—The parent plasmids used in this study are derivatives of plasmid pDF163, which contains the wild type \(\Delta\)uncBEFH-55\(\Delta\)uncBEFH operon and which was constructed to facilitate overexpression of the \(b\)-c subunit of \(F_0\)\(_b\)\(c\)-ATP synthase. Plasmid pNOC (19), a derivative of plasmid pDF163 with a C21S substitution in subunit c, was cloned between the \(Hin\)III and \(Sph\)I sites of plasmid pBR322 (59). Plasmid pNOC (19), a derivative of plasmid pDF163 with a C21S substitution in subunit b and subunit \(\delta\) of \(F_1\) have been demonstrated in solution (52), and interactions between subunit b and subunits \(\delta\) and \(\alpha\) at the top of the \(F_1\) molecule were recently demonstrated in \(F_1\)\(_b\) (53, 54). To reach the top of the \(F_1\) molecule, subunit b is estimated to extend 110 Å from the surface of the membrane (53).

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**EXPERIMENTAL PROCEDURES**

**Membrane Preparations and Biochemical Assays**—Membrane vesicles were prepared and stored in TMG buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl\(_2\), 1 mM diithiothreitol, 10% (v/v) glycerol) at 20 mg/ml after passage of cells through a French press (63). ATPase activity and protein were determined as described (63). ATP-driven ACMA quenching and NADH-driven quinacrine quenching assays were carried out in HMK assay buffer (10 mM HEPES-NaOH, pH 7.5, 5 mM MgCl\(_2\), 300 mM KCl) as described (63).

**Cross-linking**—Cross-linking was carried out using membranes in TMG buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl\(_2\), 10% (v/v) glycerol) using CuP as an oxidant or via disulfide interchange catalyzed by DTNB. All reactions were carried out at room temperature. Specific conditions are given in the figure legends. DTNB-catalyzed reactions were terminated by treatment with 25 mM NEM for 10 min, and CuP-catalyzed reactions were terminated by incubation with 25 mM NEM and 50 mM EDTA for 10 min. Samples were then mixed with an equal volume of 2× SDS sample buffer (125 mM Tris-\(\mathrm{HCl}\), pH 6.8, 4% SDS, 20% (v/v) glycerol, and 0.02% bromphenol blue), and membranes were solubilized by incubation for 1 h at room temperature immediately before electrophoresis. The solubilized membrane proteins were separated by SDS-polyacrylamide gel electrophoresis using the Tris-Tricine buffer system of Schägger and von Jagow (64) with 12.5% acrylamide slab gels of 0.75-mm thickness, and proteins were transferred electrophoretically to polyvinylidene difluoride membrane (65). Immunostaining was carried out using the ECL system (Amersham Pharmacia Biotech). Rabbit antisera specific to subunit c (66) and subunit b (67) were pretreated as described (68) and diluted 1:10,000 and 1:20,000, respectively, prior to use. Rabbit antiserum to subunit b was a generous gift of D. S. Perlin and A. E. Senior (University of Rochester).

**Effect of DCCD Inhibition of Rotary Movement on b-c Cross-link Formation**—To test whether the \(c\)-ATP synthase that was cross-linked with the \(b\)-c subunit could exchange with other subunits in the \(c\)-oligomer, as would be expected during rotary catalysis, the following experiment was devised. \(b\)-cATP synthase membranes suspended in TMG buffer were treated with 25 mM DTNB for 60 min at room temperature to promote maximal b-c cross-link formation. Maximal cross-linking was confirmed by SDS-electrophoresis and immunoblotting (data not shown). The DTNB-treated sample was then split into two parts and incubated with or without 10 mM NEM for 30 min at room temperature. Following centrifugation and resuspension in TMG buffer, the two samples were each divided into three subsets, which were then treated at room temperature as follows: subset 1 membranes were treated with 10 mM DTT for 90 min; subset 2 membranes were treated with 50 mM DTT for 30 min, and then 50 mM DCCD was added for an additional 60 min; and subset 3 membranes were treated with 50 mM DCCD for 60 min, and then 10 mM DTT was added for an additional 30 min. Each subset of membranes was divided into two aliquots, one of which was treated with 1.5 mM CuP for 20 min to promote re-formation of b-c cross-links. The reaction with CuP was terminated with 50 mM EDTA and 20 NEM, and the sample then dissolved in SDS sample buffer. Control membranes, not treated with CuP, were treated with 20 mM NEM and diluted directly into SDS sample buffer.

**RESULTS**

Cross-linking of \(b\)-N2C Subunit b with Cys in the C-terminal Region of Subunit c—The C-terminal helix of subunit c is predicted to lie at the periphery of the oligomeric \(c\)-ring with the end of the helix extending to the periplasmic surface of the membrane, where it should lie close to the N-terminal region of 1
2
subunit b. Cys were introduced into the C-terminal region of subunit c, and the substituted mutant proteins were expressed from a plasmid also expressing an N2C-substituted subunit b. In the case of the bN2C/cV78C combination, cross-links were formed by autoxidation in the absence of dithiothreitol, either in vivo or during the preparation of membranes (Fig. 1). In the case of bN2C/cV74C and bN2C/cV75C, significant b-c cross-link formation was only observed after oxidative treatment of the mutant membranes with CuP (Fig. 1). In all three cases, cross-linking appears to be maximized with approximately 50% of the subunit b cross-linked in a b-c heterodimer and 50% remaining monomeric. Also, in each case, the formation of b-c dimers occurs much more readily than b-b homodimer formation. The results support the proposed periplasmic localization of the N terminus of subunit b and C terminus of subunit c and also may suggest that only one of the N-terminal helices of the two b subunits lies proximal to the c-ring of F0. The bN2C/cV78C combination was selected for further functional studies because of the ease of b-c heterodimer formation by autoxidation and because minimal c-c homodimers were formed under these conditions (19).

Assessing the Functional Effects of Cross-linking of Subunits bN2C and cV78C—As shown in Fig. 1, disulfide cross-links form spontaneously between Cys-2 of subunit b and Cys-78 of subunit c when bN2C/cV78C membranes were prepared without dithiothreitol. To assess the effect of b-c cross-linking on F0 function, membranes were typically prepared in TMG buffer containing 1 mM DTT, and then, to begin each experiment, the membranes were centrifuged and resuspended in TMG buffer containing 25 μM DTT. CuP and DTNB were compared as oxidants in promoting b-c cross-link formation. CuP had previously been shown to promote b-b dimer formation with bN2C membranes (48) and c-c dimer formation with cV78C membranes (19). DTNB proved to be the more useful of the two reagents in studies directed at assessing the effect of b-c dimer formation on F0 function. Less cV78C dimer formation was observed with DTNB than with CuP, and because c-c dimer formation by itself proved to be inhibitory to F0 function, the experiments done with DTNB proved to be simpler to interpret. Immunoblots from a typical cross-linking experiment are shown in Fig. 2. The bN2C/cV78C dimeric product was most easily detected with antiserum to subunit b (A) or antiserum to subunit c (B). IA, immunoartifact.
at higher concentrations of DTNB or with CuP. The cross-linked b-c product was also detected with antiserum to subunit c (Fig. 2B). The b-c dimer appears on the Western blot just below a prominent immunoartifact (Fig. 2B, IA). The formation of c-c dimers is also apparent in both bN2C/cV78C and cV78C membranes. Minor amounts of b-b dimers were seen in control bN2C membranes but not in the bN2C/cV78C membranes (Fig. 2A). In the latter case, b-c dimer formation may prevent the less frequently observed b-b dimer formation.

**Inhibitory Effect of b-c Dimer Formation on ATPase-coupled Proton Pumping**—The effect of b-c subunit cross-linking on ATP-driven ACMA quenching was assessed with bN2C/cV78C membranes, using bN2C and cV78C membranes as controls. Results from a representative experiment are presented in Fig. 3. First, note in Fig. 3A that the ATP-driven quenching response of bN2C membranes was only marginally affected by any of the treatments, the treatments being exactly equivalent to those already described in Fig. 2. Similarly, as shown in Fig. 3B, the small amounts of c-c dimer formation seen in cV78C membranes under the mild oxidizing conditions (traces 2–4) caused only minor diminutions of the quenching response, although a more marked inhibition was observed after oxidation with 25 μM DTNB (trace 5). The pattern of inhibition observed in Fig. 3C with bN2C/cV78C membranes is quite different. Progressively greater inhibition was observed as the extent of b-c cross-linking increased, shown in the progression of traces 2–5. The progressive inhibition can be attributed to b-c dimer formation rather than c-c dimer formation because the amount of c-c dimer formation in cV78C and bN2C/cV78C membranes was nearly equivalent in the experiment shown here (Fig. 2B) and in other documenting experiments. We note again that the increased c-c dimer formation seen in cV78C membranes under conditions 2, 3, and 4 (Fig. 2B) did not lead to significant differences in inhibition of function (Fig. 3B).

It seemed possible that the increased inhibition observed with bN2C/cV78C versus cV78C membranes was due to the initial lower ACMA quenching activity of the nonoxidized bN2C/cV78C membranes. The bN2C/cV78C membranes also exhibited lower ATPase activity, as shown in Table 1.2 To examine this possibility, the sensitivity of ACMA quenching response of cV78C membranes to oxidation was checked at a lower concentrations of membrane, i.e., conditions under which the fluorescence quenching response was greatly reduced. As shown in Fig. 4, the ATP-driven quenching response of cV78C membranes was little affected by treatment with 10 μM DTNB and only marginally affected by 25 μM DTNB. We conclude that the major inhibitory effects of DTNB seen in Fig. 3C result from b-c cross-linking and not from the minor extent of c-c cross-linking.

The b-c dimer formation observed in Fig. 2 would be expected to inhibit membrane ATPase activity as well as ATP-driven proton pumping if the extrinsic F1 sector remains tightly coupled to the rotary motor. Surprisingly, cross-linking with DTNB led to very modest inhibition of ATPase activity, and the inhibition was as great for cV78C membranes as for bN2C/cV78C membranes (Table 1). In the case of the bN2C/cV78C membranes, the DTNB-resistant ATPase activity is clearly not coupled to H+ pumping (see Fig. 3C). These results raised the possibility that DTNB-catalyzed cross-linking of b to c reduced the ATP-driven ACMA quenching response indirectly, by increasing the proton permeability of the membrane. This question is examined in the experiments shown in Fig. 5. NADH-driven quenching of quinacrine fluorescence was measured following resuspension of whole membrane vesicles in TMG buffer plus 25 μM DTNB. The quenching of fluorescence, which is indicative of formation of a transmembrane pH gra-

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4 The reduced ATPase activity of bN2C/cV78C membranes, seen after lauryldimethylamine oxide activation, suggests that less F0F1 is incorporated into these membranes relative to the single Cys-substituted membranes. This possibility is consistent with Western blot analysis, which suggests an approximately 2-fold reduction of α, β, and b subunits into bN2C/cV78C membranes versus bN2C or cV78C membranes.
ATP-driven quenching of ACMA fluorescence was measured by concentration of 62.5 μM DTT prior to assay (trace 4) and 25 μM DTNB (trace 5) is shown.

**Fig. 4. Effect of c-c cross-linking on ATP-driven proton pumping by cV78C membranes under conditions of a reduced quenching response.** ATP-driven quenching of ACMA fluorescence was measured using the membrane samples treated as described in Figs. 2 and 3. Membranes were suspended in HMK assay buffer at a protein concentration of 62.5 μg/ml. Control membranes were kept on ice in TMG buffer with 25 μM DTNB. Similar results were seen with LDAO 1% of untreated control. Specific activity was reduced with DTT and then reoxidized with CuP, the cV78C sulfhydryl group should remain proximal to the bN2C sulfhydryl and maximal cross-link re-formation is predicted. These predictions were borne out in a typical experiment, shown in Fig. 6B. In Fig. 6B, lane 1, the NEM-treated enzyme was reduced with DTT and then reoxidized with CuP, a treatment that should result in maximal b-c cross-link reformation. In Fig. 6B, lane 2, DTT reduction followed by DCCD treatment severely reduced the amount of b-c cross-link formed in the final CuP treatment. In Fig. 6B, lane 3, DCCD treatment prior to DTT reduction led to maximal b-c cross-link re-formation on treatment with CuP. Fig. 6B, lanes 4–6, shows that the DCCD treatment, by itself, does not have major effects on re-formation of the b-c cross-link in control membranes not treated with NEM. Lanes 6–9 indicate that DTT treatment used was sufficient to completely reduce the b-c cross-link formed in the initial oxidative treatment. The same pattern of cross-linking was observed in six other, similarly designed experiments, in which the protocol was varied in minor ways. In two of these experiments, the amount of b-c cross-linked product seen in control lane 5 (Fig. 6B) was reduced significantly from that seen in control lane 6, which may indicate that DCCD-inhibitable thermal motion helps to place subunits b and c in optimal cross-linking proximity. (A slight reduction of cross-linking in lane 5 is also apparent in the experiment shown in Fig. 6B.) In other experiments, the diminution of b-c cross-link formation observed in lane 2 was shown to be unaff
A.

FIG. 6. Movement of subunit c from the b-c interface is demonstrated by bN2Cc/V78C cross-linking. A strategy for measuring movement of subunit c after initial cross-link to subunit b. In the initial step, the c₁₂ oligomeric ring has been cross-linked to subunit b via a bN2Cc/V78C disulfide linkage, and the remaining free sulfhydryl groups were then reacted with NEM. Following reduction of the disulfide linkage with DTT (arrow to left), the ring is expected to move away from the b-c interface by thermal motion (or conceivably in an ATP dependent reaction), and the free subunit c sulfhydryl group is expected to be fixed in the position shown by DCCD treatment. Subsequent oxidative treatment with CuP should not result in re-formation of the b-c cross-link. In the control experiment indicated by the right arrow, the enzyme is treated with DCCD prior to DTT reduction, a treatment that should prevent movement of the c/V78C sulfhydryl away from the b-c interface. Subsequent oxidative treatment with CuP should result in maximal re-formation of the b-c cross-link. B. Experiment testing the effect of DCCD on re-formation of b-c cross-link following reduction of the initial cross-link with DTT. All treatments were carried out at room temperature. Membranes were initially centrifuged and resuspended in TMG buffer and treated with 25 μM DTNB for 1 h to promote maximal b-c cross-link formation and then treated with 10 mM DCCD for 30 min (lanes 1–3 and 7–9) or not treated with NEM (lanes 4–6). Following centrifugation and resuspension in TMG buffer, membranes were treated with 10 mM DTT for 90 min (lanes 1, 4, and 7); treated with 10 mM DTT for 30 min, and then 50 μM DCCD for an additional 60 min (lanes 2, 5, and 8); or treated with 50 μM DCCD for 60 min, and then 10 mM DTT was added for an additional 30 min (lanes 3, 6, and 9). Membranes in lanes 1–6 were treated with CuP to promote re-formation of b-c cross-links, and the reaction was terminated with EDTA and NEM prior to SDS gel electrophoresis. Control membranes in lanes 7–9 were treated with NEM and diluted directly into SDS sample buffer as a control to show that DTT effectively reduced the initial cross-link. Complete reduction of cross-links in the (NEM) control samples was also observed (not shown). An immunoblot with antiserum to subunit b is shown.

affected by inclusion of ATP in the DTT reduction stage of the experiment.

DISCUSSION

The cross-linking of Cys at position 2 of subunit b with Cys at positions 74, 75, and 78 of subunit c supports the previously assumed topological arrangement of these subunits, in which the N-terminal end of subunits b and the C-terminal end of subunit c extend to the periplasmic surface of the membrane (Fig. 7). Lötcher et al. (69) had previously demonstrated cross-linking of the α-carboxyl group of the C-terminal residue of subunit c, i.e. Ala-79, with the amino group of phosphatidylethanolamine in a reaction catalyzed by the water-soluble carbodiimide, 1-ethyl-[3-(dimethylamino)propyl]-carbodiimide. According to the measurements of Wiener and White (70), the ethanolamine moiety of the phospholipid should lie 20–25 Å from the center of the membrane. The Ala-79 α-carboxyl lies 29 Å from the Asp-61 side chain carboxyl in the NMR model (23), which would place the Asp-61 carboxyl group in the fatty acyl region of the cytoplasmic leaflet near the center of the lipid bilayer. The cross-linking of Cys positions 74 and 75 of subunit c with Cys-2 of subunit b suggests that a more substantial region of the C-terminal segment of subunit c may pack in the periplasmic, polar head group region of the membrane. The positioning of Asp-61 toward the center of the membrane differs from the suggested placement for the Na⁺ binding carboxyl group of the Propionigenium modestum subunit c at the cytoplasmic surface (36) and would lead us to predict the necessity of H⁺ access channels from both sides of the membrane. In a study reporting the NMR structure of the N-terminal region of subunit b, together with an analysis of b-b dimer formation with Cys-substituted subunits, the TMHs of subunit b were proposed to associate at a preferred 20° packing angle to cross the membrane as a dimer. The cross-linking results shown here indicate that only one of the two subunit b can dimerize with subunit c and suggest that the second b TMH may lie more to the periphery of the complex, perhaps in association with subunit α, or that a single subunit c interacts simultaneously with both of the b subunit transmembrane domains.

The cross-linking of subunit bN2C with subunit cV78C can be causally related to inhibition of ATPase-coupled proton pumping. Such inhibition is expected in the rotary model. A small amount of cV78C-cV78C cross-linked product was observed in these experiments, but by use of appropriate controls, the major effects on proton pumping could clearly be attributed to b-c cross-link formation. Unexpectedly, b-c cross-link formation did not result in substantial inhibition of ATPase activity, which suggests an uncoupling of ATPase function and proton-translocating function. The bN2C/cV78C mutant F,F,F is unusual in that the membrane ATPase activity, in its reduced state, was markedly inhibited relative to the lauryldimethylamino oxide activated state. The combined effect of the double Cys substitutions at the periplasmic side of the membrane, and

FIG. 7. Topological organization of subunit b and the oligomeric c rotor in the phospholipid bilayer of the E. coli inner membrane. Three units of the oligomeric c ring are shown, with TMH-2 on the outside and TMH-1 on the inside. The bottom of the γ and ε subunits are shown to extend to the top surface of the c-ring, and the cytoplasmic domain of the b subunit is shown to extend up the side of the α,β, hexamer of F₁. The relative positions of the N2C cysteine at the N terminus of subunit b and the V78C cysteine at the C terminus of subunit c at the periplasmic surface of the membrane and of cAsp-61 at the center of the membrane are indicated.
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to their cross-linking, thereby perturb the interaction of F_{1} with F_{0} at the other side of the membrane.

DCCD-inhibitable movement of subunit c relative to the subunit b stator was demonstrated in the experiment described schematically in Fig. 6A. Following bN2C6/V78C disulfide bond formation and NEM modification of the remaining free sulfhydryl groups, the disulfide bond was reduced, and the c-oligomer was allowed to reposition itself relative to the b-stator. Modification of the c-oligomer with DCCD to inhibit further movement prevented re-formation of the disulfide bond. The experiment proves, first, that the subunit c in disulfide linkage with subunit b can move away from subunit b by thermal motion and, second, that DCCD reaction with the c-oligomer prevents movement of the subunit c back to the original position. The lack of an ATP requirement for movement of subunit c away from subunit b, after reduction of the disulfide bond, indicates that the repositioning is occurring by thermal motion rather than as a part of the ATP-coupled transport cycle. However, in order to see an ATP effect, the ATP induced movement would have to be rate-limiting relative to the disulfide reduction step.

In summary, this study provides the important supportive evidence consistent with the rotary mechanical model for F_{0}F_{1} ATP synthase. In the recent studies of Sambongi et al. (37) and Pänke et al. (38), the rotation of the c-oligomer was directly observed by microscopy following attachment of an actin filament to the c-ring by independent approaches. However, Tsunoda et al. (39) have recently questioned whether the rotation observed is with a non-disrupted F_{0}F_{1} complex. The results presented here provide biochemical evidence that subunit c can move relative to the subunit b stator by DCCD-inhibitable thermal motion. Previously, independent evidence was provided in support of a second tenet of the rotary catalytic mechanism, i.e. that the eγ subunit complex should remain fixed to the polar loops of a specific set of subunit c during enzyme function (71, 72). Although a case is building for a fixed interaction between the c12-rotor and γe shaft and for the movement of the c12-rotor relative to a b2 stator, other seemingly contradictory evidence remains to be explained (73), and additional evidence is required for a complete proof of the rotary catalytic mechanism.

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