Structure of the Membrane Domain of Subunit \( b \) of the

Escherichia coli \( F_0F_1 \) ATP Synthase

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The structure of the N-terminal transmembrane domain (residues 1–34) of subunit \( b \) of the Escherichia coli \( F_0F_1 \) ATP synthase has been solved by two-dimensional \( ^1H \) NMR in a membrane mimetic solvent mixture of chlo-roform/methanol/H\( _2 \)O (4:4:1). Residues 4–22 form an \( \alpha \)-helical structure. The helical structure is interrupted by a rigid bend in the region of residues 23–26 with \( \alpha \)-helical structure resuming at Pro-27 at an angle offset by 20° from the transmembrane helix. In native subunit \( b \), the hinge region and C-terminal \( \alpha \)-helical segment would connect the transmembrane helix to the cytoplasmic domain. The transmembrane domains of the two subunit \( b \) in \( F_0 \) were shown to be close to each other by cross-linking experiments in which single Cys were substituted for residues 2–21 of the native subunit and \( b-b \) dimer formation tested after oxidation with Cu(II)(phenanthroline)\( _2 \). Cys residues that formed disul-fide cross-links were found with a periodicity indicative of one face of an \( \alpha \)-helix, over the span of residues 2–18, where Cys at positions 2, 6, and 10 formed dimers in highest yield. A model for the dimer is presented based upon the NMR structure and distance constraints from the cross-linking data. The transmembrane \( \alpha \)-helices are positioned at a 23° angle to each other with the side chains of Thr-6, Gln-10, Phe-14, and Phe-17 at the interface between subunits. The change in direction of helical packing at the hinge region may be important in the functional interaction of the cytoplasmic domains.

During oxidative and photo phosphorylation ATP is synthesized by a \( H^+ \)-transporting \( F_0F_1 \)-ATP synthase. In mitochondria, chloroplasts, and eubacteria, the enzyme consists of an \( \alpha \)-helical protein that is likely to span the hydrophobic domain of the lipid bilayer to anchor the largely hydrophilic subunit \( b \) in the membrane. The helical structure is interrupted by a rigid bend in the region of residues 23–26 with an \( \alpha \)-helical structure resuming at Pro-27 at an angle offset by 20° from the transmembrane helix. In native subunit \( b \), the hinge region and C-terminal \( \alpha \)-helical segment would connect the transmembrane helix to the cytoplasmic domain. The transmembrane domains of the two subunit \( b \) in \( F_0 \) were shown to be close to each other by cross-linking experiments in which single Cys were substituted for residues 2–21 of the native subunit and \( b-b \) dimer formation tested after oxidation with Cu(II)(phenanthroline)\( _2 \). Cys residues that formed disulfide cross-links were found with a periodicity indicative of one face of an \( \alpha \)-helix, over the span of residues 2–18, where Cys at positions 2, 6, and 10 formed dimers in highest yield. A model for the dimer is presented based upon the NMR structure and distance constraints from the cross-linking data. The transmembrane \( \alpha \)-helices are positioned at a 23° angle to each other with the side chains of Thr-6, Gln-10, Phe-14, and Phe-17 at the interface between subunits. The change in direction of helical packing at the hinge region may be important in the functional interaction of the cytoplasmic domains.

The mechanism by which proton translocation through \( F_0 \) is coupled to rotary catalysis in \( F_1 \) remains to be elucidated. Subunit \( c \) is believed to play the central role in proton transport via protonation-deprotonation of an essential Asp-61 carboxylate from alternate access channels on either side of the membrane (2). The structure of subunit \( c \), the smallest subunit in \( F_0 \), has been solved by heteronuclear NMR (11), and a ring-like organization of the \( c \) oligomer in \( F_0 \) is believed to be mediated by cross-linking approaches (12, 13). Low resolution electron microscopic and atomic force microscopic images also suggest a ring-like arrangement of the \( c \) oligomer with subunits \( a \) and \( b \) lying at the periphery of the ring (14–16). The placement of subunits \( a \) and \( b \) at the outside of the ring is supported by cross-linking studies (13, 17). To couple \( H^+ \) transport to rotary catalysis in \( F_1 \), \( H^+ \)-flux through \( F_0 \) is proposed to drive rotation of the \( c \) oligomeric ring relative to the stationary subunits \( a \) and \( b \) at the periphery of the complex (9, 18–20). In such a model, subunit \( b \) is proposed to play the role of a stator, holding the \( \alpha \beta \beta \gamma \) complex of \( F_1 \) fixed to the stationary \( F_0 \) subunits as the \( \epsilon \) subunits rotate as a unit. The \( \gamma \) and \( \epsilon \) subunits are known to project below the surface contact with the surface of the \( c \) oligomer in \( F_0 \)(21–23). Recent electron microscopy now indicate a second stalk at the periphery of the \( F_1F_0 \) interface, which is presumed to represent a dimer of \( \beta_2 \) subunits extending from \( F_0 \) to \( F_1 \)(7, 24, 25). Little is known about the structure of subunit \( b \).

Subunit \( b \) is an amphipathic protein of 156 residues. The N-terminal 33-residue segment is highly hydrophobic and the presumed membrane anchor (26). Indeed, residues in this N-terminal segment were readily labeled with 3-(trifluoromethyl)-3-(\( m \)-(\( ^{129} \)F)iodophenyl)diazirine, a lipid soluble, photo-activatable carbene precursor (27). The remainder of the protein is highly hydrophilic and thought to extend from the membrane surface to bind \( F_1 \). The cytoplasmic domain lacking residues 1–24, termed \( b_{\text{sol}} \), has been expressed and characterized. Soluble form shown to bind to \( F_1 \)(28). The cytoplasmic domain has an elongated shape with high \( \alpha \)-helical content and associates to form a homodimer in solution. Dimerization appears to be a necessary prerequisite for \( F_1 \) binding (29, 30). The soluble domain binds to subunit \( \delta \) of \( F_1 \) in solution (31), and interactions between subunit \( b \) and subunits \( \delta \) and \( \alpha \) at the top of the \( F_1 \) molecule have been demonstrated in \( F_1F_0 \)(32). To reach the top of the \( F_1 \) molecule, subunit \( b \) is estimated to extend 110 Å from the surface of the membrane (32). It may be possible to solve the structure of the individual domains of subunit \( b \) by NMR methods as a means of circumventing solubility problems inherent in approaches with deter-
gent solubilization of the whole subunit. The aqueous solution structures of subunit e and portions of subunit δ are already in hand (33, 34), and the structure of subunit c in chloroform/methanol/H₂O solvent agrees well with predictions made from the biochemical and genetic experiments on the protein in situ (2, 11, 13). Further, subunit c retains its function after passage through chloroform/methanol/H₂O solvent when reconstituted into liposomes with subunits a and b (35). Based on its hydrophobicity, the membrane anchoring segment of subunit b was expected to be soluble in the chloroform/methanol/H₂O mixture used in studies of subunit c. We report here on the structure of the membrane anchoring segment of subunit b in chloroform/methanol/H₂O solvent and its possible relevance to the structural organization of the native subunit b dimer in F₀.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—A 34-residue peptide corresponding to the N-terminal sequence of the E. coli subunit b was synthesized at the University of Wisconsin Biotechnology Center on an Applied Biosystems Synergy 432A instrument using Fmoc (N-(9-fluorenyl)methoxy-carbonyl) chemistry. The C-terminal carboxyl was amidinated. The peptide was purified from the crude synthesis mixture by reverse phase high pressure liquid chromatography on a Dynamax C-4 column eluted with a linear 55–72% gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. The identity of the purified peptide was confirmed by amino acid analysis and electrospray mass spectrometry. The final product was judged to be ≥99% pure based on analytical high pressure liquid chromatography.

NMR Spectroscopy—Samples for NMR were 2 mM peptide in either CDCl₃:CD₃OD:Η₂O (4:4:1 by volume) or CDCl₃:CD₃OD:Η₂O (4:4:1 by volume) containing 50 mM NaCl and 1 mM dithiothreitol. The pH of the solution was measured with a glass electrode and adjusted to pH 6.0 without correction for the deuterium isotope effect. DQF-COSY, TOCSY, and NOESY experiments were performed on a DMX-600 spectrometer (Bruker) with a triple axis gradient capability. Double pulse field gradient echo solvent suppression (36) was used for recording TOCSY and NOESY in protic solvent. Magic angle gradients (37) were used for coherence pathway selection and water suppression in DQF-COSY experiments. TOCSY used the DIPSI-2 spin-lock sequence (38) and a mixing time of 75 ms. A mixing time of 160 or 80 ms was used in NOESY experiments. Data was collected using 640 (NOESY, TOCSY) or 800 (DQF-COSY) increments in t₁. Time domain data was also extended in t₁ by linear prediction. Squared sine apodization and zero filling to 2,048 points was applied in each dimension before Fourier transformation. Spectra were processed and analyzed using Felix 95.0 software (Molecular Simulations Inc., Palo Alto, CA) on a Silicon Graphics O₂ computer. Coupling constants (|J_HN,HN|) were calculated from DQF-COSY and NOESY spectra essentially as described (39). The structure was calculated from 275 NOE-derived inter- and intra-residue distance constraints and 20 angle constraints derived from coupling constants. Distance calibration and structure calculation was performed with the DYANA software package (40) by simulated annealing. The MOLMOL program (41) was used for visual analysis of the structure and for preparing molecular graphics figures.

Mutagenesis, Expression, and Cross-link Analysis—A two stage PCR-based mutagenesis procedure was used with plasmid pNOC for the introduction of single cysteine residues between residues 2 and 20 of subunit b (12). Mutagenic primers corresponding to 21–25 base sequence of the sense strand were designed with a single codon changed based mutagenesis procedure was used with plasmid pNOC for the introduction of single cysteine residues between residues 2 and 20 of subunit b (12). Mutagenic primers corresponding to 21–25 base sequence of the sense strand were designed with a single codon changed for preparing molecular graphics figures.

1 The abbreviations used are: DQF-COSY, double-quantum filtered correlation spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; TOCSY, total correlation spectroscopy; PCR, polymerase chain reaction.

2 The nucleotide numbering system is from the sequence given by Walker et al. (26).
RESULTS

NMR Spectra of Peptide b₁₋₃₄ in Chloroform/Methanol/H₂O Solvent—Peptide b₁₋₃₄ is quite hydrophobic and it proved to be very soluble in a mixture of chloroform/methanol/H₂O (4:4:1 by volume). The DQF-COSY spectrum of b₁₋₃₄ in this solvent mixture demonstrated a good dispersion of HN and Hα chemical shifts (Fig. 1). The distribution of chemical shifts of the HN and Hα protons is typical for a protein containing α-helical and coiled segments (43). Proton chemical shifts were assigned by standard procedures (44). Main chain chemical shifts assignments for all residues were complete with the exception of Asn-2, where HN was not observed. Most of the side chains protons have been assigned except for a few aliphatic side chains where complete assignment was not possible due to spectral overlap and chemical shift degeneracy. A table of chemical shift assignments has been deposited in the BMRB data bank (http://www.bmrb.wisc.edu). NOE analysis revealed a pattern of sequential and medium range NOEs, which is characteristic of an α-helix (Fig. 2A). No long range NOEs were observed (Table I), indicating that the peptide does not form tertiary folds. The Hα-HN cross-peaks of the residues 12–21 and 25 were still readily observable by DQF-COSY in completely deuterated solvent in an experiment where data was collected from 6 to 14 h after dissolving the peptide. These regions of the peptide must therefore have a particularly stable hydrogen bonded secondary structure.

General Features of the b₁₋₃₄ Solution Structure—The three-dimensional structure of b₁₋₃₄ was calculated by simulated annealing with the DYANA package of NMR software (40). Of the initial collection of 200 calculated structures, 180 had a similar overall folding pattern. In the 20 remaining structures there was no apparent clustering of structures into an alternative fold. The 10 lowest energy structures were energy minimized using AMBER forcefield as implemented in DISCOVER (Molecular Simulations Inc.). The atomic coordinates of the 10 final structures have been deposited as entry 1b9U at the Protein Data Bank, Rutgers, New Jersey. The best fit superposition of the 10 final structures is shown in Fig. 3. Mean

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**TABLE I**

| Statistics for 10 final structural models from DYANA calculations |
|---------------------------------------------------------------|
| NOE-derived distance constraints (total = 275)               |
| Intraresidue                                                  |
| Sequential (i = 1)                                           |
| Short range (1 < |i – j| ≤ 4)                                |
| Long range (|i – j| > 4)                                           |
| Coupling constant derived ithedral angle constraints (total = 20) |
| ϕ                                                             |
| Mean global pairwise backbone RMSD 0.37 ± 0.14 Å             |
| Mean global pairwise all heavy atom RMSD 0.89 ± 0.14 Å       |
| Number of consistent constraint violations                   |
| Distance violations ≥0.2 Å                                   |
| Angle violations ≤5°                                         |
pairwise root mean square deviation between individual structures for residues 3–33 was 0.4 ± 0.1 Å and 0.9 ± 0.1 Å for the backbone and all heavy atoms, respectively. There were no distance constraint violations exceeding 0.2 Å. The distribution of angles in a Ramachandran plot were 74% in the “most favored” region, with 23% in the “additionally allowed” and 3% in “generously allowed” regions. The definitions of the Ramachandran plot regions are those used in DYANA. Statistics on the structure calculation are presented in Table I and Fig. 2B.

The b1–34 peptide forms a well defined α-helix from residues 4–22, which is interrupted by a bend region from residues 23–26 with resumption of the α-helix from residue 27 to the C terminus. A large stretch of the initial α-helical segment is unusually stable as judged by the very slow HN exchange in deuterated solvent. The uninterrupted stretch of hydrophobic side chains from residues 11 to 20 may stabilize the hydrogen bonded secondary structure in either a lipid bilayer or a membrane-mimetic solvent by forming a nonpolar sheath around the protein backbone. Sequential proline residues at positions 27 and 28, which would break the \((i,i+4)\) pattern of hydrogen bonding in an α-helix, correlate with the position of the bend in the structure. This region is well ordered in solution despite the absence of hydrogen bonds. Such a rigid conformation probably results from the combination of restricted torsional mobility of the backbone of the two proline residues and spatial constraints imposed by the bulky side chains of residues 22–26.

A more extended α-helical segment of the cytoplasmic domain.

**Dimerization of the Transmembrane Domain of Subunit b in Situ**—The cytoplasmic domains of subunit b forms a functionally important homodimer in F0, which suggests a possible proximity of transmembrane segments as well. Single Cys residues were introduced from position 2–20 of a cysteine-free subunit b (bC21S) to test this possibility by cross-linking. The bC21S mutant was shown to be functionally equivalent to wild type (12). Each of the single Cys mutants grew on a succinate carbon source, indicating a functional oxidative phosphorylation system. Most mutants grew similarly to wild type (1.5–2-mm colony diameter after 3 days at 37 °C) with the exception of bI7C (0.5 mm), bG9C (0.8 mm), and bI12C (1 mm). Membrane vesicles from each mutant were analyzed for b-b homodimer formation by disulfide bridge formation following treatment with Cu(II)-(1,10-phenanthroline)2 (Fig. 4). Relatively intense high yield b-b dimer were observed for b-b homodimer formation by disulfide bridge formation following treatment with Cu(II)-(1,10-phenanthroline)2 (Fig. 4). Relatively intense high yield b-b dimer were observed for the bN2C, bT6C and bQ10C substitutions. Less intense dimer formation was observed with Cys substitutions at residues 3, 4, 8, 9, 11, 13, 14, 17, and 18. The periodicity of high yield cross-linking seen in Fig. 4 mimics that expected for one face of an α-helix. The less intense cross-linking seen over consecutive stretches of residues suggest that the helices may be relatively mobile in the membrane.

**Possible Arrangement of the Transmembrane Domains of Two Subunits b in F0**—Intersubunit distance restraints derived from the cross-linking pattern were used to envision the orientation of the membrane domains of the two b subunits in the native F0 complex. Two minimized mean b1–34 structures were docked to each other using distance constraints from the cross-linking data. The distances between the α-carbons of residues 2, 6, and 10 of two different b subunits were con-
strained to 4–8 Å, the distance usually found for natural disulfide bridges in proteins (45). The distances between α-carbons of residues forming lower yield cross-links were constrained to 4–11 Å. Backbone angles of residues 3 to 33 were restrained to the values in the mean structure, and side chain angles left unrestrained. A dimer structure was calculated using molecular dynamics and energy minimization with DISCOVER (Molecular Simulations Inc.). The fit of the cross-linking distance constraints to the model are shown in Table II. The modeling indicates that the membrane anchoring segments of the two b subunits are positioned at a 23° angle to each other with interacting helical faces making Van der Waals contact at the side chains of residues 6 and 10 (Fig. 5).

### DISCUSSION

Before this work, Girvin et al. (11) used solution NMR to solve the structure of F0 subunit c dissolved in chloroform/methanol/H2O (4:4:1) solvent. Importantly, subunit c folds in a helical hairpin, as it is predicted to fold in the membrane, with a number of side chains interacting in accord with the predictions of genetic and biochemical studies of F0 in situ. The solvent mixture used may be a good membrane mimetic, because it can organize heterogeneously around polar and apolar surfaces of amphiphatic proteins. We have shown previously that purified subunit c, prepared in chloroform/methanol/H2O solvent, can be reconstituted with subunits a and b to form an F0 with normal proton translocating function (35). The experiment indicated that the protein was not denatured by the solvent treatment. We have attempted similar experiments here in reconstituting peptide b1–34 with purified subunits a and c and were unable to reconstitute proton-translocating activity. This negative result is in agreement with earlier observations that partial removal of small segments of the C-terminal domain of the subunit b disrupted the assembly of an active F0 complex (46, 47).

The structure of the transmembrane region of subunit b derived here by NMR analysis of the protein in chloroform/methanol/H2O solvent fits well with features of the protein expected in a native lipid bilayer. The N-terminal α-helical segment (residues 4–22) is followed by hinge region from residues 23–26 before resumption of the α-helix. Because an α-helix of 20 amino acids is required to traverse the fatty acyl hydrocarbon interior of a phospholipid bilayer, we expect the initial N-terminal helix to be the hydrocarbon spanning region of the protein. This would place Asn-2 at the periplasmic hydrocarbon/polar interface and the hinge region (residues 23–26) at the cytoplasmic hydrocarbon/polar interface of the phospholipid bilayer. The distance between α-carbons of Asn-2 and Trp-26 is 34 Å in the structure, which is close to the distance of 32 Å predicted between fatty acyl carbonyls in opposing leaflets of a palmitoyloleylphosphatidylcholine bilayer determined by x-ray and neutron diffraction (48, 49). The positioning of these residues near the glycerol moiety of the phospholipid was previously indicated by labeling studies with a photoactivatable phospholipid analog (50).

Trp and Tyr residues are preferentially found at the hydrocarbon/polar interface of the lipid bilayer in transmembrane proteins of known structure (49, 51–53). The Tyr-24 and Trp-26 residues in the b1–34 structure would also be predicted to organize in the interfacial region. In the case of the Trp-26 side chain, the aromatic rings lie parallel to the predicted surface of the lipid bilayer but in a region of the protein devoid of other protein contacts (Fig. 3). Trp-26 can be replaced with either an acidic or a basic residue without impairing function (54), indi-

#### TABLE II

| Residue pair | Distance constraint range Final distance |
|--------------|-----------------------------------------|
| a            | A                                       |
| 2–2'         | 4–8                                     | 8.1  |
| 3–3'         | 4–11                                    | 4.5  |
| 4–4'         | 4–11                                    | 10.9 |
| 6–6'         | 4–8                                     | 5.2  |
| 8–8'         | 4–11                                    | 11.6 |
| 9–9'         | 4–11                                    | 10.6 |
| 10–10'       | 4–8                                     | 5.2  |
| 11–11'       | 4–11                                    | 10.5 |
| 13–13'       | 4–11                                    | 10.4 |
| 14–14'       | 4–11                                    | 7.6  |
| 17–17'       | 4–11                                    | 10.3 |
| 18–18'       | 4–11                                    | 11.1 |

a Prime designates residue of second subunit in dimer.
cytoplasmic domains believed critical in $F_1$ binding function to be adjacent to each other and the interactions between unrestrained.  

Reorient in a phospholipid bilayer in a more perpendicular manner to optimize hydrogen bonding between the indole NH and fatty acyl carbonyl groups (55).3

The aromatic ring interactions of Phe-14-Phe-17, respectively.  

Fig. 5. Model for possible interaction of transmembrane domains of subunit b based on the cross-linking constraints and NMR model. Side chains of residues discussed in text are shown in bold color, i.e. Asn-2, Thr-6, and Gln-10 (magenta), Phe-14 and Phe-17 (green), and Trp-26 (yellow). Van der Waals contacts are made between the pairs of Thr-6 and Gln-10 side chains. The rings of Phe-14, Phe-17, and Phe-17 interact in an aromatic cluster. The aromatic rings of Trp-26 and Trp-26' project toward the front and back of the structure, respectively.

3 Such a reorientation does occur in the molecular dynamics calculation illustrated in Fig. 5, where side chain angles were left unrestrained.
Transmembrane Domain of Subunit b in $F_0$

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