Dimerization Interactions of the b Subunit of the Escherichia coli F1F0-ATPase*

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Site-directed mutagenesis and N-terminal truncations were used to examine dimerization interactions in the b subunit of Escherichia coli F1F0-ATPase. Individual cysteine residues were incorporated into bsyn, a soluble form of the protein lacking the membrane-spanning N-terminal domain, in two main areas; the heptad repeat region and the hydrophilic portion of the F1F0-ATPase* which begins at residue Val-124. The tendencies of these cysteine residues to form disulfide bonds with the corresponding cysteine in the bsyn dimer were tested using disulfide exchange by glutathione and air oxidation catalyzed by Cu2++. Within the heptad repeat region, only cysteines at residues 59 and 60, which occupy the b and c positions of the heptad repeat, showed significant tendencies to form disulfides, a result inconsistent with a coiled-coil model for bsyn. Mixed disulfide formation most readily occurred with the S60C + L65C and A61C + L65C pairs. Cysteines at positions 124, 128, 132, and 139 showed strong tendencies to form disulfides with their mates in the dimer, suggesting a parallel α-helical interaction between the subunits in this region. Deletion of residues N-terminal to either Glu-34 or Asp-53 had no apparent effect on dimerization as determined by sedimentation equilibrium, while deletion of all residues N-terminal to Lys-67 produced a monomeric form. These results imply that residues 53–66 but not 24–52 are essential for bsyn dimerization. Taken together the results are consistent with a model in which the two b subunits interact in more than one region, including a parallel alignment of helices containing residues 124–139.

The F1F0-ATP synthase, or ATPase, is responsible in oxidative phosphorylation and photophosphorylation for the translocation of protons across a membrane with concomitant synthesis of ATP. The enzyme complex consists of a membrane-spanning sector (F0) that conducts protons, and a membrane-peripheral sector (F1) that uses energy from proton movement to synthesize ATP (for reviews, see Refs. 1–4). The mechanism by which energy coupling from F0 to F1 takes place is currently being investigated. Some believe that the c subunits are clustered together with a and b on the periphery (5–7) while others propose that a and the two b subunits are surrounded by a ring of c subunits (8, 9). b has a single transmembrane region at its N terminus; the remainder of the 156-residue protein is hydrophilic with the exception of a short stretch of hydrophobic amino acids at residues 124–132. Based on its sequence the protein is predicted to be largely a-helical in nature, and a heptad repeat has been discerned between residues 33 and 79, suggestive of a coiled-coil interaction in this region. Previously one of us has constructed a gene encoding the hydrophilic portion of b(10). The product of this gene, bhol, is soluble, forms a dimer in solution, is mostly a-helical as measured by circular dichroism, and competes with the F0 sector for binding to F1. Therefore it is likely that bhol adopts a structure similar to the hydrophilic region of full-length b.

Proteolysis studies have shown that the hydrophilic portion of b is required for the binding of F1 to the membrane (11–13), and mutation of the glycine at position 131 to aspartate prevents proper assembly of the F1F0 complex (14). Therefore the b subunit provides a critical link between the two sectors, but its role in energy transduction has yet to be determined.

Two regions of b have been suggested to play a role in its dimerization: the heptad repeat region (residues 33–79; Ref. 10) and the hydrophilic region near the C terminus (residues 124–132; Ref. 15). To investigate these regions, and to obtain information about their relative orientation, we have introduced individual cysteine mutations into the soluble hydrophilic portion of b. By estimating the relative propensities of cysteines introduced at various positions to form an intersubunit disulfide bond, one may gain insight as to the proximity of the residues. In addition, several forms of b that have been truncated at the N terminus were analyzed by ultracentrifugation to clarify the role of the N-terminal region in dimerization.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—Molecular biological techniques were performed essentially as described in Sambrook et al. (16). Strain JM103 of E. coli (17) was used as the host for plasmids based on pUC8. Strain MM294 was used as the host for plasmids based on pSD80. The uncF strain KM2 (18), generously provided by Kimberly McCormick and Brian Cain, is based on strain 1100 and was used for expression of full-length b constructs.

To construct the gene encoding bsyn, four pairs of overlapping single-stranded synthetic oligonucleotides were made double-stranded by annealing and subsequent treatment with Klenow fragment. The oligonucleotides were inserted one at a time into the multiple cloning region of pUC8 (19) to produce pDM3. The sequence of the gene was verified by DNA sequencing using the Sanger method. Cysteine mutations were incorporated into pDM3 by two different methods. In some cases, degenerate oligonucleotides with the potential to encode mutations at several sites were synthesized and incorporated into the plasmid. For other mutations, mutagenic primers were used directly in vivo.

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along with either the forward or reverse M13/prl sequencing primer in polymerase chain reactions; the polymerase chain reaction products were incorporated into pDM3 using appropriate restriction enzymes. The resulting E. coli transformants were screened for expression of bsyn containing cysteine as described previously (20). DNA sequencing by the dideoxynucleotide method was used to verify the sequence of the entire mutagenic insert.

Plasmid pSD80 (21) carries a tac promoter upstream of the multiple cloning site, an unc transcription terminator downstream of the HindIII site at the end of the multiple cloning region, and the lacI* gene elsewhere on the plasmid. The single MscI site of pSD80 was removed by cutting with that enzyme and ligating the unique SacI site to produce plasmid pSD82, which served as the basis for pSD100.

The unc sequence inserted in pSD51 (10) spans residues A-2144 through C-2940, numbered as in Ref. 22, beginning 43 bases upstream from uncF, which encodes b, and extending about halfway through the next gene, uncH. Polymerase chain reaction mutagenesis was used to amplify this entire insert from pSD51 and to introduce a translation initiation region and an MscI restriction site upstream of the unc sequence. The product was cloned into pSD82, using the EcoRI and HindIII site to yield plasmid pSD84. Subsequent replacement of part of the non-unc upstream sequence with a synthetic double-stranded fragment gave plasmid pSD100, which contains the following sequence beginning at the EcoRI cloning site: GTAATGCGAGGATTTAAGGTCCTAAGCTGATACCC. With an alanine-Dalargno sequence and a start codon (both shown in bold) for a peptide beginning with amino acid sequence MSYWPEGLT. Within the sequence encoding the peptide is a series of sites specific for restriction endonucleases MscI, SacI, and KpnI. The MscI site (underlined), which was used extensively in subsequent work, spans the codons for the tryptophan and proline residues. The sequence shown is linked to unc residue A-2144 located upstream from the uncF start codon.

Plasmid pSD100 was originally designed for the production of plasmids encoding N-terminal deletions of b through the use of exonuclease III. In the current work, however, it was used for two different purposes. First, the MscI and HindIII sites were used for subcloning of segments of uncF to produce plasmids pKK1, pSD111, and pSD114, which have in-frame fusions between the leader sequence and the inserted uncF sequence. In the construction of plasmid pKK1, which encodes b395–150, the 578-base pair PvuII-HindIII fragment of pSD59 (10) was inserted. The 322-base pair insert used for the construction of pSD111, which encodes b393–150, was obtained by cutting pDM3 with BglII, filling the end with Klenow fragment, then cutting with HindIII. The 373-base pair fragment used for the construction of pSD114, which encodes protein b360–150, was obtained by cutting pDM3 with MfeI, removing overhanging bases with mung bean nuclease, then cutting with HindIII. Constructs were verified by restriction enzyme mapping and, for pSD111 and pSD114, sequencing through the site of the in-frame fusion. The protein products were shown to be recognized by b-specific monoclonal antibodies.

Second, the plasmid pDM8, expressing the entire b subunit, was generated by subcloning the SnaBII-HindIII fragment of pDM5 into pSD100. Thus pDM8 carries the wild type uncF gene sequence from the initiation codon to the SnaBI site, and the synthetic gene sequence from the SnaBI site to the termination codon. The mutations R138C and S139C were subcloned individually from their pDM3-like plasmids into pDM8. Both mutants were expressed from the synthetic gene sequence from the HindIII site to the termination codon. The mutations R138C and S139C were subcloned individually from their pDM3-like plasmids into pDM8. Both mutants were expressed from the synthetic gene sequence from the HindIII site to the termination codon.

Protein Purification—The expression and purification of bsyn was carried out essentially as described earlier for b393–150, with the exception of the final stage. In this step, the protein was loaded onto a column of DEAE-Sepharose instead of a column of Sepharose 4-B-300, and was eluted with a linear gradient of 0–250 mM NaCl in 250 mM of 25 mM imidazole-HCl, pH 6.4, 1 mM EDTA. The resulting column fractions containing bsyn were typically pooled, concentrated using an Amicon Diaflo apparatus with a YM-10 membrane, dialyzed against 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and stored at -80 °C. Purification of mutant proteins containing cysteine was identical except that 1 mM dithiothreitol (DTT)1 was added to all buffers after the 40% (NH4)2SO4 precipitation step.

bSyn, which is expressed from plasmid pSD114, was purified by procedures similar to those used for b393–150, except that the protein was precipitated with 45% saturated ammonium sulfate, and size exclusion chromatography on a column of Sepharose S-200 was added as a final step. Protein b393–150, which is expressed from plasmid pSD111, was purified by procedures similar to those used for bsyn, except that the protein was precipitated with 45% saturated ammonium sulfate, and that this was followed by a pH precipitation at pH 5.1 before DEAE-Sepharose chromatography. The precursor of protein b53–156 was expressed from plasmid pKK1, was found in the pellet from the initial ultracentrifugation. The pellet was resuspended in buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, stored at 4 °C overnight, and the ultracentrifugation was repeated. Analysis of the supernatant and pellet fractions by SDS-PAGE revealed the protein to have been completely solubilized with a slight increase in electrophoretic mobility. The protein was precipitated with 90% saturated ammonium sulfate, then purified by ion exchange chromatography on DEAE-Sepharose and size exclusion chromatography on Sephadex G-75. The protein was followed by SDS-PAGE and by absorbance at 280 nm, as it lacked absorbance at 280 nm.

Disulfide Bond Formation Induced by Glutathione (GSH)—Oxidized and reduced glutathione were purchased from Sigma. Samples of the proteins to be tested were converted to the fully oxidized form by dialysis at 4 °C for 24 h in buffer containing 0.1 mM NaHCO3 and 10 mM CuCl2. The buffer was then changed by dialysis to 0.1 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, 1 mM EDTA (glutathione reaction buffer, GRB). Other samples, to be tested in parallel, were not oxidized with CuCl2 but were converted to the fully oxidized form by dialysis at 4 °C for 1 h in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT. The protein concentrations of all samples were measured by the Bradford assay (23), and were adjusted to a uniform level (usually in the range of 0.5–1.0 mg/ml or 32–64 μM). In cases where two proteins containing different mutations were used, the proteins were first adjusted to the same concentration, and then equal volumes were mixed. To the pre-oxidized samples were added 2 volumes of a glutathione mixture containing 15 mM GSSG and 12 mM GSH. To the pre-reduced samples were added 2 volumes of a mixture containing 13.75 mM GSSG and 11.25 mM GSH. The different ratios of GSSG:GSH allow for reduction of GSSG by the 1 mM DTT present in the pre-reduced samples; the final ratios were identical at GSSG:GSH = 1.08:1. The reactions were incubated at room temperature. At various time points (e.g. 30 min, 1 h, 2 h, 4 h and 4 h), 15-μl aliquots were quenched with 10 μl of 0.1 M N-ethylmaleimide (NEM), diluted into SDS-PAGE sample buffer lacking reducing agent, and heated to 100 °C for 5 min. A zero time point was prepared by quenching an aliquot before the glutathione mixtures had been added. All samples were analyzed on SDS-PAGE under nonreducing conditions.

Disulfide Bond Formation Induced by Air Oxidation Catalyzed by CuCl2—A concentration of partially or fully purified proteins were adjusted to uniformity (typically 0.5–1.0 mg/ml) with 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT. The partially purified proteins had been purified up to and including the 40% (NH4)2SO4 precipitation stage (see Ref. 10). The proteins were dialyzed at 4 °C in 1 liter of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT overnight. As a zero time point, 5-μl aliquots were then added to 45 μl of SDS-PAGE sample buffer containing 15 μM CuCl2 and heated to 100 °C. The dialysis bags were transferred to 1 liter of 0.1 M NaHCO3, 10 mM cysteine, 10 μM CuCl2; in some cases the cysteine was omitted. The bicarbonate buffer was always prepared immediately before use. Five-μl aliquots were taken and quenched as described above after 2, 4, 6, 8, 24, and 48 h of dialysis, then analyzed by non-reducing SDS-PAGE. Results obtained by this procedure were similar to those seen with the GSH/GSSG method provided the free cysteine was present; in its absence all mutant proteins containing cysteine residues exhibited essentially complete disulfide formation after 24 h. Substitution of 0.1 M Tris-HCl, pH 7.5, 0.1 mM NaCl for the 0.1 M NaHCO3 did not affect the results obtained.

Expression of Full-length b Subunit and Partial Purification of Membranes—E. coli K12 bearing pMD112 or pMD113 was grown in 1 liter of Luria broth at 37 °C with shaking. When A600 reached 0.060, isopropl β-d-thiogalactopyranoside was added to 15 μM, and growth was continued until A600 reached 0.800. The cells were harvested by centrifugation, washed with 50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, and stored at -80 °C. The cells were resuspended in a volume of 50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride equal to 10 times the original weight, then disrupted by passage through a French pressure cell at 20,000 p.s.i. After centrifugation for 10 minutes in a Beckman JA-20 rotor, the supernatant was centrifuged for 2 h at 38,000 rpm in a Beckman Ti-50 rotor. The pellet was resuspended in 10 mM triethanolamine-H2SO4, pH 7.5, 10 mM EDTA, 10% glycerol and centrifuged at 38,000 rpm as before. The pellet was resuspended in 50 mM triethanolamine-HCl, pH 7.5, 5 mM MgCl2, 10% glycerol, and centrifuged at 21234
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**RESULTS**

**Construction of b<sub>syn</sub> and Mutagenesis—**The b subunit is an integral membrane protein, with a single membrane-spanning region at the N terminus. To facilitate study of this protein, the sequence encoding the entire subunit except for the N-terminal 24 amino acids has been previously subcloned into pUC8 to produce the plasmid pSD59; the polypeptide expressed has been called b<sub>sub</sub>. To make the introduction of mutations into b<sub>sub</sub> easier, a gene containing numerous silent mutations was designed such that useful restriction endonuclease sites were added. The gene was synthesized as eight overlapping oligonucleotides and inserted into pUC8 by the IODO-GEN method (28). The amino acid sequence of b<sub>sub</sub> is based on the full-length E. coli b<sub>sub</sub> sequence. The DNA sequence of the synthetic gene encoding b<sub>syn</sub> is given below the DNA sequence; the 9-residue leader sequence is indicated in italics. Numbering is based on the full-length b<sub>sub</sub> subunit, starting at residue Tyr-24. Residues that were mutated to cysteine appear in bold type.

 Ala-128 has been implicated in b dimerization interactions (15). Each residue in the “VAILAVA” region (positions 124–132) has also been mutated to cysteine to examine this region. In addition, three residues toward the C terminus were mutated (positions 138, 139, and 146). Note that since the b<sub>syn</sub> polypeptide exists as a dimer in solution, the introduction of a single cysteine mutation into the gene results in the presence of two cysteines in the dimer. Throughout this paper we will refer to the position of the cysteine residue in one subunit as n and the position of the cysteine in the second subunit as n′.

**Disulfide Bond Formation Near the C Terminus—**One convenient method used to induce disulfide bond formation is incubation with excess glutathione (31, 32). Glutathione (GSH/GSSG) acts as a disulfide buffer and as a disulfide exchange reagent; after one cysteine residue forms a mixed disulfide with glutathione, a second nearby cysteine can displace it, forming a protein-protein disulfide bond. The second cysteine will not be able to react if it is too far away or has poor geometry for disulfide formation. Eventually an equilibrium is reached; if the disulfide formed of the protein predominates at equilibrium, then one may infer that the two cysteines are close together. Equilibrium can be assumed if the same result is obtained regardless of whether the proteins started in a fully reduced or a fully oxidized state.

The glutathione procedure is illustrated for b<sub>syn</sub> polypeptides containing cysteines in positions 128, 138, and 139, or 146 (Fig. 2). The proteins were incubated with a glutathione mixture (GSH/GSSG = 1:1) as described under “Experimental Procedures.” At various time points aliquots were reacted with excess NEM to modify all thiol groups present and thus prevent further disulfide exchange. Each polypeptide was reacted in duplicate, being either mostly oxidized or mostly reduced at the start of the experiment (Fig. 2A). Comparison of the band intensities after 2 h of incubation (Fig. 2B) revealed that equilibrium had been attained. The proteins containing the A128C and S139C mutations showed a strong tendency to dimerize.
buffer lacking reducing agent, and analyzed by SDS-PAGE. Samples of each protein were quenched with NEM, added to sample m

\[ A128C \quad R138C \quad S139C \quad S146C \quad \text{wt} \]

- \( (b_{\text{syn}})_2 \)
- \( b_{\text{syn}} \)

94 kDa - 67 kDa - 43 kDa - 30 kDa - 20.1 kDa - 14.4 kDa

while cysteines at positions 138 and 146 did not tend to form disulfides under these conditions. These results imply that positions 128 and 128' are close together in the quaternary structure of the dimer, as are positions 139 and 139', suggesting a parallel interaction between the b_{\text{syn}} subunits in this region. In contrast, cysteine residues at positions 138 and 138' lack either proximity or proper orientation, as do the residues at 146 and 146'. It is evident from Fig. 2 that it is possible to observe a wide range of propensities for disulfide bond formation using this procedure. Note that because equilibrium was reached, the lack of disulfide formation at positions 138 and 146 cannot be due to inaccessibility of the residues to the glutathione.

**Disulfide Formation within the Heptad Repeat Region—**A similar experiment was performed with b_{\text{syn}} proteins containing mutations at positions 59–65 or 68 in the heptad repeat region; results are shown for samples which began in the fully oxidized form (Fig. 3). Of these positions, the A59C (heptad b position) and S60C (heptad c position) proteins showed the highest tendency to form disulfides; however, they did not form nearly as completely as the A128C or S139C proteins. Cysteines in the other heptad positions had poor propensities to form disulfides (Fig. 3). In a coiled-coil domain, cysteines at the \( d \) positions of the heptad repeat (residues 61 and 68) would be expected to show the highest tendency to form disulfide bonds, since such bonds at these positions perturb the residue orientation and secondary structure the least (33). The result that the \( b \) and \( c \) positions in this region of b_{\text{syn}} showed the highest propensities to form disulfide bonds is inconsistent with the presence of a coiled-coil structure, in which predominantly hydrophobic residues in the \( a \) and \( d \) positions form an interface.

**FIG. 3. Disulfide bond formation in the heptad repeat region.** Purified b_{\text{syn}} proteins containing the mutations indicated were dialyzed in a buffer containing either 10 \( \mu \text{M} \text{CuCl}_2 \) or 1 \( \text{mM} \text{DTT} \) and treated with excess glutathione as described under “Experimental Procedures.” After 2 h, the reactions were quenched with excess NEM and samples were analyzed by nonreducing SDS-PAGE. It was verified that the reactions had reached equilibrium (not shown); only the proteins that had been dialyzed in the presence of \( \text{CuCl}_2 \) (i.e. were oxidized before glutathione treatment) are shown here. The position of each mutation in the heptad repeat pattern is indicated.

**Mixed Disulfide Formation in the Heptad Repeat Region—**To investigate the relationship between the subunits of the b_{\text{syn}} dimer, proteins containing single cysteine mutations in the region 59–65 were mixed in pairs representing all possible combinations of two. Glutathione was added as described earlier, and the reactions were quenched with NEM after 2 h. Selected results are shown (Fig. 4); in most cases, mixed disulfide formation did not occur to an extent greater than that seen for the individual mutations by themselves (e.g. S60C + A61C, A61C + D63C, and D63C + L65C in Fig. 4). The highest level of disulfide formation was observed with the S60C + L65C and A61C + L65C combinations. Since the proteins were pure before they were mixed, this observation implies that exchange of subunits between b_{\text{syn}} dimers must have taken place during the course of the reaction. In addition, the result suggests that residue 65 of one subunit is close to residues 60 and 61 of the other, while other combinations of positions in this region are not as close or of correct geometry for disulfide bond formation.

**Cu\textsuperscript{2+}-catalyzed Disulfide Bond Formation—**Air oxidation catalyzed by Cu\textsuperscript{2+} (34, 35) was selected as a complementary method of gauging tendencies to form disulfide bonds. For this technique the proteins of interest were first dialed in the presence of 1 \( \text{mM} \text{DTT} \); the dialysis bags were then transferred to an open beaker of buffer containing 10 \( \mu \text{M} \text{CuCl}_2 \) and 10 \( \text{mM} \) free cysteine. The formation of intersubunit disulfide bonds was then followed over a 48-h period by periodically quenching aliquots of the samples with NEM followed by non-reducing SDS-PAGE. Analysis by this method, when applied to a number of mutations examined in Figs. 2 and 3, produced results consistent with those obtained by incubation with glutathione (data not shown).

The Cu\textsuperscript{2+} dialysis technique was used to test partially purified b_{\text{syn}} proteins carrying single cysteine mutations in positions 124–132 or in position 138 (Fig. 5). At the zero time point, after dialysis in the presence of DTT, the b_{\text{syn}} proteins were completely reduced (Fig. 5A). After 2 h of dialysis in the presence of 10 \( \mu \text{M} \text{CuCl}_2 \) and 10 \( \text{mM} \) free cysteine, the most complete disulfide bond formation was observed at positions 124, 128, and 132 (Fig. 5B); the relative levels of oxidation did not change significantly upon further dialysis (not shown). The 4-residue periodicity of cross-linking is striking and suggests a parallel \( \alpha \)-helical arrangement in this region.

**Disulfide Bond Formation in Membrane-bound b—**The full-length b subunit carrying either the R138C or the S139C mutation was expressed from a plasmid in cells of strain KM2, which has a chromosomal deletion of the uncF gene (18). The
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The level of induction was such that the amount of b expressed from the plasmids was almost the same as that expressed from the chromosome of 1100 cells, as analyzed by Western blotting (data not shown). Membranes from these cells were prepared such that the F<sub>1</sub>F<sub>0</sub>-ATPase complex should remain intact, and were then treated with 10 µM CuCl₂. At various times, aliquots were quenched with NEM and analyzed by Western blotting (Fig. 6A). The wild type b subunit contains one cysteine residue (Cys-21), but this residue did not form disulfides under these conditions (Fig. 6A). Some disulfide formation was observed in the R138C protein after only 1 h of incubation, but the level of dimer did not increase with time. The S139C protein showed a consistent increase in the amount of disulfide formed, with most of the protein in the disulfide state after 4 h of incubation. To investigate the loss of signal in the S139C protein with increasing time, the samples were reduced with DTT and blotted again (Fig. 6B). After reduction, the intensity of the S139C band returned to its original level, implying that the antibody used does not recognize the disulfide-bonded form as efficiently as the monomer. Thus it is likely that the actual ratio of oxidized:reduced S139C in Fig. 6A is higher than is apparent from the relative band intensity. A 9.5-fold longer exposure of the blot shown in Fig. 6A revealed no cross-linking of b to any other proteins (data not shown).

Sedimentation Analysis of b<sub>syn</sub> and N-terminal Truncations—Previously we reported the characterization of b<sub>syn</sub> as a 31-kDa dimer by sedimentation equilibrium analysis (10). This has been confirmed with b<sub>syn</sub>, but we have observed that inclusion of data up to an A<sub>280</sub> of 0.8, which corresponds to a protein concentration of 1.6 mg/ml, resulted in an apparent molecular weight of 34,200 and a pattern of residuals indicative of aggregates at higher concentrations (data not shown). In contrast, analysis of nine data sets which were limited to concentrations below 0.7 mg/ml gave an average molecular weight of 30,600 (Table I) and random patterns of residuals.

Plasmids encoding three forms of the protein with additional residues deleted from the N terminus were constructed as described under "Experimental Procedures." In protein b<sub>34–156</sub>, the hydrophobic sequence Y<sup>24</sup>WPLMAI<sup>33</sup> was removed and a different leader sequence, MSYW, was placed at the N terminus of protein b<sub>53–156</sub>, which lacked both the hydrophobic sequence noted above and also the first portion of the heptad repeat sequence in the polar domain. Both of these proteins behaved...
similarly to $b_{\text{syn}}$ during purification and both existed as dimers at relatively low concentrations (Table I), while slight aggregation was observed at higher concentrations (data not shown).

The sedimentation coefficients of the proteins were determined in sedimentation velocity experiments at 20 °C, corrected to pure water, and used to calculate frictional ratios by standard methods. Clearly, removal of the N-terminal sequence caused the protein to become less asymmetric, although a frictional ratio of 1.63 is still high for a globular protein.

The precursor of protein $b_{67–156}$ was expressed with the same leader as $b_{34–156}$ and $b_{53–156}$, but its behavior and properties were quite different. Absorbance at 280 nm was lost during purification, indicating proteolytic removal of the entire leader sequence from the N terminus. The molecular weight of the purified polypeptide was determined by electrospray mass spectrometry to be 9,989.6, and N-terminal analysis by dansylation data. Also, the ability of residues 60 and 61 to form disulfides with residue 65 suggests that the residues could be spatially close but not tightly interacting; if so, the molecular dynamics of protein structure might frequently bring the cysteines into correct orientation for disulfide formation in $b_{\text{syn}}$.

When proteins containing cysteine residues at different positions were mixed, the strongest disulfide formation occurred between positions 60 and 65, and positions 61 and 65 (Fig. 4). The fact that disulfide formation occurred to a greater extent in the mixtures than in the pure proteins implies exchange between $b_{\text{syn}}$ dimers, and therefore the establishment of a monomer/dimer equilibrium. This equilibrium seems to lie strongly in favor of the dimeric form, as evidenced by the ultracentrifugation data. Also, the ability of residues 60 and 61 to form disulfides with residue 65 more strongly than with their counterparts in the opposing subunit suggests that some structure other than simple parallel and symmetrical $\alpha$-helices (as would be the case in a coiled-coil structure) exists in this region. It is possible that a single helix is present in each dimer, but that these helices are staggered relative to each other; alternatively a less regular structure or a turn may be present between residues 61 and 65.

Among the more C-terminal mutations, disulfide bond formation was strongest at positions 128 and 139 in the glutathione reactions (Fig. 2), and at positions 124, 128, and 132 in the air oxidation experiments (Fig. 5). These results imply that the $b$ subunits interact in a parallel fashion in this region with, for example, the residue in position 124 closely interacting with the residue in position 124. Furthermore, the periodic nature of disulfide bond formation propensities between residues 124 and 132 suggests that two interacting parallel $\alpha$-helices exist in this region. It is especially noteworthy that, among bacterial and chloroplast $b$ subunits, positions 124, 128, and 132 are well conserved (36). When the residues in this region were mutated individually to aspartate, a similar periodic effect on bacterial growth yield and ATPase activity was observed (15). The results obtained with the soluble $b_{\text{syn}}$ protein are supported by the experiment using the entire $b$ subunit in membranes, in which R138C showed a poor tendency to form disulfides while S139C formed disulfides well (Fig. 6). Because R138C disulfide formation achieved its maximal, but low, level after 1 h (Fig. 6A), it is possible that the disulfided protein

**DISCUSSION**

Our results do not support a coiled-coil model for residues 33–79 of $b$. Upon incubation with glutathione the A59C and S60C mutations formed disulfides more readily than any other position tested in the heptad repeat region (Fig. 3). These residues occupy positions $b$ and $c$ of the heptad repeat; therefore a standard coiled-coil they would be on opposite sides of the helices and would not be expected to form disulfides easily. At the same time, the two $d$ position sites tested (mutations A61C and A68C) showed little tendency to form disulfides. In addition, the sedimentation equilibrium ultracentrifugation experiments with N-terminal truncations imply that residues 53–66 are critical for $b_{\text{syn}}$ dimer formation, but that residues 24–52 are not (Table I). If a coiled-coil structure were present, one would expect a gradual shift from dimer toward monomer rather than the abrupt transition caused by removal of residues 53–66. Therefore both the disulfide formation and the sedimentation equilibrium results are inconsistent with the presence of a coiled-coil structure between residues 33–68 of the $b$ subunit.

Interestingly, at none of the positions between 59 and 68 did disulfide bond formation occur to the extent observed for several mutations toward the C terminus (e.g. 124, 128, and 139). This result, as well as the observation that disulfide formation at position 60 does not readily occur in the F$_{F_0}$ complex, raises the question of whether or not positions 59 and 60 actually form tight interactions in the dimer. It seems possible that the residues could be spatially close but not tightly interacting; if so, the molecular dynamics of protein structure might frequently bring the cysteines into correct orientation for disulfide formation in $b_{\text{syn}}$.

When proteins containing cysteine residues at different positions were mixed, the strongest disulfide formation occurred between positions 60 and 65, and positions 61 and 65 (Fig. 4). The fact that disulfide formation occurred to a greater extent in the mixtures than in the pure proteins implies exchange between $b_{\text{syn}}$ dimers, and therefore the establishment of a monomer/dimer equilibrium. This equilibrium seems to lie strongly in favor of the dimeric form, as evidenced by the ultracentrifugation data. Also, the ability of residues 60 and 61 to form disulfides with residue 65 more strongly than with their counterparts in the opposing subunit suggests that some structure other than simple parallel and symmetrical $\alpha$-helices (as would be the case in a coiled-coil structure) exists in this region. It is possible that a single helix is present in each dimer, but that these helices are staggered relative to each other; alternatively a less regular structure or a turn may be present between residues 61 and 65.

Among the more C-terminal mutations, disulfide bond formation was strongest at positions 128 and 139 in the glutathione reactions (Fig. 2), and at positions 124, 128, and 132 in the air oxidation experiments (Fig. 5). These results imply that the $b$ subunits interact in a parallel fashion in this region with, for example, the residue in position 124 closely interacting with the residue in position 124. Furthermore, the periodic nature of disulfide bond formation propensities between residues 124 and 132 suggests that two interacting parallel $\alpha$-helices exist in this region. It is especially noteworthy that, among bacterial and chloroplast $b$ subunits, positions 124, 128, and 132 are well conserved (36). When the residues in this region were mutated individually to aspartate, a similar periodic effect on bacterial growth yield and ATPase activity was observed (15).

The results obtained with the soluble $b_{\text{syn}}$ protein are supported by the experiment using the entire $b$ subunit in membranes, in which R138C showed a poor tendency to form disulfides while S139C formed disulfides well (Fig. 6). Because R138C disulfide formation achieved its maximal, but low, level after 1 h (Fig. 6A), it is possible that the disulfided protein

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**TABLE I**

**Sedimentation analysis of $b_{\text{syn}}$ and N-terminal truncations**

| Protein | Sequence* | Partial specific volume, $v_0$ | Polypeptide molecular weight | Observed molecular weight | Sedimentation coefficient, $s_{20w}$ | Frictional ratio, $f/f_0$ |
|---------|-----------|------------------------------|-----------------------------|----------------------------|--------------------------------------|--------------------------|
| $b_{\text{syn}}$ | TMITNSHY156 | 0.740 | 15,509 | 30,600 ± 2000; $n = 9$ | 1.80S | 1.87 |
| $b_{34–156}$ | SYWE156 | 0.739 | 14,018 | 27,800 ± 1500; $n = 8$ | 1.81S | 1.74 |
| $b_{53–156}$ | SYW65–156 | 0.742 | 11,899 | 25,300 ± 1000; $n = 8$ | 1.70S | 1.63 |
| $b_{67–156}$ | SYWL60–156 | 0.745 | 9,991 | 10,200 ± 530; $n = 6$ | 0.96S | 1.61 |

* Removal of the initiating methionine residue was assumed for all proteins. Residues in parentheses were removed during purification and were excluded from calculations.

* Sedimentation equilibrium data for $b_{\text{syn}}$ were obtained at a rotor speed of 30,000 rpm and initial protein concentrations of 0.48, 0.24, and 0.12 mg/ml; data sets were selected such that the maximal absorbance corresponded to a protein concentration of less than 0.75 mg/ml. Data for $b_{34–156}$ and $b_{53–156}$ were obtained at rotor speeds of 18,000, 25,000, and 30,000 rpm with an initial protein concentration of 0.2 mg/ml; data sets were selected such that the maximal absorbance corresponded to a protein concentration of less than 0.66 mg/ml ($b_{34–156}$) or 0.56 mg/ml ($b_{53–156}$). Data for $b_{67–156}$ were obtained at 241 nm using rotor speeds of 42,000 and 48,000 rpm with an initial protein concentration of 0.66 mg/ml; up to a concentration of 2.9 mg/ml were included in analyses. Data were fit using a single component model, and are presented as the average ± S.E.; $n$, number of determinations.

* Protein concentrations in sedimentation velocity experiments were 1.0 mg/ml for $b_{\text{syn}}$ and $b_{34–156}$, 0.85 mg/ml for $b_{53–156}$, and 0.82 mg/ml for $b_{67–156}$. The latter protein was observed at 235 nm. Values presented were calculated using the second moment method.

* Frictional ratios were calculated using molecular weights of 31,018 for $b_{\text{syn}}$, 28,036 for $b_{34–156}$, 23,798 for $b_{53–156}$, and 9,991 for $b_{67–156}$.

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2. A. J. W. Rodgers, R. Aggeler, M. B. Morris, R. A. Capaldi, and S. M. Howitt, unpublished data.
Dimerization of b Subunit of E. coli F1F0-ATPase

represents a minor fraction of b subunits that were not properly incorporated into the ATPase complex.

The strong tendency of residue 139 to form disulfides, coupled with the similar behavior of cysteines at positions 124, 128, and 132, suggests that an a-helix may extend from residue 124 to 139, forming an important dimerization interface between the subunits. There are hydrophobic residues (isoleucines) at positions 135 and 136 of the E. coli subunit (Fig. 1); position 136 is hydrophobic in almost all of the species listed by Blair et al. (36). Therefore such a helix would have a relatively hydrophobic face that could interact with the corresponding residues on the other b subunit. While we did not observe disulfide bond formation in b syn at position 146, which would lie on the same face of this proposed helix, Rodgers et al.² found a disulfide at this position to form relatively readily in F1F0. These results imply that a conformational change occurs in this region upon the binding of b to F1.

Previously, Howitt et al. (15) observed that an A128D mutation caused reduction of ATPase activity in membranes as well as loss of dimerization of their soluble b protein. Our present results indicate that the 128 and 128‘ positions are close together in the b syn dimer, and therefore it is likely that the effects of the A128D mutation are due to mutual repulsion of the like-charged residues incorporated at a key interaction site. A similar explanation likely holds true for the observation that a G131D mutation in b prevents proper assembly of the F1F0 complex (14).

The present studies shed substantial new light on the structure of the b subunit. Soluble forms of b that lack the transmembrane region form dimers with extended and mostly hydrophobic structures (10, 15). On the basis of the fact that a single mutation (A128D) is able to abolish dimerization of the hydrophobic portion of b, it has been suggested that interactions in the hydrophilic domain are not important in dimerization in the wild type b subunit (9). An end-to-end, rather than a side-by-side, orientation of the soluble dimer was proposed. Furthermore, a model for the structure of b was presented in which the N-terminal transmembrane helices are closely associated but the hydrophilic regions extend away from each other (9). In contrast, the work presented here implies that residues in regions 53–66 and 124–139 are involved in b syn dimer formation, and therefore that dimerization interactions depend on residues distributed throughout the soluble subunits. A model in which a pair of long a-helices extend in parallel toward F1 from the membrane is attractive, especially in light of the steadily decreasing frictional ratios observed upon removal of residues between Val-25 and Asp-53 (Table I), and yet our present results argue against association of these helices in a coiled-coil structure. The C-terminal region appears to form a more globular structure, possibly centered around hydrophobic core helices between residues 124 and 132. Residues in this putative globular domain would provide important dimerization contacts, as well as sites of interaction with F1.

Proteolysis studies have revealed that the hydrophobic portion of the b subunit is required for binding of F1 to the membrane (11–13). Cryo-electron microscopy using b syn and F1 have implied that b interacts with the C-terminal domain of one of the b subunits of F1 (37). These observations suggest that b may play a role in energy coupling between proton translocation in F0 and ATP synthesis in F1. In future studies it will be important to further explore areas of contact between b and F1, as these interactions may give insight as to the functional role played by b in the intact complex.

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