Role of the Asymmetry of the Homodimeric \( b_2 \) Stator Stalk in the Interaction with the \( F_1 \) Sector of \textit{Escherichia coli} ATP Synthase

Kristi S. Wood and Stanley D. Dunn

From the Department of Biochemistry, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario N6A 5C1, Canada

The \( b \) subunit dimer in the peripheral stator stalk of \textit{Escherichia coli} ATP synthase is essential for enzyme assembly and the rotational catalytic mechanism. Recent protein chemical evidence revealed the dimerization domain of \( b \) to contain a novel two-stranded right-handed coiled coil with offset helices. Here, the existence of this structure in more complete constructs of \( b \) containing the C-terminal domain, and therefore capable of binding to the peripheral \( F_1 \)-ATPase, was supported by the more efficient formation of intersubunit disulfide bonds between cysteine residues that are proximal only in the offset arrangement and by the greater thermal stabilities of cross-linked heterodimers trapped in the offset configuration as opposed to homodimers with the helices trapped in-register. \( F_1 \)-ATPase binding analyses revealed the offset heterodimers to bind \( F_1 \) more tightly than in-register homodimers. Mutations near the C terminus of \( b \) were incorporated specifically into either the N-terminally or the C-terminally shifted polypeptide, \( b^N \) or \( b^C \), respectively, to determine the contribution of each position to \( F_1 \) binding. Deletion of the last four residues of \( b^C \) substantially weakened \( F_1 \) binding, whereas the effect of the deletion in \( b^C \) was modest. Similarly, benzophenone maleimide introduced at the C terminus of \( b^N \), but not \( b^C \), mediated cross-linking to the \( \delta \) subunit of \( F_1 \). These results imply that the polypeptide in the \( b^N \) position is more important for \( F_1 \) binding than the one in the \( b^C \) position and illustrate the significance of the asymmetry of the \( b \) dimer in the enzyme.

The process of oxidative phosphorylation in mitochondria and bacteria, or photophosphorylation in chloroplasts, requires the enzyme \( F_1 F_0 \)-ATP synthase to utilize the energy of the transmembrane proton gradient for the production of ATP from ADP and P\(_i\). The enzyme functions as a molecular motor, with rotor and stator complexes consisting of subunits from both the membrane-peripheral \( F_1 \) and membrane-integral \( F_0 \) sectors of the protein. In the \textit{Escherichia coli} enzyme, \( F_0 \) contains three subunits in an \( ab_{25\alpha \beta \delta} \) stoichiometry, whereas \( F_1 \) has five subunits in the stoichiometry of \( \alpha_3 \beta_3 \gamma \delta \epsilon \). The \( \gamma \epsilon \delta \) subunits compose the rotor, and \( b_{2\delta} \) forms the stator. As the rotor is driven by the passage of protons through a pore formed by the \( a \) and \( \alpha \) subunits of \( F_0 \), rotation of \( \gamma \) within the \( \alpha_3 \beta_3 \) hexamer of \( F_1 \) causes conformational changes in the catalytic nucleotide-binding sites located on the \( \beta \) subunits, promoting ATP synthesis and release. One function of the \( b_{2\delta} \) stator is to hold the \( \alpha_3 \beta_3 \) hexamer against the rotational torque, as otherwise \( \alpha_3 \beta_3 \) would simply turn with the rotor rather than undergoing the conformational changes associated with the formation and release of ATP. In anaerobic or facultative bacteria, the enzyme can function as a proton pump, hydrolyzing ATP to drive protons out of the cytoplasm, against the electrochemical gradient. For recent reviews see Refs. 1–10.

The major component of the stator stalk is the 156-residue \( b \) subunit, which forms an elongated dimer extending from the periplasmic side of the cytoplasmic membrane to the top of \( F_1 \), where it interacts with \( \delta \) (6–8, 11). Clear roles for some sections of the \( b \) subunit have been determined, in particular the trans-membrane domain formed by residues 1–24 (12), the dimerization domain encompassing residues 53–122 (13, 14), and the C-terminal \( \delta \)-binding domain composed of residues 123–156 (15–18). The tether region (residues 25–52) links the transmembrane and dimerization domains; its function is not well understood, but it is known to interact with the \( a \) subunit and to play a role in coupling (19, 20).

The interactions of \( b_2 \) with \( F_1 \) occur predominantly through the C-terminal \( \delta \)-binding domain. Although interactions exist between \( b \) and \( \alpha_3 \beta_3 \) (19, 21), \( \delta \) subunit is required for the binding of \( F_1 \) to \( F_0 \) in membranes (22). The interaction of \( b \) and \( \delta \), mediated through the C-terminal regions of each subunit, appears to be key to this binding (15, 16).

Dimerization of \( b \) is essential for \( F_1 \) binding and ATP synthase function, although it can be significantly weakened through mutation in the dimerization domain before activity is lost (14, 23). The isolated dimerization domain has been characterized as an atypical, parallel, two-stranded coiled coil (13, 24, 25). Sequence analyses of this region have identified an 11-residue hendecad pattern, with positions denoted \( abcdefghi \) (6, 26). Hendecad patterns are indicative of right-handed coiled coils in which 11 residues make three turns of the helix relative to the interhelical axis (27, 28); the expected distribution of positions for a two-stranded structure of this type is shown on the helical wheel in Fig. 1A. The hendecad pattern seen in \( b \) subunit sequences is unusual in that the \( a \) and \( h \) positions at the center of the interface are usually occupied by ala-
nine or other small amino acids, whereas larger hydrophobic residues are often seen in the d and e positions that are more peripherally situated (6). In the absence of a high resolution dimeric structure, assignment of the hydrophobic strip defined by the a, d, e, and h positions as the dimerization interface in the isolated dimerization domain has been supported by recent studies of disulfide formation between cysteine residues introduced into the a and h positions between residues 61 and 90 and by assessment of the stabilities of disulfide-linked dimers (29). Results of these studies further implied that the two helices of the dimer are offset, rather than in-register as in left-handed coiled coils. In this staggered configuration, one of the helices, denoted \( b^N \), is N-terminally shifted relative to the other helix, denoted \( b^C \), by about 5.5 residues (one-half of a hendadec), making the dimer intrinsically asymmetric, as seen in Fig. 1B. A functional significance of the right-handed coiled coil in stabilizing the dimer (26), except when cysteine was inserted at residues 53–156, was suggested. The goal of this study was to explore the existence of the offset helices in \( b \) constructs containing the \( \delta \)-binding domain and to assess its functional significance in the interaction with \( F_1 \). The results we present confirm the asymmetric nature of the dimer and reveal the different roles of \( b^N \) and \( b^C \) in the binding of \( F_1 \).

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Recombinant DNA techniques were performed by standard methods. All generated sequences were verified by sequencing. Plasmids encoding \( b \) polypeptides were constructed such that all of the resultant proteins included the R83A mutation, previously shown to stabilize the dimer (26), except when cysteine was inserted at this position. Plasmids encoding \( b \) residues 53–156 were based on pSD111, which includes a leader sequence of MSYW (24), whereas plasmids encoding \( b \) residues 34–156 were based on plasmid pJB3, in which the leader sequence is MST (30). Relevant mutations were introduced into these plasmids by subcloning appropriate sections of DNA from previously described plasmids encoding desired cysteine substitutions (29) or C-terminal mutations resulting in either premature chain termination, V153Stop, or else addition of GC to the normal C-terminal Leu-156 (16).

**Protein Expression, Purification, and Production of Disulfide Cross-linked Forms**—The various mutant forms of soluble \( b \) subunit were expressed and purified by ammonium sulfate fractionation and ion-exchange chromatography, essentially as described (24, 30). Purification steps were monitored by SDS-PAGE.

To prepare disulfide-linked dimers, purified cysteine-containing \( b \) polypeptides, either individually or as equimolar mixtures, were first reduced with 2 mM dithiothreitol for 1 h and then dialyzed overnight against a buffer containing 50 mM Tris-HCl, pH 8.0. Diazoyl bags were then transferred to buffer containing 50 mM Tris-HCl, pH 8.0, and 10 \( \mu \)M CuCl2, and dialysis was continued for 3 days. In cases where one of the \( b \) subunits contained a C-terminal Gly-Cys addition, as well as a cysteine at either position 83 or 90 in the dimerization domain, a copper/cysteine disulfide exchange buffer that contained 10 \( \mu \)M CuCl2 as well as 10 mM cysteine was used to foster internal disulfide formation, while leaving the C-terminal cysteine residue disulfide-linked to the free amino acid cysteine. The resultant cross-linked forms were purified by anion-exchange chromatography using a MonoQ 5/5 column.

**Determination of Propensity to Form Disulfides**—Purified cysteine-containing \( b \) constructs were analyzed for their propensity to form disulfide-linked dimers by a previously described method in which the polypeptides are dialyzed in the presence of air against buffer containing 10 \( \mu \)M CuCl2 and 10 mM cysteine (24, 29). Equal volumes of 60 \( \mu \)M protein samples were mixed, giving each a final concentration of 30 \( \mu \)M. Mixtures were dialyzed overnight against 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 5 mM dithiothreitol at 4 °C to reduce any disulfides. Part of each sample was removed for analysis by nonreducing SDS-PAGE, and the dialysis bags were then transferred to a buffer containing 100 mM Tris-HCl, pH 8.0, 10 mM cysteine, and 10 \( \mu \)M CuCl2 and allowed to dialyze for 24 h at 4 °C with vigorous stirring. Samples were again collected and analyzed by nonreducing SDS-PAGE. To prevent any disulfide exchange, sample buffer for nonreducing SDS-PAGE contained 15 mM N-ethylmaleimide.

**Chemical Cross-linking with Benzophenone-4-maleimide (BPM)**—All steps were performed at room temperature. Samples of purified \( F_1 \) and disulfide-linked \( b \) dimers, in which one of the subunits had a C-terminal glycylcysteine addition, were separately passed through 1-ml centrifuge columns (31) containing Bio-Gel P-10 resin (Bio-Rad) equilibrated with 50 mM sodium phosphate, pH 7.5, and 1 mM EDTA. The \( b \) dimers at a concentration of 25 \( \mu \)M were incubated for 75 min with 1 mM TCEP to reduce the C-terminal cysteine of those constructs. Chemical cross-linking of the \( b \) dimers to \( F_1 \)-ATPase using BPM (Molecular Probes, Eugene, OR) was carried out using the procedure described by McLachlin et al. (16). BPM dissolved in dimethylformamide was added in a 5-fold molar excess over the \( b \) dimers and allowed to incubate for 15 min. Unreacted BPM was quenched by addition of an equimolar amount of \( \beta \)-mercaptoethanol. The \( b \) dimer was then mixed with the column-centrifuged \( F_1 \) at a molar ratio of 2 \( F_1 \) per \( b \) dimer, in the presence of 5 mM MgCl2. Controls were performed in which no BPM was added to the \( b \) solution. Samples were exposed to long wave ultraviolet light from an Ultra-Violet Products model TM-36 transilluminator for 5 min. As an additional control, some BPM-modified samples were placed on the transilluminator but removed before it was turned on. After illumination, SDS-PAGE sample buffer was added to the samples, which were then heated at 100 °C for 2 min and analyzed by SDS-PAGE and Western blotting.

**Other Materials and Methods**—Thermal denaturation of protein constructs in 10 mM sodium phosphate, pH 7.0, was followed by circular dichroism spectroscopy at 222 nm using a Jasco J-810 spectropolarimeter equipped with a Peltier temperature control unit. The temperature was increased at the rate of...
Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

1 °C per min. Data were converted to mean residue ellipticity and fitted to a two-state model as described (32–34).

$F_1$ binding activity of $b$ dimers was determined using a competitive assay described previously (16). Purified soluble $b$ dimers at the indicated concentrations were mixed with 20 μl of an $F_1$ affinity resin bearing soluble $b$ linked to the resin through a cysteine residue near its N terminus and 31.3 μg of $F_1$ (0.3 μM) for 1 h. The resin was sedimented by centrifugation; the supernatant solution was removed; the pellet was resuspended in SDS-PAGE sample buffer and then analyzed by SDS-PAGE. Bovine serum albumin was added to the competition buffer to serve as a control for trapping of liquid within the resin pellet. As a control, resin with only cysteine linked was used.

Protein concentrations were determined using the Advanced Reagent (Cytoskeleton, Inc.), and values for $b$ subunits were corrected using a factor determined by quantitative amino acid analysis as described previously (26, 29).

SDS-PAGE was carried out using the Tris-glycine system described by Laemmli (35). Sample buffer for nonreducing analysis as described previously (26, 29). Protein concentrations were determined using the Advanced Reagent (Cytoskeleton, Inc.), and values for $b$ subunits were corrected using a factor determined by quantitative amino acid analysis as described previously (26, 29).

The presence of free thiol groups in polypeptides was determined by treatment with 1.2 mM fluorescein maleimide in SDS sample buffer lacking both dithiothreitol and $N$-ethylmaleimide. Sample buffer for reducing SDS-PAGE contained 50 mM dithiothreitol. SDS-PAGE contained 15 mM $N$-ethylmaleimide. Sample buffer for reducing SDS-PAGE contained 50 mM dithiothreitol.

The presence of free thiol groups in polypeptides was determined by treatment with 1.2 mM fluorescein maleimide in SDS sample buffer lacking both dithiothreitol and $N$-ethylmaleimide for 15 min followed by SDS-PAGE (36). Before Coomassie Blue staining, the gel was exposed to UV light to visualize the fluorescein-labeled polypeptides.

Polyclonal antibodies to the peripheral domain of $b$ and to $\delta$ subunit were raised in rabbits. The anti-$\delta$ serum was treated with $\beta$ MERC resin (16) to remove antibodies that recognized $b$ subunit. The anti-$b$ serum was sufficiently specific for our studies without any special treatment. Blotting was carried out using carbonate blot buffer as described previously (37). Primary antibodies were subsequently detected using a second antibody conjugated to alkaline phosphatase as described (38).

**RESULTS**

**Preferential Formation of Intersubunit Disulfides between Positions $a$ and $h$**—The propensity for disulfide formation between cysteine residues introduced into $b$ subunit polypeptides was assessed using a previously developed method involving initial dialysis against buffer containing 5 mM dithiothreitol and 0.1 mM EDTA to reduce any existing disulfides, followed by a second dialysis against a disulfide exchange buffer containing 10 μM Cu$^{2+}$ and 10 mM free cysteine to foster selective oxygen-dependent disulfide formation. Previous studies of disulfide bond formation using dimerization domain constructs showed that mixed pairs of polypeptides with cysteines in all adjacent $a$ and $h$ positions between Ala-61 and Ala-90 had much stronger propensities to form disulfide-linked heterodimers than any of the individual cysteine-containing constructs to form disulfides (29). We asked if this result could be extended to forms of $b$ containing the entire C-terminal domain that is involved in binding $F_1$, using $b_{34-156}$ with cysteines at individual $h$ positions (68, 79, 90) and $b_{53-156}$ constructs with cysteines at individual $a$ positions (72, 83), as described under “Experimental Procedures” (Fig. 1, C and D). The difference in size between these constructs allowed differentiation between homodimers and heterodimers by their migration on SDS-PAGE. No more than a trace of dimer formed from any pure construct during the dialysis against 5 mM dithiothreitol (Fig. 2A, upper), but surprisingly significant dimer formation occurred between constructs with cysteines in adjacent $a$ and $h$ positions (i.e. 68 × 72, 72 × 79, 79 × 83, and 83 × 90) even during this step (Fig. 2A, lower panel), After oxidizing dialysis in a disulfide-exchange buffer, essentially complete dimer formation was observed in those particular mixed samples, whereas disulfide formation was substantially incomplete for the individual polypeptides or the other mixtures.

**Disulfide-linked Offset Heterodimers Are More Stable than In-register Homodimers**—Whereas the results of the disulfide formation studies indicated that interchain disulfide formation is more favorable for each of the four adjacent $a/h$ pairs tested than for any individual construct or for any of the other mixed samples, the distinction is not absolute. We therefore sought
additional evidence by determining the thermal stability of purified, disulfide-linked \( \beta \) dimers, following the change in the circular dichroism signal as the temperature was raised. Three in-register homodimers and two offset heterodimers were analyzed (Fig. 3). In comparison with the cysteineless constructs, shown in Fig. 3 as open symbols, homodimers linked through cysteines at positions 79, 83, or 90 all showed broader transitions indicative of lower cooperativity. In contrast, the two offset heterodimers melted with sharp transitions, similar to those of the cysteineless forms, implying that interhelical interactions in the native cysteineless forms are more closely approximated by those in the offset heterodimers. Because the introduction of either type of disulfide converts folding from a bimolecular to a unimolecular reaction, both result in increases in the midpoint of melting, but it is also notable that the melting midpoints of the heterodimers were about 20 °C higher, in the range of 73–75 °C, than those of the in-register homodimers that were between 49 and 54 °C. Overall, the results of these studies imply that the unusual offset relationship of the helices previously seen in the isolated dimerization domain applies also to constructs extending to the C terminus.

Offset Heterodimers Preferentially Bind \( F_1 \)—To investigate the functional relevance of the offset relationship of the \( \beta \) subunit helices, we studied the effects of either offset or in-register intersubunit disulfides on the interaction with \( F_1 \) using a semi-quantitative competitive assay, in which the ability of a soluble \( \beta \) conjugated to agarose beads for binding a limited amount of \( F_1 \) is tested (16). Results of this experiment are shown in Fig. 4. As seen in the left-hand lanes in Fig. 4 next to the molecular weight standards, control resin lacking conjugated \( \beta \) had only small amounts of \( F_1 \) trapped within the resin, whereas the affinity resin bound substantial \( F_1 \) in the absence of soluble competitor, but had only the background level when competing wild-type soluble \( \beta \) was added (upper panel). Both of the tested offset heterodimers, 79/83 and 83/90, resembled the wild-type \( \beta \) in their ability to compete for \( F_1 \), whereas the in-register homodimers formed with cysteines at any of the three positions competed less effectively. The stronger binding of the offset heterodimers to \( F_1 \) implies the relevance of the staggered conformation of \( \beta \) within the enzyme.
Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

**FIGURE 4. Comparison of F$_1$-binding affinities of noncovalent and disulfide-linked $b$ dimers.** The ability of purified homodimeric or heterodimeric disulfide-linked $b$ dimers to compete for F$_1$, with wild-type $b$ or an F$_1$ affinity resin was tested at three concentrations as described under "Experimental Procedures." The control resin was substituted with cysteine rather than $b_{MERC}$ subunit. The lanes labeled No $b$ received the affinity resin and F$_1$, but no competing soluble form of $b$. Proteins bound to the resin were analyzed by reducing SDS-PAGE. Effective competition is indicated by reduction in the amount of F$_1$ subunits bound to the resin. The bands of bovine serum albumin and soluble $b$ constructs reflect the volume of incubation buffer trapped within the resin pellet.

$b^N$ Is More Important for F$_1$ Binding—The final four residues of the $b$ polypeptide have been found previously to be required for the binding of $b$ to F$_1$ through the $\delta$ subunit; deletion of these residues completely abolished F$_1$ binding (16). Subsequent studies from the Cain laboratory showed that coexpression of a bR36l mutant, which is also nonfunctional by itself, complemented the four-residue truncation, implying that a heterodimeric form of $b$ with just one of the subunits extending to the C terminus would foster assembly and function of ATP synthase (39). Because formation of a disulfide bond between position 83 of one $b$ subunit and position 90 of the other $b$ subunit locks the polypeptide with 90C in the $b^N$ position, and the polypeptide with 83C in the $b^C$ position (see Fig. 1C), we were able to ask which of the two $b$ subunits must extend to the normal C terminus, Leu-156, by incorporating the four-residue C-terminal deletion into the constructs bearing either of the cysteine mutations. Through disulfide formation and subsequent purification, we prepared heterodimers in which the final four residues were deleted from $b^N$ only, $b^C$ only, or from both of the polypeptides. These constructs were tested for their ability to compete with F$_1$ binding using the competitive assay (Fig. 5). As before, both the wild-type control and offset disulfide-linked dimer with both C termini intact ($b^C \times b^N$) competed effectively, as indicated by the strong reduction in F$_1$ bound to the resin. Notably, the C-terminal truncation of $b^N$ strongly reduced competition for F$_1$, whereas the C-terminal truncation of $b^C$ had a much less significant effect (the truncation of polypeptides is indicated in the figure by the subscript $\Delta 4$). As expected, controls with deletion of the C-terminal residues on both subunits, either offset or in-register, showed no competition for F$_1$. These results indicate that the C-terminal residues of $b^N$ play the more significant role in binding F$_1$, but that the C-terminal residues of $b^C$ contribute to binding more weakly.

$b^N$ Is in Closer Proximity to $\delta$ than $b^C$—Previously it was shown that a form of $b$ with a glycycysteine addition at the C terminus could be cross-linked to $\delta$ through use of BPM, a photoactivable cross-linking reagent (16). Using a strategy similar to that described above for the C-terminal deletions, we asked which of the two $b$ positions had the C terminus proximal to $\delta$ by incorporating the glycycysteine addition into either $b^N$ or $b^C$. After formation of the heterodimeric forms of $b$ by dialysis against the CuCl$_2$/cysteine buffer, and subsequent purification of heterodimers, it was necessary to obtain selective reduction of the C-terminal cysteine, which had formed a mixed disulfide with free cysteine during the oxidation step, so that the cross-linking agent could be added specifically. Although this selective disulfide reduction might seem challenging, the very favorable nature of the disulfide link between the polypeptides in positions 83 and 90 not only enhances disulfide formation (Fig. 2), but it also makes reduction of the interpolypeptide disulfide difficult (29), whereas reduction of the exposed C-terminal mixed disulfide with free cysteine can be expected to occur readily.

In preliminary studies to determine conditions for selective reduction, heterodimers were incubated with concentrations of TCEP between 0.2 and 2 mM for either 10 or 75 min, and then fluorescein maleimide was added to react with thiol groups, and samples were analyzed by nonreducing SDS-PAGE. Before staining for protein, the gel was exposed to UV light to visualize the proteins that had been labeled with fluorescein. The image of the UV-illuminated gel was compared with the stained gel to determine the amount of fluorescein-labeled dimer, indicative of the intact dimer having a reduced C-terminal cysteine, and monomers, indicative of reduction of the interpolypeptide disulfide bond. As expected, the C-terminal cysteine residue was readily reduced by low concentrations of TCEP, whereas...
Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.

**DISCUSSION**

Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.

**DISCUSSION**

Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.

**DISCUSSION**

Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.

**DISCUSSION**

Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.

**DISCUSSION**

Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.

**DISCUSSION**

Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.

**DISCUSSION**

Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.

**DISCUSSION**

Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.

**DISCUSSION**

Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.

**DISCUSSION**

Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.

**DISCUSSION**

Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.

**DISCUSSION**

Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.

**DISCUSSION**

Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

Recent studies from this laboratory indicated that, in a dimerization domain construct of the $b$ subunit of *E. coli* ATP synthase, the two helices interact through a previously identified right-handed surface, implying that they formed a right-handed coiled coil, and that the relationship of the helices in this structure further differed from that seen in the more common left-handed coiled coil in that they were offset along the helical axes rather than in register (26, 29). The goal of this study was to determine whether the offset is also observed in constructs that extend to the C-terminal $\delta$-binding domain and to see if the offset holds functional significance for the interaction with the $F_1$ sector.
Asymmetry of the $b_2$ Stator Stalk of ATP Synthase

One possible explanation for these effects is that binding to $F_1$ may be mediated almost entirely by one of the $b$ subunits rather than by a surface formed by their interaction. The primary function of the second subunit would then be to support the structure of the first one, and even the suboptimal in-register alignment of the homodimers may fulfill this function at the ambient temperature used in the binding assay. We feel that caution should be exercised in accepting this explanation of the $F_1$-binding properties, because some adjustment of the interhelical relationship over the distance between the disulfide and the $\delta$-binding domain could make the difference between homodimers and heterodimers in the latter region smaller than expected. The larger difference in the effect of C-terminal truncation on $F_1$ binding and the apparently complete specificity of $b^N$ for cross-linking to $\delta$, however, indicate that such adjustment is partial at most.

The Two $b$ Subunits Play Different Roles in ATP Synthase—
The C terminus of $b$ is essential to interaction with $F_1$, likely through $\delta$, and mutations to this region have detrimental effects on $F_1$ binding (15, 16). However, Grabar and Cain (39) observed functional complementation of two defective $b$ monomers, one of which was missing the four C-terminal residues, implying that these residues are essential for only one of the two $b$ subunits. By disulfide-linking two different $b$ subunits together, we were able to incorporate a C-terminal mutation specifically into either $b^N$ or $b^C$. This enabled us to determine that $\delta^N$ plays the more important role, and only $b^N$ could be cross-linked to $\delta$ using BPM. The more modest effect seen with truncation of $b^C$ could indicate the supporting role suggested above. In this regard, previously reported results imply that the C-terminal helical segment of at least one of the two $b$ subunits folds back to form a larger helical bundle in the $\delta$-binding domain (13, 16, 42).

Relationship of the Offset Right-handed Coiled Coil of $b_2$ to the Intrinsic Asymmetry of ATP Synthase—The intrinsic asymmetry of ATP synthase, which is unavoidable given its subunit stoichiometry, has a number of interesting facets. Regarding this study, the homodimeric $b$ subunit found in most eubacterial enzymes is the only polypeptide that occurs with a stoichiometry of two per enzyme complex. The two $b$ subunits cannot have identical interactions with their key binding partners, the $a$ subunit of $F_0$ and the $\delta$ subunit of $F_1$, which are both present as single copies. How the $b_2$ homodimer interacts within the asymmetric enzyme has therefore been a question.

Our results address both general and specific aspects of this question. First, it is the normal expectation that a homodimer will be symmetric with one 2-fold axis of symmetry, but this is not necessarily true because a number of asymmetric homodimers have been reported (43). The offset helices in the right-handed coiled coil of $b_2$ make the homodimer intrinsically asymmetric. For any given position, the residues on the polypeptides occupying the $b^N$ and the $b^C$ positions will be in different environments and in proximity to different side chains of the partner helix. The most compelling reason for the preferential adoption of the offset configuration by the two-stranded right-handed coiled coil is steric hindrance at the $a$ and $h$ positions, because their proximity to the interhelical axis prevents knobs-into-holes packing. Our results show that the asymmetry precedes interaction with other subunits, rather than being induced by such interactions. Although the subunits in the free soluble $b_2$ are probably dynamic with respect to their position, especially because of the reversible monomer-dimer equilibrium in this system, it seems likely that interaction with $F_1$ will stabilize the positions, with the polypeptide in the $b^N$ position interacting more strongly with $\delta$ subunit than the polypeptide in the $b^C$ position. The polypeptides in the two positions can also be expected to have different interactions with the $a$ subunit.

The ATP synthases of chloroplasts and some eubacterial species, particularly photosynthetic organisms, contain single copies of two similar but nonidentical $b$-type subunits, called subunits I and II in chloroplasts or $b$ and $b'$ in eubacteria (44, 45). It seems likely to us that the development of the heterodimeric system represents an adaptation in which, following a gene duplication event, one subunit evolved to function more efficiently as $b^N$ and the other as $b^C$, whereas in the homodimeric systems the single $b$ sequence must be capable of filling both roles. Previous work has shown that the soluble domains of $b$ and $b'$ subunits of Synechocystis formed heterodimers but not homodimers (45), and recently portions of the $b$ and $b'$ subunits of Thermosynechococcus elongatus have been incorporated into chimeric forms of $E. coli$ $b$, so that ATP synthase complexes with heterodimeric $b$ subunits are produced (46). We anticipate that this system will make it possible to further develop the functional significance of the $b^N$ and $b^C$ positions in the future.

Acknowledgments—We thank Drs. Derek McLachlin and Paul Del Rizzo for scientific advice and discussions and Yumin Bi for technical assistance. Some of the experiments described were carried out at the Biomolecular Interactions and Conformations Facility, in the Schuylkill School of Medicine & Dentistry of the University of Western Ontario; the assistance of the Facility Manager, Lee-Ann Briere, is also gratefully acknowledged.

REFERENCES

1. Senior, A. E., Nadanaciva, S., and Weber, J. (2002) Biochim. Biophys. Acta 1553, 188–211
2. Wilkens, S. (2005) Adv. Protein Chem. 71, 345–382
3. Duncan, T. M. (2004) in The Enzymes: Energy Coupling and Molecular Motors (Hackney, D. D., and Tamao, F., eds) 3rd Ed., pp. 203–275, Elsevier Academic Press, New York
4. Nakanishi-Matsui, M., and Futai, M. (2006) IUBMB Life 58, 318–322
5. Feniouk, B. A., Suzuki, T., and Yoshida, M. (2006) Biochim. Biophys. Acta 1757, 326–338
6. Dunn, S. D., Cipriano, D. J., and Del Rizzo, P. A. (2004) in Handbook of ATPases: Biochemistry, Cell Biology, Pathophysiology (Futai, M., Wada, Y., and Kaplan, J. H., eds) pp. 311–318, Wiley-VCH, Weinheim, Germany
7. Weber, J. (2007) Trends Biochem. Sci. 32, 53–56
8. Weber, J. (2006) Biochim. Biophys. Acta 1757, 1162–1170
9. Walker, J. E., and Dickson, V. K. (2006) Biochim. Biophys. Acta 1757, 286–296
10. Richter, M. L., Samra, H. S., He, F., Giessel, A. J., and Kuczera, K. K. (2005) J. Bioenerg. Biomembr. 37, 467–473
11. Dunn, S. D., Revington, M., Cipriano, D. J., and Shilton, B. H. (2000) J. Bioenerg. Biomembr. 32, 347–355
12. Dmitriev, O., Jones, P. C., Jiang, W., and Fillingame, R. H. (1999) J. Biol. Chem. 274, 15598–15604
13. Revington, M., McLachlin, D. T., Shaw, G. S., and Dunn, S. D. (1999) J. Biol. Chem. 274, 31094–31101
14. Sorgen, P. L., Bubb, M. R., McCormick, K. A., Edison, A. S., and Cain, B. D. (1998) Biochemistry 37, 923–932
15. Takeyama, M., Noumi, T., Maeda, M., and Futai, M. (1988) J. Biol. Chem. 263, 16106–16112
16. McLachlin, D. T., Bestard, J. A., and Dunn, S. D. (1998) J. Biol. Chem. 273, 15162–15168
17. Bhatt, D., Cole, S. P., Grabar, T. B., Claggett, S. B., and Cain, B. D. (2005) J. Bioenerg. Biomembr. 37, 67–74
18. McLachlin, D. T., and Dunn, S. D. (2000) Biochemistry 39, 3486–3490
19. McLachlin, D. T., Coveny, A. M., Clark, S. M., and Dunn, S. D. (2000) J. Biol. Chem. 275, 17571–17577
20. Caviston, T. L., Ketchum, C. J., Sorgen, P. L., Nakamoto, R. K., and Cain, B. D. (1998) FEBS Lett. 429, 201–206
21. Weber, J., Wilke-Mounts, S., Nadanaciva, S., and Senior, A. E. (2004) J. Biol. Chem. 279, 11253–11258
22. Futai, M., Sternweis, P. C., and Heppel, L. A. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 2725–2729
23. Cipriano, D. J., Wood, K. S., Bi, Y., and Dunn, S. D. (2006) J. Biol. Chem. 281, 12408–12413
24. McLachlin, D. T., and Dunn, S. D. (1997) J. Biol. Chem. 272, 21233–21239
25. Revington, M., Dunn, S. D., and Shaw, G. S. (2002) Protein Sci. 11, 1227–1238
26. Del Rizzo, P. A., Bi, Y., Dunn, S. D., and Shilton, B. H. (2002) Biochemistry 41, 6875–6884
27. Lupas, A. (1996) Trends Biochem. Sci. 21, 375–382
28. Lupas, A. N., and Gruber, M. (2005) Adv. Protein Chem. 70, 37–78
29. Del Rizzo, P. A., Bi, Y., and Dunn, S. D. (2006) J. Mol. Biol. 364, 735–746
30. Dunn, S. D., and Chandler, J. (1998) J. Biol. Chem. 273, 8646–8651
31. Penefsky, H. S. (1979) Methods Enzymol. 56, 527–530
32. Briere, L. K., and Dunn, S. D. (2006) Biochemistry 45, 8607–8616
33. Santoro, M. M., and Bolen, D. W. (1988) Biochemistry 27, 8063–8068
34. Swint, L., and Robertson, A. D. (1993) Protein Sci. 2, 2037–2049
35. Laemmli, U. K. (1970) Nature 227, 680–685
36. McLachlin, D. T., and Dunn, S. D. (1996) Protein Expression Purif. 7, 275–280
37. Dunn, S. D. (1986) Anal. Biochem. 157, 144–153
38. Agheler, R., Capaldi, R. A., Dunn, S., and Gogol, E. P. (1992) Arch. Biochem. Biophys. 296, 685–690
39. Grabar, T. B., and Cain, B. D. (2004) J. Biol. Chem. 279, 31205–31211
40. Diez, M., Borsch, M., Zimmermann, B., Turina, P., Dunn, S. D., and Graber, P. (2004) Biochemistry 43, 1054–1064
41. Krebstakies, T., Zimmermann, B., Graber, P., Altendorf, K., Borsch, M., and Greie, J. C. (2005) J. Biol. Chem. 280, 33338–33345
42. Dunn, S. D., Bi, Y., and Revington, M. (2000) Biochim. Biophys. Acta 1459, 521–527
43. Harrison, C. J., Hayer-Hartl, M., Di Liberto, M., Hartl, F., and Kuriyan, J. (1997) Science 276, 431–435
44. Peng, G., Bostina, M., Radermacher, M., Rais, I., Karas, M., and Michel, H. (2006) FEBS Lett. 580, 5934–5940
45. Dunn, S. D., Kellner, E., and Lill, H. (2001) Biochemistry 40, 187–192
46. Claggett, S. B., Grabar, T. B., Dunn, S. D., and Cain, B. D. (2007) J. Bacteriol. 189, 5463–5471