Characterization of a $b_2\delta$ Complex from Escherichia coli ATP Synthase*

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The $\delta$ subunit of Escherichia coli ATP synthase has been expressed and purified, both as the intact polypeptide and as $\delta_i$, a proteolytic fragment composed of residues 1–134. The solution structure of $\delta_i$ as a five-helix bundle has been previously reported (Wilkens, S., Dunn, S. D., Chandler, J., Dahlquist, F. W., and Capaldi, R. A. (1997) Nat. Struct. Biol. 4, 198–201). The $\delta$ subunit, in conjunction with $\delta$-depleted F$_1$-ATPase, was fully capable of reconstituting energy-dependent fluorescence quenching in membrane vesicles that had been depleted of F$_1$. A complex of $\delta$ with the cytoplasmic domain of the $b$ subunit of F$_0$ was demonstrated and characterized by analytical ultracentrifugation using p$_{ST34-156}$, a form of the $b$ domain lacking aromatic residues. Molecular weight determination by sedimentation equilibrium supported a $b_2\delta$ subunit stoichiometry. The sedimentation coefficient of the complex, 2.1 S, indicated a frictional ratio of approximately 2, suggesting that $\delta$ and the $b$ dimer are arranged in an end-to-end rather than side-by-side manner. These results indicate the feasibility of the $b_2\delta$ complex reaching from the membrane to the membrane-distal portion of the F$_1$ sector, as required if it is to serve as a second stalk.

Recent work has strongly indicated that hydrolysis of ATP by F$_1$ is accompanied by rotation of the $\gamma$ and $\epsilon$ subunits relative to the $\alpha_3\beta_3$ hexameric ring (5–11), consistent with proposals from Paul Boyer’s laboratory (12). The high resolution structure of the mitochondrial F$_1$ (13) reveals that the N and C termini of the $\alpha_3$ subunits are responsible for coupling their catalytic activities.

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The proton-translocating ATP synthases couple the generation of ATP to the protonotive force present across membranes involved in energy transduction (for reviews, see Refs. 1–4). These complex enzymes consist of a peripheral F$_1$ sector, which catalyzes ATP synthesis and hydrolysis, and an integral F$_0$ sector, which catalyzes movements of protons across the membrane. In the relatively simple ATP synthase of Escherichia coli, F$_1$ contains five types of subunits in a stoichiometry of $\alpha_3\beta_3\gamma_6\epsilon_6$, while F$_0$ contains three types of subunits in a stoichiometry of $\alpha_{b_9-12}\delta$. Subunit interactions at the interface of the two sectors are responsible for coupling their catalytic activities.

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In all systems, $\delta$ or the analogous mitochondrial protein called oligomycin sensitivity conferral protein (OSCP),$^1$ is essential for the coupling of the catalytic activities of the two sectors. The $\delta$ subunit (reviewed in Ref. 14) has no significant effect on steady-state ATP hydrolysis rates by isolated F$_1$-ATPase but does alter unisite hydrolysis (15). $\delta$ binds to F$_1$ through interactions with the external surface of the N-terminal third of the $a$ subunit (16–20). In some systems, $\delta$ alters the proton permeability of F$_0$ (21). This effect is not seen in E. coli, but here $\delta$ is essential for the interaction of F$_1$ and F$_0$, implying a link between $\delta$ and F$_0$ (22). The physical and functional nature of the $\delta$-F$_0$ interaction is currently the subject of intense interest. In recent work, an interaction of $\delta$ or OSCP with the $b$ subunit of F$_0$ has been demonstrated (23–25). Nearest neighbor analysis by chemical cross-linking had not revealed cross-links between $b$ and $\delta$ in the E. coli system (26), but they had been reported for the corresponding subunits in the chloroplast (27) and mitochondrial (28) enzymes. The importance of the cytoplasmic domain of $\delta$ to the F$_1$–F$_0$ interaction has also been demonstrated through proteolysis (29–31) and direct binding (32) studies.

E. coli $\delta$ purified following pyridine treatment of F$_1$-ATPase was shown to be an elongated monomer (33), but the low yield of the preparation limited the scope of work that could be carried out. The current studies were undertaken to produce recombinant $\delta$ in quantities appropriate for high resolution structural analysis and to permit the characterization of interactions of $\delta$ with other subunits in ATP synthase. Here we describe the preparation of recombinant $\delta$ and a proteolytic fragment called $\delta_i$ as well as the hydrodynamic analysis of a complex of $\delta$ with the cytoplasmic domain of the $b$ subunit of F$_0$. The solution structure of $\delta_i$ has been previously reported (34).

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids—**Recombinant DNA procedures were carried out as described by Sambrook et al. (35) using E. coli strain MM294 (36) as the host. Plasmid pHN2 (37), which carries the lac promoter, lacI*, and the unc transcription terminator, was used as the vector. The E. coli uncH DNA sequence encoding the $\delta$ subunit was amplified, and the translation initiation region was altered using the expression cassette polymerase chain reaction procedure of MacFerrin et al. (38). The upstream primer, CGCGGATTCTGGAGGAGATTAAAAATTTGTTGTA,

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1 The abbreviations used are: OSCP, oligomycin sensitivity conferral proteins; $b_{\alpha_{\gamma-12}}$ and $b_{\alpha_{\gamma-156}}$, forms of the cytoplasmic domain of the $b$ subunit containing residues Val$^{12}$_-Leu$^{156}$ and Tyr$^{24}$_-Leu$^{156}$, respectively; $b_{\alpha_{\gamma-156}}$ and $b_{\alpha_{\gamma-156}}$, forms of the cytoplasmic domain of the $b$ subunit containing residues Glu$^{32}$_-Leu$^{156}$ and Asp$^{54}$_-Leu$^{156}$, respectively, preceded by the leader sequence Ser-Tyr-Trp, assuming removal of the initiating methionine; $b_{\alpha_{\gamma-156}}$, a form of the cytoplasmic domain of the $b$ subunit containing residues Glu$^{32}$_-Leu$^{156}$ preceded by a leader sequence Ser-Thr, assuming removal of the initiating methionine; $s_{\alpha_{\gamma-12}}$, the sedimentation coefficient observed under the experimental conditions.
TTATTACCGG, contained an EcoRI cloning site, a Shine-Dalgarno sequence, an AT-rich spacer region, and the first 19 bases of the uncH coding sequence. The downstream primer, GCATCCGCGGTTAACG- TCGAAGGCTGCTG, contained a Smal cloning site and the last 20 bases of the uncH coding sequence. The PCR product was cut with EcoRI and Smal and then ligated into pH12 that had been digested with the same enzymes, to produce plasmid pJC1. The uncH gene in pJC1 was sequenced and compared with the published sequence (39) to confirm that no mutations had been introduced within the coding region.

A form of the b subunit lacking aromatic residues, b_{ST34–156}, was expressed from plasmid pD3. This plasmid was constructed using polymerase chain reaction to mutate the N-terminal sequence of b_{uncH}, encoded by plasmid pDM3 (40). The mutagenic primer, CCGGCATA GGTTAAAGCCCGCCAAG, included successive NdeI and ScaI sites, which encoded a Met-Ser-Thr leader followed by 16 bases encoding the b sequence beginning with residue Gln1; the M13 universal primer was used as the downstream primer. The product was cut with NdeI and BglII and inserted into pIB2, a derivative of pSD80 (41) containing a modified b_{uncH} coding sequence (40), in which an NdeI site had been placed at the start codon. The insert was sequenced to ensure that no undesired mutations had been introduced.

Purification of δ—Strain MM294/pJC1 was grown at 30 °C in LB broth with vigorous shaking. When the cells reached a density that gave an A_{600} of 0.8, isopropylthiogalactoside was added to a concentration of 1 mM, and growth was continued for 3–4 h. After harvesting and washing, the cell pellet was suspended in 10 volumes of cold 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂. All subsequent steps were carried out at 4 °C. Phenylmethylsulfonyl fluoride was added to a concentration of 1 mM, and the cells were broken by one passage through a French pressure cell at 20,000 p.s.i. Cell debris, ribosomes, and membranes were removed by centrifugation for 1.5 h at 40,000 rpm in a Beckman Ti-60 rotor. Soluble proteins were fractionated by ammonium sulfate precipitation and column chromatography. Material precipitating between 20 and 32% of saturation was collected, redissolved in one-half volume of 2.7 ml. In one set of experiments, the rotor speed was 60,000 rpm, and scans were taken at 10-min intervals. Data were analyzed using the software provided by Beckman.

Other Materials and Methods—b_{ST34–156} was purified using techniques similar to those employed for other forms of the b subunit by size exclusion chromatography on Sephadex G-75. Fractions used in analytical ultracentrifugation experiments had absorbance at 280 nm of less than 0.005/mg/ml. The expression and purification of two other forms of the b subunit, b_{D4–156} and b_{D5–156}, have been described previously (40). δ-Depleted F₁-ATPase was the generous gift of Drs. S. Wilkens and R. Capaldi of the University of Oregon (Eugene, OR).

RESULTS

Design of pJC1 and Expression of δ—Previous plasmids carrying uncH, which encodes δ, and its natural translation initiation region gave poor expression of δ, even when transcription was from a high level promoter. Two factors that may have contributed to this low expression were corrected in the construction of pJC1. First, evidence that an mRNA secondary structure encompassing the region of uncH from the Shine-Dalgarno to residue 36 of the coding sequence strongly reduces expression has been presented by Pati and co-workers (47). In the construction of plasmid pJC1, the expression cassette polymerase chain reaction strategy of MacFerrin et al. (38) was used to replace C and G residues in the spacer region between the Shine-Dalgarno and the initiation codon with A or T to weaken this secondary structure, making the translation initiation region more accessible to ribosomes. Second, in pJC1 the unc transcriptional terminator was placed downstream of the uncH sequence. We have previously shown that the terminator placed immediately after a coding sequence can increase expression by stabilizing the transcript against exonucleolytic attack (48).

Induction of pJC1 with isopropyl-1-thio-β-d-galactopyranoside at 37 °C led to the synthesis of large amounts of δ, but essentially all of the expressed subunit was in the form of insoluble inclusion bodies (data not shown). Growth and induction of cells at 30 °C led to the expression of good levels of soluble δ (see Fig. 1A), although if the induction period was extended beyond 3 h, some of the subunit was insoluble. The soluble portion of δ was purified to homogeneity using the standard procedures of ammonium sulfate precipitation and using ion exchange and size exclusion chromatography, as described under “Experimental Procedures.” Samples of the preparation at various stages were examined by SDS-polyacrylamide gel electrophoresis (Fig. 1A).

The δ subunit was unstable in the crude extract. Coincident with the loss of δ was the appearance of a new 14-kDa polypeptide, designated δ′ because it could be identified immunologically as a fragment of δ (data not shown). For the purification of intact δ, proteinase was minimized by designing the ammonium sulfate fractionation to provide higher purification in exchange for a lower yield. Nevertheless, a portion of δ was cleaved during the subsequent dialysis in preparation for DEAE-Sepharose chromatography, and δ′ eluted from this column just after δ with some overlap, as can be seen in the samples of sequential column fractions in Fig. 1B (also note the material at position of the arrow in the DEAE pool of Fig. 1A). The residual δ′ and the higher molecular weight impurities were removed from δ by size exclusion chromatography on Sephadex G-75. The δ′ fragment has also been purified using Sephadex G-75.

The recombinant δ was capable of fulfilling the role of the subunit in energy transduction. In the experiment shown in Fig. 2, the indicated amounts of either δ or δ-depleted F₁-ATPase were mixed with an excess of the complementary component and then reconstituted with membrane vesicles depleted of their endogenous F₁, and ATP-dependent quinacrine quenching was measured. Essentially the same molar amounts of δ or δ-depleted F₁-ATPase were required to restore maximal quenching.

2 S. D. Dunn, unpublished observations.
3 S. Wilkens and R. A. Capaldi, personal communication.
quenching, indicating that the recombinant δ is folded properly and fully functional.

Interaction of β and δ—Preliminary experiments conducted by techniques such as size exclusion chromatography indicated that any interaction between δ and β (32), the cytoplasmic domain of β, would be relatively weak. To see such an interaction, we carried out sedimentation velocity experiments in the analytical ultracentrifuge. This technique has the advantages that relatively high concentrations may be analyzed, the sample is not significantly diluted during the experiment, and information about the size and shape of the complex can be obtained.

Isolated recombinant δ sedimented at 20 °C with sobs = 1.67 S (Table I), a value similar to that of the expressed cytoplasmic domain of β, which forms dimers (32, 40). These proteins sediment slowly considering their molecular weights, indicating that both, especially β, are extended. A complex of the two proteins should sediment more rapidly because of its greater molecular weight, and the extent of the increase will depend on the overall shape of the complex. Experiments summarized in Table I show that mixtures of the soluble δ domains b34–156 or b53–156 with δ sedimented slightly more rapidly than either component alone, indicating complex formation to some extent. The complex did not sediment rapidly enough to form a boundary that could be resolved from that of the excess β domains that were present in these experiments, so the observed sedimentation coefficients reflect contributions from both complexed and unassociated species. Accordingly, it was difficult to ascertain whether the observed s values reflected substantial formation of a complex that sedimented only marginally faster than either individual component or weaker formation of a complex that would sediment substantially faster.

Characterization of a (δST34–156)2β Complex by Sedimentation Velocity.—To eliminate the contribution of the excess free β subunit to the observed s value, we produced a form of β lacking aromatic residues (see “Experimental Procedures” for details). The only tryptophan and tyrosine residues present in b34–156 and b53–156 were added at their N termini during plasmid construction to make them detectable at 280 nm, so removing them would not be expected to affect the interaction with δ. In the new polypeptide, a leader sequence Met-Ser-Thr was fused to residues Glu34 to Leu156 of β; since the N-terminal methionine is ordinarily removed, this polypeptide was called bST34–156. Lacking aromatic residues, bST34–156 is transparent at 280 nm but can still be observed at wavelengths below 250 nm due to absorbance by the peptide bond and certain side chains. Sedimentation equilibrium experiments showed bST34–156 to be essentially dimeric.
Molecular weight determinations by sedimentation equilibrium analysis

Sedimentation equilibrium analysis was carried out at 20 °C in six-sector cells as described under "Experimental Procedures."

| Component, inferred molecular weight | Rotor speed | Concentration; wavelength | Analysis method, a molecular weight ± S.D.; number of determinations |
|-------------------------------------|-------------|---------------------------|---------------------------------------------------------------------|
| \((b_{ST34–156})_2\)                | 27,504      | 30,000                | 0.5, 1.0, or 2.0 mg/ml; 240 nm                                       | One component, 28,500 ± 1,400; 8                                    |
| \(\delta\)                          | 19,204      | 15,000                | 1 mg/ml; 280 nm                                                   | One component, 22,500 ± 400; 3                                     |
| \((b_{ST34–156})_2\delta\)          | 46,708      | 15,000                | 1 mg of \(\delta\) ml, 4 mg of \((b_{ST34–156})_2\) ml; 280 nm | One component, 40,100 ± 500; 5                                      |
|                                     |             |                       | Two components, 44,900 ± 1600; 5                                  |

a More details of this experiment can be found in the legend to Fig. 4.

b The partial specific volumes of \((b_{ST34–156})_2\) and \(\delta\) were calculated to be 0.740 and 0.742 cm³ per g, respectively.

c The same data were fitted for either one or two components. For the two-component fit, the first component was taken to be free \(\delta\), and the best fit for the molecular weight of a second, noninteracting component was determined.

(see observed and inferred molecular weights in Table II) but with a slight tendency to become monomeric at low protein concentrations. In sedimentation velocity analysis, a low concentration of \((b_{ST34–156})_2\) (0.5 mg/ml) sedimented at 1.47 S, and the sedimentation coefficient increased to 1.71 at higher concentrations (Fig. 3, A). Again, this pattern suggests some degree of dissociation into monomers at low protein concentration, so the value observed at the higher concentrations is more likely an accurate reflection of the sedimentation coefficient of the dimer.

A set of sedimentation velocity experiments was conducted in which \(\delta\) at a concentration of 0.5 mg/ml was sedimented in the presence of various concentrations of \((b_{ST34–156})_2\) (Fig. 3, B). Three or four determinations were made at each concentration, with the S.D. indicated by the error bars. The addition of the \((b_{ST34–156})_2\) caused the observed \(s\) value of \(\delta\) to rise rapidly from 1.67 to 2.08 S and then to rise more slowly with further increases in \(b\) concentration. Calculation of \(s_{20,\mu}\) by correction of the data for solvent density provides a value of 2.07–2.16, depending on whether one uses the \(s_{obs}\) value obtained at 4 mg/ml \((b_{ST34–156})_2\) or extrapolates to zero \(b\) concentration as shown in Fig. 3. Regardless of the choice, these results demonstrate that the complex sediments only moderately faster than either component alone.

Sedimentation coefficients were also measured for \((b_{ST34–156})_2\delta\) mixtures in the presence of 50 mM Mg²⁺, since this ion is particularly effective in maintaining the F₁₁F₅₆ interaction. At low concentrations of the \(b\) domain, Mg²⁺ caused a slight reduction in the rate of sedimentation, but there was little effect at \((b_{ST34–156})_2\delta\) concentrations of 3 or 4 mg/ml (data not shown).

**Sedimentation Equilibrium Analysis of the \((b_{ST34–156})_2\delta\) Interaction**—Sedimentation equilibrium experiments were carried out to confirm the subunit stoichiometry of the complex as \((b_{ST34–156})_2\delta\). In this experiment, the starting concentrations of \(\delta\) and \((b_{ST34–156})_2\) were 1 and 4 mg/ml, respectively; this represents a 2:8-fold molar excess of \((b_{ST34–156})_2\) dimer over \(\delta\). With these high concentrations and the low rotor speeds used (12,000–18,000 rpm), substantial concentrations of all species were maintained even near the meniscus so that complex formation would be favored. As in the sedimentation velocity analyses, the distribution of \(\delta\) and complexes containing \(\delta\) could be determined at 280 nm without any direct contribution from \((b_{ST34–156})_2\) representative data are shown in Fig. 4. The apparent concentration gradient of \(\delta\) in the presence of the \(b\) domain (A) was significantly steeper than when the subunit was present alone (B), indicative of formation of a complex with higher molecular weight. The results were analyzed in two ways (Table II). Determination of the molecular weight that gave the best fit for a single component provided a value of just over 40,000, which can be compared with the inferred weight of 46,708 for the \((b_{ST34–156})_2\delta\) complex. Alternatively, the data could be fitted to two noninteracting components, \(\delta\) and a second component of unknown molecular weight. The best fit for the second component was about 45,000, which closely approaches the expected value for the \((b_{ST34–156})_2\delta\) complex.

**Summary of Properties of \((b_{ST34–156})_2\delta\)**—Information about both the shape of the complex and the affinity of the subunits can be obtained from the ultracentrifugation data shown in Fig. 3 and Table II. Given a stoichiometry of two copies of \((b_{ST34–156})_2\) to one of \(\delta\), which was indicated by the sedimentation equilibrium results, the sedimentation coefficient allows calculation of a frictional ratio of 2.03–2.13 (Table III). In comparison, the frictional ratio of \(\delta\), a moderately extended protein, was 1.42, while that of the highly extended \((b_{ST34–156})_2\) was 1.76. These results indicate that the complex is more extended than either the \(b\) dimer or the \(\delta\) subunit alone.

Three of the sets of velocity data shown in Fig. 3 were collected at \((b_{ST34–156})_2\) concentrations of 0.5, 0.72, and 1.0 mg/ml, which correspond to molar ratios of \((b_{ST34–156})_2\) dimer to \(\delta\) of 0.7, 1.0, and 1.39. Calculation of the \(K_d\) of the complex based on the fraction of \(\delta\) migrating as a complex gave values of 5–10 μM.
and energy coupling. These results imply that essentially all of the preparations with very high activity for reconstitution of enzyme were soluble, permitting an analysis of size and shape. Sedimentation velocity and equilibrium experiments using the $b_{ST34-156}$ construct showed that they produce an extended complex with a subunit stoichiometry of $b\delta$. While this stoichiometry might be expected, given the fact that the cytoplasmic domain of $b$ forms dimers in solution (32), it was possible that $\delta$ might displace one of the subunits of the $b$ dimer to produce a $b\delta$ heterodimer. The molecular weight of such a complex containing the $b_{ST34-156}$ construct would be 32,956. In contrast, we determined that the average molecular weight of species containing $\delta$ in mixtures of such a complex was 46,708, which is expected for the $b\delta$ complex.

The interaction of $\delta$ with the cytoplasmic domain of $b$ appears to be rapidly reversible and relatively weak, since no interaction could be detected by size exclusion chromatography. The $K_d$ calculated from the sedimentation rate of $\delta$ in the presence of subsaturating levels of $b_{ST34-156}$ was in the range of 5–10 $\mu$m, if $b$ concentrations are expressed as the dimer. However, our results suggest that a small fraction exists as monomer at the low protein concentrations used, so this must be taken as a rough estimate of affinity. Although somewhat weak, the $b\delta$ interaction nevertheless appears to be essential for the proper binding of $E. coli$ F$_1$-ATPase to $F_0$, since $\delta$ is essential for this assembly and it seems unlikely to interact with any other subunit of $F_0$.

The shape of the $b_{ST34-156}\delta$ complex was revealed by sedimentation velocity ultracentrifugation to be highly extended, with a frictional ratio of 2.1. The high value of this frictional ratio reveals the complex to be at least as asymmetric as $b_{ST34-156}$, the more asymmetric of its parts. If the $\delta$ dimer and $\delta$ were to interact side-by-side, one would expect the complex to be less asymmetric with a lower frictional ratio. Instead, the $b\delta$ interaction could be detected by size exclusion chromatography. The $K_d$ calculated from the sedimentation rate of $\delta$ in the presence of subsaturating levels of $b_{ST34-156}$ was in the range of 5–10 $\mu$m, if $b$ concentrations are expressed as the dimer. However, our results suggest that a small fraction exists as monomer at the low protein concentrations used, so this must be taken as a rough estimate of affinity. Although somewhat weak, the $b\delta$ interaction nevertheless appears to be essential for the proper binding of $E. coli$ F$_1$-ATPase to $F_0$, since $\delta$ is essential for this assembly and it seems unlikely to interact with any other subunit of $F_0$.
the γ subunit rotates inside. The weakness of the interaction, however, suggests to us that the complex must be stabilized in the entire enzyme, possibly through interactions of b with other subunits in the F₈ sector.

From data presented here, the first 52 residues from the N terminus of b are not essential for the interaction of b and δ. The lack of effect of δ domains on the NMR signal of δ’ (25) and the effect of proteolytic digestion of δ on the binding of F₈ to F₉ (18) suggest that δ will interact with the C-terminal part of the δ subunit. Further analysis of the regions of b and δ involved in the interaction is currently under way in this laboratory.

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