Disulfide Linkage of the *b* and δ Subunits Does Not Affect the Function of the *Escherichia coli* ATP Synthase†

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ABSTRACT: The ATP synthase of *Escherichia coli* is believed to act through a rotational mechanism in which the $b_2\delta$ subcomplex holds the $\alpha\beta$ hexamer stationary relative to the rotating γ and ϵ subunits. We have engineered a disulfide bond between cysteines introduced at position 158 of the δ subunit and at a position just beyond the normal C-terminus of the *b* subunit. The formation of this disulfide bond verifies that the C-terminal region of *b* is proximal to residue 158 of δ. The disulfide bond does not affect the ability of the F_1F_0 complex to hydrolyze ATP, couple ATP hydrolysis to the establishment of a proton gradient, or maintain a proton gradient generated by the electron transport chain. These results are consistent with a permanent association of b_2 with δ as suggested by the rotational model of enzyme function.

ATP synthase (reviewed in refs *1*−*4*) acts in oxidative phosphorylation to couple the dissipation of a transmembrane proton motive force to the synthesis of ATP from ADP and inorganic phosphate. The enzyme is composed of two interacting sectors: F_0, which is membrane-integral and acts as a proton pore, and F_1, which is membrane-peripheral and houses the three catalytic sites. In *Escherichia coli*, F_0 has three subunits of stoichiometry $a_1b_2c_{9-12}$, and F_1 contains five subunits of stoichiometry $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$. When detached from the membrane, F_1 acts strictly to hydrolyze ATP, but the F_1F_0 complex can either use proton movement to synthesize ATP or act as an ATP-dependent proton pump.

The crystal structure of most of the $\alpha_3\beta_3\gamma$ complex from bovine heart mitochondria (*5*) and electron micrographs of F_1F_0 (*6*) have provided much insight into the structure of ATP synthase. The α and β subunits alternate in a ring surrounding two long helices from the γ subunit. The rest of γ and the ε subunit form a central stalk structure that connects the αβ hexamer to F_0 (*7*). A peripheral stalk structure, composed of the two *b* subunits along with δ, also joins α and β to F_0 (*8*, *9*).

A variety of evidence indicates that the energy from proton movement across the membrane is transferred to the catalytic sites through rotation of the γ and ε subunits relative to α and β (*10*−*18*). It is thought that the *b* and δ subunits act as a stator to prevent $\alpha_3\beta_3$ from rotating along with γ and ε (*3*, *4*). An interaction between the *b* and δ subunits has been demonstrated by a number of techniques, including the yeast two-hybrid system (*19*), nuclear magnetic resonance spectroscopy (*20*), and analytical ultracentrifugation (*21*). The *b* and δ subunits or their homologues have been chemically cross-linked in the mitochondrial (*22*), chloroplast (*23*), and *E. coli* (*24*) enzymes. The cross-link in chloroplast ATP synthase was obtained using the cross-linker 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC)[1] and was localized to the C-terminal region of subunit I (homologous to *E. coli b*) and the N-terminal 165 residues of the 187-residue δ subunit (*23*). In the *E. coli* system, the cross-link took place between cysteine residues introduced near the C-terminus of the 156-residue *b* subunit and a site within the C-terminal 29 residues of δ, possibly at residue Met-158 (*24*).

The EDC treatment of chloroplast ATP synthase resulted in inhibition of photophosphorylation and ATP hydrolysis by F_1F_0 (*23*). These effects could have been caused by the cross-link between δ and subunit I or by modification and/or cross-linking of other subunits of the complex. In this work we used the information gained from our previous chemical cross-linking studies to engineer a disulfide link between the *b* and δ subunits of the *E. coli* enzyme and measured the effect of this disulfide bond on enzyme function.

EXPERIMENTAL PROCEDURES

General Methods. Recombinant DNA techniques were carried out essentially as described in Sambrook et al. (*25*). Protein concentrations were assayed by the method of Lowry et al. (*26*). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) was performed by the method of Laemmli (*27*) using 15% separating gels, and proteins were stained with Coomassie Brilliant Blue R-250. Western blotting was carried out using carbonate blot buffer as

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[1] Abbreviations: EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMG buffer, buffer containing 50 mM sodium phosphate pH 7.0, 5 mM $MgCl_2$, and 10% glycerol; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MOPS−sucrose buffer, buffer containing 10 mM MOPS pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, and 10% methanol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid).

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described previously (*28*). Antibodies were labeled with ^{125}I by the IODO-GEN method (*29*). The anti-*b* monoclonal antibody was a generous gift of Drs. Karlheinz Altendorf and Gabriele Deckers-Hebestreit of Universität Osnabrück. Anti-*δ* antibodies were prepared as described (*24*).

Construction of Plasmids. Plasmid pACWU1.2 (*30*), which expresses the entire *unc* operon with all endogenous cysteine residues mutated to alanine, was the parent for plasmids used to express F_1F_0 bearing cysteine mutations in both *b* and *δ*. These plasmids were constructed as follows. The *uncF* and *uncH* genes were first subcloned in two steps into pTZ18u (*31*). In the first step of the subcloning, most of the *uncF* gene was amplified by polymerase chain reaction (PCR) using pACWU1.2 as a template; during this step a silent mutation was added to introduce an *Eco*RV restriction site at the codon for Asp-147 of *b*. This fragment was cloned into the multiple cloning region of pTZ18u to give pDM58. In the second step of the subcloning, the rest of *uncF* (including the introduced *Eco*RV site) and the entire *uncH* gene was amplified by PCR using pACWU1.2 as a template. This fragment was inserted into pDM58 using the *Eco*RV site to give pDM59, which encoded both *uncF* and *uncH* containing three mutations derived from pACWU1.2 (*b*C21A, *δ*C65A, and *δ*C141A) as well as a silent *Eco*RV site at the codon for Asp-147 of *b*. These background mutations will hereafter be considered "wild type".

Mutagenic PCR was carried out using either pDM59 or pACWU1.2 as the template to generate the *δ*M158C mutation along with either the *b*E155C mutation or a Gly−Cys extension (hereafter referred to simply as *b*158C) onto the C-terminus of *b*. The PCR products were inserted into the unique *Eco*RV and *Bsi*WI sites of pDM59 (wt) to give pDM60 (*b*E155C/*δ*M158C) or pDM61 (*b*158C/*δ*M158C). Part of the *uncF* gene (including the introduced *Eco*RV site) and the entire *uncH* gene from plasmids pDM59, pDM60, and pDM61 were subcloned into pACWU1.2 using either *Nar*I or *Ppu*MI along with *Afl*II. The resulting plasmids were named pDM62 (wt), pDM63 (*b*E155C/*δ*M158C), and pDM64 (*b*158C/*δ*M158C). These plasmids were identical to pACWU1.2 except for the cysteine mutations introduced into the *uncF* and *uncH* genes and the silent *Eco*RV site in *uncF*.

Preparation of Membranes. Plasmids pDM62, pDM63, and pDM64 were transformed into *E. coli* strain DK8, from which the *unc* operon has been deleted (*32*). Cells were grown at 37 °C in 1 L of 2×YT medium supplemented with 20 mM sodium phosphate pH 7.0 until A_{600} reached approximately 3. The cells were then harvested, washed twice with 50 mM sodium phosphate pH 7.0 and 5 mM $MgCl_2$, resuspended in a volume of 50 mM sodium phosphate pH 7.0, 5 mM $MgCl_2$, and 10% glycerol (PMG buffer) corresponding to two times their wet weight, and stored at −80 °C. To prepare membranes, cells were thawed and a further five volumes of PMG buffer were added along with 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride. The cells were passed once through a French pressure cell at 20 000 psi, and cell debris was removed by centrifugation at 10 000 rpm for 10 min. The supernatant was subjected to further centrifugation at 38 000 rpm for 90 min to collect the membrane fraction. The pellet from this centrifugation step was washed with 10 mM MOPS pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, and 10% methanol (MOPS−sucrose buffer) containing 1 mM DTT, centrifuged again, and washed a second time with MOPS−sucrose buffer without DTT. After another centrifugation step the membranes were resuspended to about 30 mg of protein/mL in MOPS−sucrose buffer and stored at −80 °C.

Formation of b−δ Disulfide Bonds. Membranes bearing F_1F_0 expressed from pDM62 (wt), pDM63 (*b*E155C/*δ*M158C), or pDM64 (*b*158C/*δ*M158C) were diluted to 1.11 mg of protein/mL in MOPS−sucrose buffer at room temperature. Copper(II) chloride was added to either 10 or 100 *μ*M such that the final protein concentration was 1 mg/mL. The membranes were allowed to stand at room temperature for various time periods, but typically 20 min, before EDTA was added to 1 mM to chelate the Cu^{2+}. Five minutes after the addition of EDTA, samples were taken for use in assays or for analysis by Western blotting.

Measurement of ATPase Activity. ATPase activity was measured at 37 °C using the colorimetric assay of Futai et al. (*33*), except that the final reaction mixture contained 10 mM HEPES−KOH pH 7.5, 4.5 mM $MgCl_2$, 150 mM KCl, and 4 mM ATP.

Energy-Dependent Fluorescence Quenching. The establishment of a proton gradient was monitored by observing the quenching of quinacrine fluorescence essentially as described by Fraga and Fillingame (*34*). Membrane samples containing 150 *μ*g of total protein were diluted to 2 mL with 10 mM HEPES−KOH pH 7.5, 5 mM $MgCl_2$, and 300 mM KCl to which 5 *μ*L of 0.15 mg/mL quinacrine in ethanol had been added immediately beforehand. The samples were monitored at room temperature using a Hitachi F-4101 fluorescence spectrophotometer. The excitation wavelength was 422 ± 2.5 nm, and the emission wavelength measured was 520 ± 5 nm. After the sample had achieved a stable signal, ATP was added to a final concentration of 2 mM (to measure ATP-driven fluorescence quenching), or NADH was added to a final concentration of 0.5 mM (to measure NADH-driven fluorescence quenching). The fluorescence was monitored for 150 s after addition of ATP or NADH.

RESULTS

Disulfide Bond Formation between b and δ. Previously, we modified cysteine residues introduced at the penultimate position of *b* (*b*E155C) or as a Gly−Cys addition to the C-terminus (*b*158C) with a chemical cross-linker and observed formation of *b*-*δ* cross-links in F_1F_0 (*24*). Peptide mapping and sequence analysis showed that the site of cross-linking within *δ* was C-terminal to residue Met-148 and suggested that Met-158 was involved. To confirm the proximity of *δ*Met-158 to the C-terminal region of *b*, and to test the effect of covalent linkage of *b* and *δ* on enzyme function, we sought to engineer a disulfide bond between the two subunits at position 158 of *δ*. The M158C mutation was introduced along with either *b*E155C or *b*158C into plasmid pACWU1.2, which expresses the entire *unc* operon with all of the endogenous cysteine residues mutated to alanine (*30*). This plasmid was used to avoid complication of the experiment by the possibility of disulfide bond formation among naturally occurring cysteine residues within the complex. An *unc* deletion strain bearing either pair of mutations (*b*E155C/*δ*M158C or *b*158C/*δ*M158C) grew as well using succinate as the sole carbon/energy source as the same strain complemented with wild-type pACWU1.2.

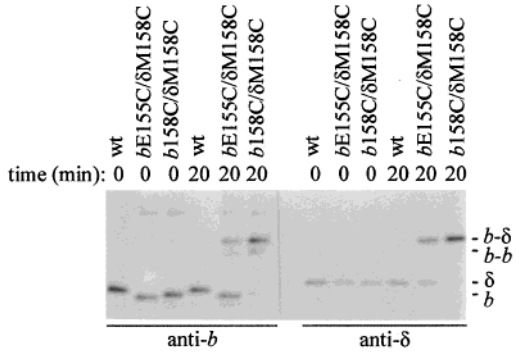

FIGURE 1: Disulfide formation between b and δ. Membranes were prepared from *unc E. coli* cells complemented by plasmid pDM62 (wt), pDM63 (*b*E155C/δM158C), or pDM64 (*b*158C/δM158C). The membranes were treated with 10 μM $CuCl_2$ in open tubes for 20 min at room temperature before samples were quenched with *N*-ethyl maleimide and analyzed by nonreducing SDS−PAGE followed by Western blotting. The blot was probed with a monoclonal antibody raised against *b* or polyclonal antibodies raised against δ. The zero time points were taken immediately before the addition of $CuCl_2$.

Membranes prepared from these cells were treated with $CuCl_2$ to induce disulfide bond formation. Treatment with 10 μM $CuCl_2$ for 20 min resulted in almost complete conversion of δ to $b-\delta$ in *b*158C/δM158C membranes, as estimated from the disappearance of the *b* and δ bands and the appearance of the cross-linked band (Figure 1), while disulfide bond formation in the *b*E155C/δM158C membranes was not as efficient. The anti-δ polyclonal antibodies recognized δ that had been cross-linked to *b* much more strongly than un-cross-linked δ. In addition to the $b-\delta$ cross-link, a small amount of $b-b$ dimer was observed with the *b*158C protein. Treatment of membranes with concentrations of $CuCl_2$ lower than 10 μM, with 10 μM $CuCl_2$ in the presence of 10 mM free cysteine, or with other reagents such as glutathione, dithionitrobenzoate, or dibromobimane resulted in cross-linking that was less efficient than observed in Figure 1 (data not shown).

Effect of Disulfide Bond Formation on Enzyme Function. Membranes treated with $CuCl_2$ were tested for their ability to hydrolyze ATP and to generate a proton gradient using either ATP or NADH as a substrate. In addition to treatment with 10 μM $CuCl_2$ as shown in Figure 1, the membranes were also treated with 100 μM $CuCl_2$ to ensure maximal disulfide bond formation. Hydrolysis of ATP by membranes treated with either level of $CuCl_2$ was unimpaired (Table 1), and since the vast majority of the ATPase activity of the membranes is derived from F_1F_0, this observation indicates that the formation of the cross-links had no significant effect on ATP hydrolysis by F_1F_0.

To determine whether ATP hydrolysis was coupled to proton translocation, the membranes were tested for their ability to quench the fluorescence of quinacrine when supplied with ATP. Treatment with 10 or 100 μM $CuCl_2$ had no significant effect on the ability of the membranes to generate a proton gradient (Figure 2A,B). Disulfide bond formation in the samples tested was found to be nearly complete (Figure 3), and therefore $b-\delta$ disulfide bond formation did not significantly inhibit coupling of ATP hydrolysis to proton pumping. Treatment with 100 μM $CuCl_2$ gave rise to a faint, diffuse new band on SDS−PAGE that migrated between the positions of unlinked *b* and the *b* dimer.

Table 1: Effect of $b-\delta$ Disulfide Formation on ATPase Activity[a]

membrane preparation	$[CuCl_2]$ (μM)	relative activity ± SD (%)[b]
wild type	0	100
	10	97 ± 5
	100	99 ± 5
*b*158C/δM158C	0	100
	10	98 ± 5
	100	103 ± 7

[a] Membranes were prepared and treated with various concentrations of $CuCl_2$ as described in Experimental Procedures. [b] Specific activities are expressed relative to the activity observed for no $CuCl_2$ treatment. The mean of three determinations ± standard deviation is shown. Individual activity determinations ranged from 0.8−0.9 U/mg for the wild-type membranes and 1.1−1.2 U/mg for the *b*158C/δM158C membranes. One unit of activity is defined as the amount of enzyme required to hydrolyze 1 μmol of ATP in 1 min.

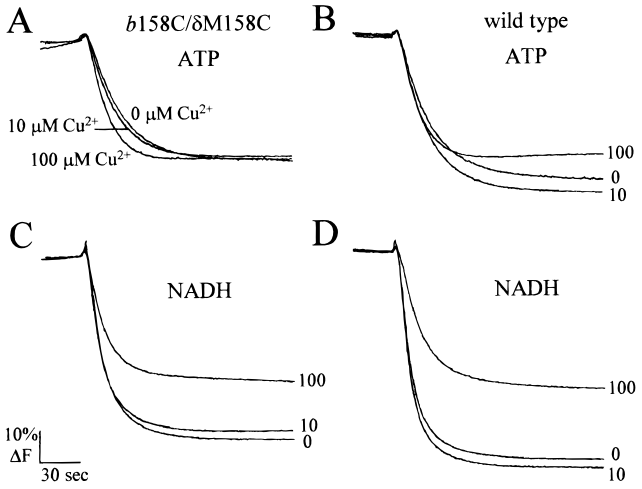

FIGURE 2: ATP- and NADH-driven fluorescence quenching. Membranes were prepared from *unc E. coli* cells complemented by plasmid pDM64 (*b*158C/δM158C). The membranes were treated with 0, 10, or 100 μM $CuCl_2$ in open tubes for 20 min at room temperature. Samples were then diluted into buffer containing quinacrine, either 2 mM ATP (panel A) or 0.5 mM NADH (panel C) was added, and the change in quinacrine fluorescence was monitored. The experiments were repeated using wild-type enzyme (panels B and D).

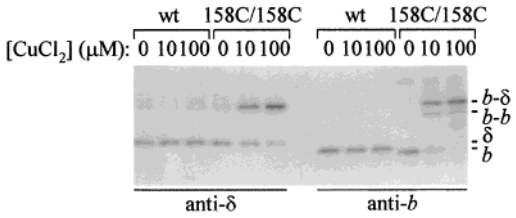

FIGURE 3: Disulfide bond formation in membranes assayed in Figure 2A,B. Samples of the membranes assayed in Figure 2A,B were placed in an SDS−PAGE sample buffer containing 15 mM *N*-ethyl maleimide and analyzed by Western blotting. The blot was probed with antibodies against δ and *b* as indicated.

The band was recognized by monoclonal antibodies raised against the *b* subunit, but its exact origin is unknown.

The ability of the membranes to maintain a proton gradient when supplied with NADH was also tested. When disulfide bond formation was not induced, the *b*158C/δM158C membranes were able to maintain a proton gradient as effectively as the wild-type membranes (Figure 2C,D). Treatment of the *b*158C/δM158C membranes with 10 μM

$CuCl_2$ resulted in no significant change in the proton gradient in either wild type or mutant membranes (Figure 2C,D). Treatment with 100 μM $CuCl_2$ caused a similar decrease in the strength of the proton gradient in both types of membranes (Figure 2C,D). Because it occurred in both wild type and *b*158C/δM158C membranes, this decrease was probably due to effects of the $CuCl_2$ on components of the membrane other than the F_1F_0 complex. These results show that the ability of the *b*158C/δM158C membranes to maintain a stable proton gradient was not affected by disulfide bond formation between *b* and δ.

DISCUSSION

The results described here demonstrate that the C-terminus of the *b* subunit is in close proximity to residue Met-158 of the δ subunit and that disulfide bond formation between *b* and δ has no significant effect on the ATP hydrolysis or proton pumping activities of ATP synthase or its permeability to protons.

The observation of efficient disulfide bond formation between *b*158C and δM158C at a relatively low concentration of $CuCl_2$ (Figure 1) supports the conclusion drawn previously (*24*) that residue M158 of δ is located close to the C-terminus of the *b* subunit. It is probable that poorer disulfide bond formation was observed with the *b*E155C mutation than with the *b*158C mutation due to structural constraints imposed on *b* in the F_1F_0 complex. Disulfide bond formation might have been more efficient in the 158C protein because the cysteine was linked to the rest of *b* by a glycine residue, which was included to provide conformational flexibility. The previously observed cross-linking between *b*E155C and δM158 may have been possible due to the distance between the reactive groups on the cross-linker used (approximately 11 Å).

Recently other workers have replaced the C-terminal residue of *b*, Leu-156, with a cysteine residue, and have obtained cross-linking between this cysteine and the endogenous Cys-90 of the α subunit (*35*). Using the crystal structure of bovine heart mitochondrial F_1 as a guide, Cys-90 is predicted to be within the N-terminal β-barrel domain of α. This domain has been implicated in the binding of δ (*36*, *37*). These results suggest that residues *b*Leu-156, αCys-90, and δMet-158 are close to each other in the F_1F_0 complex.

Together the *b* and δ subunits are believed to reach from the membrane to near the top of the F_1 sector to form the peripheral stalk which may function to hold the $\alpha_3\beta_3$ hexamer stationary during rotation of γ and ε driven by proton flow through F_0 (*8*, *9*). The lack of a significant effect of disulfide bond formation on F_1F_0 activity is consistent with the idea that the interaction between *b* and δ is stable through the reaction cycle and not transient as some have suggested (*38*). It is possible that the *b* and δ subunits change their conformation or shift their position relative to each other during catalysis, but they appear to remain in direct contact at all times.

Others have reported that treatment of chloroplast ATP synthase with EDC resulted in a cross-link between δ and subunit I, which is homologous to *E. coli b*, as well as inhibition of photophosphorylation and F_1F_0-catalyzed ATP hydrolysis (*23*). It was unclear whether the effects on activity were caused by the δ−I cross-link or by modification or linking of other subunits by the cross-linker. In contrast, the cross-linking reported here is specific to the *b* and δ subunits. Because resistance to passive proton movement across the membrane by F_1F_0 was not affected by cross-linking of *b* and δ (Figure 2C,D), it appears that the cross-linking had no major structural consequences for the F_0 sector.

The disulfide bond between *b*L156C and αCys-90 obtained by Rodgers and Capaldi was shown not to affect ATPase activity but did inhibit ATP-dependent proton pumping (*35*). The authors ascribed the latter effect to a need for conformational flexibility in the region joining the second stalk to $\alpha_3\beta_3$. In other work it was shown that an engineered disulfide bond between the α and δ subunits had no significant effect on the activity of F_1F_0 (*37*). This result and the work presented here imply that a stable $b_2\delta$ complex remains associated with a single α subunit throughout the catalytic cycle. While consistent with the proposed function of the second stalk as a stator, a permanent association of the second stalk with one particular α necessitates a permanent asymmetry among the three α subunits. Recent spin-labeling studies have shown that binding of the hydrophilic portion of *b* to F_1 causes conformational changes at the active site.[2] Whether these conformational changes result in functional asymmetry of the catalytic sites remains to be determined.

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