Site-directed Cross-linking of b to the α, β, and α Subunits of the
Escherichia coli ATP Synthase∗

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The b subunit dimer of the Escherichia coli ATP synthase, along with the δ subunit, is thought to act as a stator to hold the αβ3 hexamer stationary relative to the α subunit as the γε3φ9-12 complex rotates. Despite their essential nature, the contacts between b and the α, β, and α subunits remain largely undefined. We have introduced cysteine residues individually at various positions within the wild type membrane-bound b subunit, or within b24–156, a truncated, soluble version consisting only of the hydrophilic C-terminal domain. The introduced cysteine residues were modified with a photocross-linking cross-linking agent, and cross-linking to subunits of the F1 sector or to complete F1F0 was attempted. Cross-linking in both the full-length and truncated forms of b was obtained at positions 92 (to α and β), and 109 and 110 (to α only). Mass spectrometric analysis of peptide fragments derived from the b24–156A92C cross-link revealed that this dimer interacts with the α subunit near a non-catalytic αβ interface. A cysteine residue introduced in place of the highly conserved arginine at position 36 of the subunit could be cross-linked to the α subunit of F0 in membrane-bound ATP synthase, implying that at least 10 residues of the polar domain of b are adjacent to residues of α. Sites of cross-linking between b24–156A92C and β as well as b24–156I109C and α are proposed based on the mass spectrometric data, and these sites are discussed in terms of the structure of b and its interactions with the rest of the complex.

ATP synthase, or F1F0-ATPase, utilizes a transmembrane proton gradient to synthesize ATP and is responsible for the final step in oxidative phosphorylation and photophosphorylation. The enzyme (reviewed in Refs. 1–3) is composed of two sectors. The membrane-integral F0 sector is a proton pore, and in Escherichia coli has a subunit composition of αβ2γε9-12. The membrane-peripheral F1 sector has a subunit stoichiometry of α3β3γε. A key feature of the F1 sector, as seen in the bovine heart mitochondrial crystal structure (4), is that the α and β subunits alternate in a ring around a lengthy pair of α-helices of γ. Each β subunit bears one catalytic nucleotide-binding site, while non-catalytic nucleotide-binding sites are found on the α subunits. These nucleotide-binding sites are located close to the interfaces between α and β subunits, with one site near each of the six interfaces.

Subunits from each sector contribute to the formation of two stalks that join F1 and F0. The γ and ε subunits form the central stalk, rotation of which is believed to be caused by translocation of protons across the membrane by the α and c subunits. This rotation is thought to cause conformational changes in the catalytic sites, driving synthesis of ATP (1). It is believed that the α and β subunits are prevented from rotating by a peripheral stalk consisting of δ and the two b subunits (5, 6) that joins the αβ3 complex to the α subunit.

In recent years the interaction of the b dimer and δ has been well established by a variety of evidence (7–10). The δ subunit appears to be located near the crown of the F1 complex, the part of F1 furthest from the membrane (11–15). Because b has a single membrane-spanning region at its N terminus, the remainder of the subunit must span a distance of over 100 Å to come in contact with δ. Consistent with this proposed arrangement, the region of interaction between b and δ has been localized to the C terminus of b (10, 16). The hydrophilic domain of b by itself is mostly α-helical as measured by circular dichroism (17), and an isolated complex composed of δ with the hydrophilic domain of b was demonstrated by sedimentation velocity ultracentrifugation to be highly extended (9). The dimerization domain of b, encompassing residues 53–122, was also shown to be highly extended (18). Therefore the b,δ complex seems to have a shape consistent with its proposed identity as the peripheral stalk of ATP synthase.

To fulfill its putative role as a key component of the stator, the b,δ complex must resist the torque generated by rotation of γ and ε. Due to the relatively weak interaction between the b and δ subunits (Kd = 5–10 μM; Ref. 9), and the apparent structural flexibility of the hydrophilic domain of b (6, 10, 19), it seems necessary that the binding of b to F1 must be stabilized through additional interactions. Rodgers and Capaldi (20) have reported the formation of a disulfide bond between a cysteine residue introduced at the C-terminal position of the 156-residue b subunit and the endogenous Cys-90 of α. However, little other information exists regarding the position of b relative to the α and β subunits, the closeness of the interaction, or the sites of contact.

Exactly how the b subunits interact with α and/or c is also unknown, but it is generally believed that the transmembrane domains of the b subunits lie on the outside of the ring of c subunits (1, 21). Chemical cross-linking of purified ATP synthase (22) or F0 (23) indicates that α and b are proximal, and analysis of second-site revertants to a G9D mutation identified residue 240 of the α subunit, within the fifth putative transmembrane helix (24, 25), as a site that may be close to b within the membrane (26). The propensity of cysteines introduced within residues 2–18 of b to form disulfides suggests that...
the two b subunits are adjacent to one another in the membrane (27).

More information about contacts of the second stalk with F$_1$F$_0$ is essential for an understanding of its function in the enzyme complex. In the present work we have introduced cysteine residues into the b subunit at various positions to allow the attachment of chemical cross-linkers. Activation of these cross-linkers and analysis of the resultant products has allowed us to develop a model of the interactions between the b subunits and the rest of the ATP synthase.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Recombinant DNA techniques were performed by standard techniques. Wild type b$_{24–156}$ was expressed from plasmid pDM3 (28). Mutations encoding cysteine were incorporated into pDM3 by polymerase chain reaction mutagenesis using appropriate restriction endonuclease sites. The sequence of DNA produced using polymerase chain reaction was confirmed by DNA sequencing by the Sanger method. Forms of full-length b bearing the A92C, 1109C, or E110C mutations were expressed in E. coli strain KM2 (29) from plasmids based on pDM8 (28), into which the mutations encoding cysteine were subcloned from pDM3-based plasmids.

For analysis of a possible b-a cross-link, we constructed a set of plasmids encoding the ATP synthase carrying a mutation of residue bArg-36 to cysteine and/or a fusion of the gene encoding the soluble E. coli cytochrome b$_{562}$ (30, 31) to the 3’ end of uncB, which encodes the a subunit. Plasmid pACWU1.2 (32), which encodes a cysteine-less ATP synthase b-subunit. Plasmid pACWU1.2 (32), which encodes a cysteine-less ATP synthase, was amplified from plasmid pSC7 using the unique restriction sites to produce pDM65. The cytochrome b$_{562}$ gene was amplified from plasmid pACWU1.2 extending from within uncB to within uncF was subcloned into pTZ18u (33) which had been cut with BamHI and HindIII, producing plasmid pSC5. The b$_{562}$ mutation was generated by polymerase chain reaction mutagenesis and cloned into plasmid pSC5 using the SnaBI and PpuMI sites within the uncF gene to produce plasmid pSC7. The b$_{562}$ mutation was moved from pSC7 into pACWU1.2 using the unique BamHI and PpuMI restriction sites to produce pDM65. The cytochrome b$_{562}$ gene was cloned into plasmid placYCH10 (34) and cloned into plasmid pVFI72 (24), using the BgIII site which had been introduced at the 3’ end of uncB. The polymerase chain reaction primers were designed to produce an in-frame fusion of the a subunit and cytochrome b$_{562}$ with the sequence of amino acids at the site of the fusion reading SEIDHG-SADL, where Ser, Glu, Asp, and His are residues 268–271 of the a subunit with the E270D mutation introduced during insertion of the BgIII site, the GS is a linker inserted to provide for some flexibility between the fused domains, and the Ala, Asp, and Leu are the first three residues of the mature cytochrome b$_{562}$ sequence. Finally, using three-part ligations, the PvuII/Aval fragment of pSD151 carrying the cytochrome b$_{562}$ fusion to the 3’ end of uncB and the 5’ end of uncE was joined with the AvaI/PvuII fragment of pSC5 (b$_{562}$) or pSD151 (b$_{562}$) carrying the 3’ end of uncE and the 5’ end of uncF site and inserted into pACWU1.2 which had been cut with PstI and PvuII. The resulting plasmids were similar to pACWU1.2 except that pSD152 carried the subunit a-cytochrome b$_{562}$ fusion and pSD153 carried both the subunit a-cytochrome b$_{562}$ fusion and the b$_{562}$ mutation. All plasmid constructions were confirmed by mapping with restriction endonucleases.

**Expression and Purification of Proteins**—Wild type b$_{24–156}$ (formerly known as b$_{562}$) or versions of b$_{24–156}$ containing cysteine mutations were expressed and purified as described previously (28). F$_1$ was prepared by standard methods (35).

Membranes bearing F$_1$F$_0$ containing wild type or mutant b expressed in uncF strain KM2 (29) from pDM8-based plasmids were prepared as described (28). Membranes from which F$_1$ was to be removed were prepared as usual except that, following the first 38,000 rpm centrifugation step, the pellet was washed either once or twice with 1 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 10% glycerol. The membranes were then resuspended to a concentration of about 20 mg of total protein/ml and stored at −80 °C.

The unc strain DK8 (36) was used to express F$_1$F$_0$ from pACWU1.2-based plasmids. The cells were grown at 37 °C in 1 liter of 2 × YT medium until A$_{600}$ reached approximately 3, at which point they were harvested, washed with 50 mM Tris-HCl, pH 8.0, 10 mM MgCl$_2$ (50-10 buffer), and stored at −80 °C. To prepare membranes, the cells were resuspended in a volume of 50-10 buffer containing 1 mM phenylmethylsulfonyl fluoride equal to 10 times their wet weight, then disrupted by passage through a French pressure cell at 20,000 p.s.i. After removal of cell debris by centrifugation for 10 min at 10,000 rpm in a Beckman JA-20 rotor, the supernatant was centrifuged for 2 h at 38,000 rpm in a Beckman Ti50 rotor. The pellet was washed once with 50-10 buffer containing 1 mM dithiothreitol and once with 50-10 buffer lacking dithiothreitol, and then the membranes were suspended in 50-10 buffer to a concentration of about 30 mg of total protein/ml and stored at −80 °C.

**Chemical Cross-linking of Membranes**—Cross-linking of b1109C and bE110C to F$_1$, in membrane preparations was carried out as described previously (10), except that 0.5 mM p-azidophenacyl bromide (APB) (Sigma) dissolved in methanol was used instead of 1 mM benzophenone-4-maleimide (BPM). Cross-linking of bA92C to F$_1$ in membrane preparations was also carried out essentially as described (10), with the following exceptions. Membranes from which F$_1$ had been removed were diluted to 1 mg of total protein/ml, and then BPM (Molecular Probes, Eugene, OR) was added as described. After the 30-min BPM incubation, excess maleimide groups were quenched by adding 2 mM β-mercaptoethanol. After a 5-min incubation, purified F$_1$ was added to a final concentration of 22.9 μg/ml and the membranes were allowed to stand for 10 min. The membranes were then exposed to ultraviolet light and analyzed by SDS-PAGE and Western blotting.

**RESULTS**

Cross-linking of a92c to F$_1$—Previously, we expressed the hydrophilic domain of the b subunit, consisting of residues 24–156, as a protein called b$_{24–156}$. This protein is soluble, exists as a dimer, and contains no endogenous cysteine residues (28). To explore its interactions with the F$_1$ sector, we introduced cysteine residues at various positions within b$_{24–156}$ to allow site-specific modification by heterobifunctional cross-linking agents such as BPM. Upon addition of F$_1$ to the modified b$_{24–156}$ cross-linking was induced by ultraviolet light, and the products were analyzed by SDS-PAGE and Western blotting. When cross-linking of the soluble domain of b in α or β was observed, the mutation was inserted into the full-length b subunit for analysis in membrane-bound ATP synthase. Only those mutations which yielded cross-links in the latter system are represented here.

When b$_{24–156}$ bearing the A92C mutation was modified with BPM, reconstituted with F$_1$ and exposed to ultraviolet light, one major cross-linked band appeared (Fig. 1A). This product was an appropriate size for b$_{24–156}$ or b$_{562}$. On Western blots the cross-linked product was recognized by both anti-b and anti-α antibodies, but recognition by an anti-β antibody was poor (Fig. 1B). In order to test the cross-linking in F$_1$F$_0$, a plasmid encod...
Cross-linking of b subunit with the A92C mutation was used to complement KM2, an uncF (b subunit) deletion strain of E. coli (29). Membranes from these cells were prepared, modified with BPM, and exposed to ultraviolet light. No cross-linking was observed (not shown). However, removal of F1 from the membranes by treatment with low ionic strength buffer followed by modification of the membranes with BPM, re-addition of F1 to the membranes, and exposure to ultraviolet light, gave rise to new bands that were recognized by antibodies directed against both a and b as well as b (Fig. 1C). Controls demonstrated that the cross-linking did not occur in the absence of either the cross-linker or ultraviolet light, or with wild type b (not shown). The fact that b was linked to both a and b suggests that position 92 of b is near an interface between the a and b subunits. An additional band appeared after cross-linking that was recognized by antibodies against a (Fig. 1C) and d (not shown). This a-d cross-link probably arose due to reaction of the cross-linker with residual F1 that was not released from the membrane during the stripping step.

Cross-linking of I109C and E110C to F1—The mutations I109C and E110C were incorporated individually into b24–156, and these proteins were purified, modified with the cross-linker APB, reconstituted with purified F1, and exposed to ultraviolet light. This treatment gave rise to cross-linked bands that migrated in positions consistent with a-b or b-b; only the result for the I109C mutation is shown (Fig. 2A). The cross-linked proteins were recognized by antibodies against a (Fig. 2C) and d (not shown). The I109C and E110C mutations were transferred into a plasmid expressing the full-length b subunit, and membranes containing ATP synthase were prepared. The samples were

![Fig. 1. Cross-linking of bA92C to F1-A](image1)

![Fig. 2. Cross-linking of I109C in b24–156 to F1-A](image2)
Cross-linking of \(b\) in E. coli ATP Synthase

**Analysis of the A92C Cross-linked Product by Mass Spectrometry**—The cross-linked product observed in the \(b_{24–156}\) complex with the A92C mutation (Fig. 1) was analyzed by matrix-assisted laser desorption ionization time of flight mass spectrometry in an attempt to determine which regions of the \(a\) and \(b\) subunits were involved in the cross-link. To effect the analysis, the cross-linked product was extracted from a Coomassie Blue-stained gel and digested with trypsin, and the mass spectrum of the tryptic fragments was obtained (this procedure will hereafter be referred to as the “trypsin mass spectrometry” analysis). In theory, assuming there is only one site of cross-linking within the \(a\) subunit, one should observe several regions of these subunits were not represented (Fig. 3). The efficiency of cross-linking appeared to be slightly higher with the \(b_{24–156}\) subunit, both in \(F_{1}F_{0}\) membranes bearing \(F_{1}F_{0}\) containing the I109C mutation (panel A) or the \(b_{E100C}\) mutation (panel B) were modified with APB and exposed to ultraviolet light as described under “Experimental Procedures.” Controls were performed in which the cross-linker or the ultraviolet light were omitted, and using \(F_{1}F_{0}\) containing wild type \(b\). Samples were analyzed by SDS-PAGE followed by Western blotting, probing with \(^{125}\)I-labeled antibodies directed against \(b\) or \(\alpha\).

Modified with APB and exposed to ultraviolet light, resulting in cross-linking between \(b\) and \(\alpha\) in each case (Fig. 3). The efficiency of cross-linking appeared to be slightly higher with the I109C mutation. The cross-linked products were not observed when wild type \(b\) subunit was used or when the cross-linker or ultraviolet light were omitted. These results indicate that positions 109 and 110 of \(b\) are close to the \(\alpha\) subunit, both in \(F_{1}F_{0}\) and in the complex between \(F_{1}\) and \(b_{24–156}\).

**Analysis of the I109C Cross-linked Product by Mass Spectrometry**—Analysis of the I109C cross-linked product obtained using the \(b_{24–156}\) subunit should contain the site of cross-linking. Again, several peaks were observed that did not appear to originate from \(b_{24–156}\) or \(\alpha\), but it was not possible to identify them as cross-linked tryptic fragments.

The A92C cross-linked products were treated with CNBr and analyzed by SDS-PAGE (Fig. 5). Due to the scarcity of methionine residues in the \(b\) subunit, CNBr treatment gives rise to a large fragment, distinguishable from untreated \(b_{24–156}\) by SDS-PAGE, that encompasses residues 31–156 of the protein. This fragment contains the site of cross-linking, and therefore it should be shifted to a higher apparent molecular weight in the cross-linked sample. The cross-linked sample gave rise to a slowly migrating band that must correspond to a partial CNBr cleavage product, resulting in a mass of 1519.75 Da, corresponding to residues 483 of \(b_{24–156}\) and the mass spectrum of the tryptic fragments observed by mass spectrometry. The numbers and open segments denote residues that were not observed by mass spectrometry. A, results obtained from the A92C mutation. B, results obtained from the I109C mutation.

**Fig. 4. Subunit coverage in the trypsin mass spectrometry experiments.** The cross-linked bands obtained using \(b_{24–156}\) containing the A92C mutation (see Fig. 1) or the I109C mutation (see Fig. 2) were extracted from SDS-polyacrylamide gels, treated with trypsin, and analyzed by mass spectrometry. The results are depicted on rectangles, which represent the amino acid sequences of \(b_{24–156}\) \(\alpha\), or \(\beta\) as indicated. Regions found among the tryptic fragments observed by mass spectrometry are denoted by filled segments. The numbers and open segments denote residues that were not observed by mass spectrometry. A, results obtained from the A92C mutation. B, results obtained from the I109C mutation.

1B), simple tryptic digestion of the bands gave rise to fragments originating from \(b_{24–156}\) \(\alpha\) and \(\beta\) (Fig. 4A), indicating that cross-linker at position 92 formed links to both the \(\alpha\) and \(\beta\) subunits. Fragments representing all residues of \(b_{24–156}\) were observed in the mass spectrum with the exception of the region containing the cross-linker (Ser-84 to Lys-100). Tryptic fragments corresponding to most residues of \(\alpha\) and \(\beta\) were observed, but several regions of these subunits were not represented (Fig. 4A). If one assumes there was a single site of cross-linking on each of \(\alpha\) and \(\beta\), then one of these unobserved regions from each subunit should contain the site of cross-linking. Again, several peaks were observed that did not appear to originate from \(b_{24–156}\) \(\alpha\) or \(\beta\), but it was not possible to identify them as cross-linked tryptic fragments.

**Analysis of the I109C Cross-linked Product by Mass Spectrometry**—Analysis of the I109C cross-linked product obtained using the I109C mutation (Fig. 2) by the trypsin mass spectrometry procedure described for the A92C cross-link revealed tryptic fragments originating from both \(b_{24–156}\) and \(\alpha\) (Fig. 4B). Fragments representing all residues of \(b_{24–156}\) were
observed except for the region between residues Ile-101 and Arg-117, which contains the site of cross-linking. Several regions of α were not represented among the tryptic fragments (Fig. 4B). These unobserved regions may contain the site(s) of cross-linking on the α subunit. Several peaks were observed in the mass spectrum that did not correspond to any tryptic fragment from either b24–156 or α. However, we were unable to conclusively match any of these peaks with the calculated masses of possible cross-linked trypsin fragments.

In an attempt to gain more information about the I109C cross-link, an excised gel slice containing the cross-linking product was treated with CNBr and subjected to SDS-PAGE. Unmodified b24–156 and α were treated in the same way for comparison. Unfortunately no bands were observed with the cross-linked product that were not observed with the unmodified b24–156 and α (not shown).

Cross-linking of R36C in F1F0—Arg-36 is conserved in b subunits of ATP synthase from many species (43, 44), and certain mutations at this position in E. coli ATP synthase cause functional defects in the enzyme (44). Because a transmembrane domain exists at the N-terminal end of b, the region of the subunit around residue 36 may be close to the surface of the membrane and may make important contacts with subunits of F0. To explore the interactions of the b subunit with the F0 sector, we introduced a cysteine residue at position 36 and attempted cross-linking with BPM. The mutation was made in the plasmid pACWU1.2 (32), which expresses a form of ATP synthase in which all endogenous cysteine codons have been mutated to alanine. The bR36C plasmid was able to complement the unc strain DK8 when tested for growth using succinate as the sole carbon/energy source, although the growth rate of these cells was reduced relative to cells complemented with pACWU1.2. This finding is consistent with the observations of Caviston et al. (44) using the bR36C mutation.

Membranes bearing ATP synthase containing the bR36C mutation were treated with BPM and exposed to ultraviolet light. A cross-linked band migrating with an apparent molecular mass of 47 kDa was recognized by a monoclonal antibody against the b subunit (lower arrow in Fig. 6). The cross-linked band was not observed in the absence of cross-linker or ultraviolet light, or when the bR36C mutation was not present. The apparent size of the cross-linked product was too large for a b-b cross-link, but was consistent with linkage of b to either the α subunit of F0 or else the γ subunit of F1. Either one of these potential linkages would be consistent with current knowledge of the enzyme structure. Two experimental results that are not shown suggested that linkage to γ was unlikely. First, the cross-link was not recognized by reliable anti-γ antibodies (41, 42), and second, stripping the F1 from membranes did not prevent subsequent formation of the cross-link. Unfortunately, we were also unable to detect the α subunit on blots probed with an anti-a antibody obtained from a collaborating laboratory.

To obtain convincing evidence that the cross-link was between the b and α subunits, the gene for E. coli cytochrome b562, a soluble, cysteine-less protein, was fused in-frame to the 3′ end of the uncB gene, which encodes the α subunit. The molecular mass of the product of this gene fusion was calculated to be 42 kDa, as compared with 30 kDa for wild type a. The fused gene was incorporated into pACWU1.2 with either uncF having the normal arginine at position 36 of b, or else with the bR36C mutation. The presence of the cytochrome b562 fused to the C terminus of α did not affect the ability of ATP synthase to support growth of transformed cells of strain DK8 using succinate as the carbon/energy source. When membranes bearing both the a-cytochrome b562 fusion protein and the bR36C mutation were treated with cross-linker and ultraviolet light, a new band with an apparent molecular mass of 65 kDa (upper arrow in Fig. 6) replaced the 47-kDa band on Western blots probed with antibodies directed against the b subunit. This band did not appear in the absence of ultraviolet light, the cross-linker, or the bR36C mutation. The shift in the size of the cross-linked species observed with the subunit a-cytochrome b562 fusion confirms that the cysteine residue introduced at position 36 of b can be cross-linked to the α subunit in F1F0.

**DISCUSSION**

In the work described here, chemical cross-linking was used to identify sites within the b subunit of E. coli ATP synthase that interact with the α, β, and α subunits. The results indicate that the b subunit makes contacts with α through the region.
spans residues 92–110, along a non-catalytic α/β interface, and with α at a position 10 residues removed from the membrane-spanning domain of b.

Modification of a cysteine introduced into b_{24-159} at position 92 with BPM resulted in cross-linking within the region Ile-464 to Met-483 of α. The activated benzophenone is reactive to methylene groups, particularly those adjacent to nitrogen or sulfur, and the cross-linking span of BPM is about 10 Å (45). For comparison, F1 is about 100 Å across and 80 Å high, not sulfur, and the cross-linking span of BPM is about 10 Å (45).

Although the location of the cross-link between modified I109C and α is within one of the regions 212–250 or 102–106. In particular, the residues corresponding to E. coli α-(213–220) (white in Fig. 7) are solvent-exposed near the non-catalytic α/β interface, approximately 25 Å above the site of cross-linking between bA92C and α. Residues 102–106 (yellow in Fig. 7) are also positioned at the surface of the α subunit, slightly further away from the site where the cross-link to bA92C was formed. The other regions implicated in Fig. 4B, with one exception, are either not exposed to the surface or were judged to be an inappropriate distance from the site of the bA92C cross-link. The one exception was the region between residues 368 and 391, which is on the surface of the α subunit. However, this region is located at the catalytic interface between α and β, and it seems unlikely that the b subunit crosses over to that interface in this area of the complex.

The location of the bA92C cross-link to α, 55–65 Å from the surface of the membrane assuming a stalk length of 45 Å (46), has implications for the structure of b near the membrane. If the model of residues 53–122 as a continuous α-helix (18) is accepted, and if this helix extends in a relatively straight line directly toward the membrane, then the N-terminal residue of the helix (residue 53) would be close to the surface of the membrane. Since the b subunit is thought to emerge from the membrane around residue 26 (27), there appear to be about 25 more residues than required to span the distance between the membrane and the site of cross-linking at position 92. The presence of these “extra” residues could partially account for the observation that a functional ATP synthase complex could be formed despite deletions of up to 11 residues within the region of residues 50–75 of b (19). In light of our ability to cross-link position 36 of b to the α subunit (Fig. 6), it is probable that at least some of the residues of b between positions 26 and 52 interact with the cytoplasmic loops of α. There are a number of possible functions for these interactions. First, they may be important for anchoring the b subunits to α, maintaining the integrity of the stator complex. Second, given the observation that the b subunit is required for the formation of a functional proton pore upon reconstitution of F_{0}, these regions could play a role in assembly and organization of F_{0}. This idea is further supported by evidence implicating the second stalk in the maturation of F_{0} to a proton-conducting state (49, 50). Finally, the effects of mutations at residue bArg-6 on proton pumping (44) suggest the possibility that the interaction of b...
and $a$ may play a specific functional role, rather than a purely structural role, in coupling.

It is also noteworthy that the positioning of the $b$ dimerization helices as shown in Fig. 7 places residues 80–84, which in many species are predicted to form a bend, at the base of the $\alpha_5\beta_3$ hexamer. A flexible joint at this location would allow some angular movement of $\alpha_5\beta_3$ relative to $a$ and the membrane-integral N-terminal helices of the $b$ subunits, as might be expected during stages of rotation.

The proposed sites of cross-linking imply that the $b$ subunit dimer contacts the $\alpha_5\beta_3$ hexamer near a non-catalytic interface. Recently, a cysteine residue introduced at the C terminus of $b$ was found to form a disulfide bond with the endogenous Cys-90 of $a$ (20); the corresponding residue of the bovine heart mitochondrial crystal structure, Arg-90, lies near the catalytic interface between $a$ and $b$ (see Fig. 7, residue in magenta). The number of residues between the cross-linked positions described here and the C terminus of $b$ is more than sufficient to span the distance from the proposed sites of cross-linking to the top of $F_1$, as an $\alpha$-helix. Previous evidence has suggested that the region of $a$ C-terminal to residue 122 may form a more globular structure (10, 18). Therefore a location of the C-terminal residue of $b$ close to the catalytic interface is consistent with our current picture of the $b$ subunit.

The work presented here suggests a close interaction of the $b$ subunits with $a$, $\alpha$, and $\beta$. It remains unclear what structural perturbations, if any, take place within the $b$ subunit during the course of catalysis. Since the rotary mechanism of catalysis predicts that the second stalk resists considerable torque, elasticity must be expected during stages of rotation.

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