Quantitative Determination of Direct Binding of b Subunit to F1 in Escherichia coli F1F0-ATP Synthase*

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The stator in F1F0-ATP synthase resists strain generated by rotor torque. In Escherichia coli, the β3 subunit complex comprises the stator, bound to subunit α in F0 and to the αβγ hexagon of F1. To quantitatively characterize binding of b subunit to the F1 αβγ hexagon, we developed fluorimetric assays in which wild-type F1, or F1 enzymes containing introduced Trp residues, were titrated with a soluble portion of the b subunit (bST34–156). With five different F1 enzymes, Kd(bST34–156) ranged from 91 to 157 nM. Binding was strongly Mg2+ dependent; in EDTA buffer, Kd(bST34–156) was increased to 1.25 μM. The addition of the cytoplasmic portion of the b subunit increases the affinity of binding of b subunit to δ-depleted F1. The apparent Kd(bST34–156) for this effect was increased from 150 nM in Mg2+ buffer to 1.36 μM in EDTA buffer. This work demonstrates quantitatively how binding of the cytoplasmic portion of the b subunit directly to F1 contributes to stator resistance and emphasizes the importance of Mg2+ in stator interactions.

F1F0-ATP synthase is the enzyme responsible for ATP synthesis by oxidative phosphorylation or photophosphorylation in mitochondria, chloroplasts, and bacteria and for ATP-driven generation of proton or sodium gradients in bacteria. Even before the discovery that it acts a molecular rotary motor (1), it was clear that this was a protein of considerable structural complexity. In Escherichia coli, the F1 moiety lies peripheral to the membrane, with the subunit composition αβγ(αβγ)ε, and the F0 moiety, consisting of subunits αβ(αβ)n (where n is not established at time of writing), is intrinsic to the membrane. Subunit complements in higher organisms can be considerably more complex, and there is notable variation in the number of c subunits between species (2–5).

Until 1994, the consensus based on electron microscopy (for one example, see Ref. 6) and biochemical studies (for one example, see Ref. 7) was that F1 was bound to F0 by a single “central stalk” presumed to contain γ, ε, δ, and b subunits. The x-ray crystallography studies of F1 by Walker and colleagues (8, 9) changed that view by showing that the central stalk did contain γ and ε subunits, but contrary to previous ideas, could not accommodate δ and b subunits for lack of space. Subsequently, electron microscopy studies (10) and biochemical studies (11–14) have established that the δ and b subunits form a separate stalk structure, at the periphery of F1. With the emerging view of ATP synthase as a rotary motor, the central stalk is now referred to as the rotor (consisting of γε and the ring of ε subunits) and the peripheral stalk (consisting of δ and b subunits) as the stator.

The role of the stator (b,δ) is to hold one portion of the enzyme (αβγε) while the other portion rotates (γεring), thus allowing the very efficient transfer of energy between the proton “channel” and the catalytic sites (15). Rotary strain can amount to ~50 kJ/mol (15, 16); therefore, strong interactions between stator subunits are required. δ subunit is known to interact with the N-terminal region of α subunit at the very “top” of F1 (i.e. most distant from the membrane surface) (17–19). The binding affinity (Kd) between δ and F1 is ~1 nM, equivalent to 50 kJ/mol (20–22) when measured directly, and is increased considerably, to possibly Kd ~1 μM when the cytoplasmic portion of the b subunit is included in the binding assay (22). The N-terminal domain of δ interacts with α (19, 23). The b subunit interacts with the C-terminal domain of δ (24–26), with the C-terminal residues of the b subunit playing an important role. It is an interesting question whether the b subunit also interacts directly with F1 α and/or β subunits. There is evidence that this is the case. For example, cross-linking studies showed that Cys residues introduced at positions 92, 109, or 110 in b could be cross-linked to α and/or β subunits (27), and other cross-linking studies indicated close proximity of b to α (28). Native gel electrophoresis studies of F1 and the cytoplasmic portion of b showed formation of a complex (24). Also, the addition of the cytoplasmic portion of the b subunit altered the conformation of the F1 catalytic sites as shown by spin-labeling studies (29). However, these approaches could not quantitatively determine the strength of interaction between b and F1.

In this report, we titrated soluble F1 containing introduced Trp residues in the α or β subunit with a soluble cytoplasmic portion of the b subunit. By following the changes in fluorescence, we ascertained the Kd for direct interaction between b and F1. We then studied the influence of Mg2+ cation and of the presence or absence of b subunit on the b/F1 interaction. In related studies, we studied the effects of Mg2+ cation on the ability of the cytoplasmic portion of b subunit to promote binding of δ subunit to F1, and we introduced Trp residues into the b subunit at residues 92 and 109 to test the effects on interaction with F1.

EXPERIMENTAL PROCEDURES

Purification of F1, Purification of β subunit, Preparation of δ-depleted F1, Purification of bST34–156, Competition Assay of Binding of bST34–156 to F1 Using ATP-driven Proton Pumping in Reconstituted Membrane Vesicles, Routine Procedures—These were all as described previously (21, 22).
Fluorescence Binding Assay—The buffer for fluorescence titrations was 50 mM HEPES/NaOH, pH 7.0, room temperature, with either 5 mM MgSO4 or 0.5 mM EDTA. Excitation was at 295 nm. For measurement of binding of purified δ subunit to δ-depleted F1, the δ-depleted F1 was the βW107 F1, from strain pSWM86/DKs as described previously (21). βW107 F1 contains only the native Trp at residue β107, it has no normal functional properties, and it has a low Trp background signal in the δ-binding assay. Binding of δ subunit was monitored by enhancement of fluorescence at 325 nm (21, 22). For measurement of binding of $b_{\beta\delta\delta_{s}}$ to F1, a series of different F1 enzymes was used in which novel Trp residues had been inserted into otherwise Trp-free F1 (see below). The fluorescence signal at 335 nm was monitored. For most of the enzymes used, this was close to a max. No significant shift (3–5 nm) in $b_{\beta\delta\delta_{s}}$ was observed upon the addition of $b_{\beta\delta\delta_{s}}$ to any F1. It was ascertained that the presence of EDTA or Mg2+ had no effect on F1 fluorescence spectra, nor did deletion of the subunit. F1 concentration in the cuvette was varied from 100 to 500 nM. $b_{\beta\delta\delta_{s}}$ was added incrementally, usually up to 1.5 μM concentration (equivalent to 0.75 μM b dimers) but higher when necessary to reach saturation. All of the enzymes are expected from previous work to have fully occupied non-catalytic sites. To be sure that the signal changes seen on the addition of $b_{\beta\delta\delta_{s}}$ were not due to changes in nucleotide occupancy of catalytic sites, we conducted experiments on enzyme that had been passed consecutively through two 1-m1 Sephadex G-50 centrifuge columns in Tris/EDTA, pH 8.0, which removes all catalytic site nucleotide while leaving the noncatalytic sites fully occupied (30). The results were not altered in any way by this precaution. All fluorescence values were routinely corrected for by added $b_{\beta\delta\delta_{s}}$.

Results

Strains for purification of Trp-containing F1 enzymes were described in Refs. 21 and 22. Strains for purification of Trp-containing F1 enzymes were described in Refs. 38, 39, and references therein. Wild-type F1 was from strain SWM1 (40), and δW25L F1, was from strain pSWM92/DKs (21). Oligonucleotide-directed mutagenesis (41) of $b_{ST34}$ was used to generate the mutations bA292W and bI109W. The template was M13mp19 containing the EcoRI-HindIII fragment from plasmid pJBI3 (34), which contains a synthetic gene encoding a Met-Ser-Thr leader sequence (34). The oligonucleotide for bI109W, in which the italicized bases encode the Trp mutation and the underlined bases introduce a BspI site. The oligonucleotide for bI110W was 5'-GGCGAAGCGAAGAAATGGGGAGTCGAGGCGAACACGCTC-3', in which the italicized bases encode the Trp mutation and the underlined bases introduce a BspI site. The oligonucleotide for bI110W was 5'-GGCGAAGCGAAGAAATGGGGAGTCGAGGCGAACACGCTC-3', in which the italicized bases encode the Trp mutation and the underlined bases introduce a BspI site.

E. coli Strains and bST34 Mutagenesis—Strains for purification of wild-type and mutant δ subunits were described in Refs. 21 and 22. E. coli strains and mutagenesis of δ ST34—Strains for purification of Trp-containing F1 enzymes were described in Refs. 38, 39, and references therein. Wild-type F1 was from strain SWM1 (40), and δW25L F1, was from strain pSWM92/DKs (21). Oligonucleotide-directed mutagenesis (41) of $b_{ST34}$ was used to generate the mutations bA292W and bI109W. The template was M13mp19 containing the EcoRI-HindIII fragment from plasmid pJBI3 (34), which contains a synthetic gene encoding a Met-Ser-Thr leader sequence (34). The oligonucleotide for bI109W, in which the italicized bases encode the Trp mutation and the underlined bases introduce a BspI site. The oligonucleotide for bI110W was 5'-GGCGAAGCGAAGAAATGGGGAGTCGAGGCGAACACGCTC-3', in which the italicized bases encode the Trp mutation and the underlined bases introduce a BspI site. The oligonucleotide for bI110W was 5'-GGCGAAGCGAAGAAATGGGGAGTCGAGGCGAACACGCTC-3', in which the italicized bases encode the Trp mutation and the underlined bases introduce a BspI site.

RESULTS

Fluorescence Titration of F1 with the Soluble Cytoplasmic Portion of the b Subunit—The cytoplasmic portion of the b subunit used here was $b_{ST34}$, as described in Ref. 34. It consists of residues 34 through 156 (C terminus) of the b subunit with an additional Ser-Thr- sequence at the N terminus. We had previously confirmed (22) that it yields a single band on SDS gels after purification in soluble form and that it is fully functional as determined by the assay of Dunn (32) in which binding of F1 to stripped $E. coli$ membranes is competed for by added $b_{ST34}$. Although theoretically $b_{ST34}$ contains no Trp residue, we found from its fluorescence spectrum in 6 mM guanidine chloride that Trp contamination was present, in the amount of 0.09 mol/mol, and we routinely corrected for this. Titrations were carried out in either 5 mM MgSO4 or 0.5 mM EDTA-containing buffer.

The fluorophore in the titrations was either the native Trp residues in wild-type F1 or Trp inserted at a single residue position in otherwise Trp-free F1. Wild-type F1 contains nine native Trps, comprised of three in α, three in β, two in γ, and one in δ subunit. We found that there was fluorescence enhancement on the addition of $b_{ST34}$ of maximally 8.6% (average of six experiments). This allowed calculation of $K_{f}$ values for binding of $b_{ST34}$ to F1. Since the fluorescence enhancement seen in the βW107 enzyme (below) was +5.3%, it appears that this residue contributes significantly to the fluorescence enhancement seen in wild-type F1.

A series of F1 variants in which all the native Trp had been substituted (43) and a novel F1 had been incorporated at a single residue position in the α or β subunit was also tested. In each case, we confirmed that an E. coli strain containing the mutant enzyme showed normal growth on succinate plates and normal growth yield in limiting glucose, to ensure that enzyme function was not impaired. None of these F1 enzymes showed a fluorescence quench. Several of the enzymes gave no change in fluorescence when titrated with $b_{ST34}$ at concentrations up to 3.0 μM and F1 concentration of 100 nM. These were: αF291W, αL346W, βF148W, βY331W, and βF398W. A further group gave a fluorescence enhancement of ≤6% at the highest $b_{ST34}$ concentration. These were: αF409W, βF418W, βY331W, and βW107 (the last contains just the single natural Trp at residue β107, with the other natural Trp residues substituted (21)). A third group of F1 enzymes gave larger fluorescence changes on the addition of $b_{ST34}$. These were: αL55W (+14.5%), αF406W (+8.5%), βF269W (+16%), and βF410W (+24%) (average values for between 4 and 20 experiments). We decided to use this last group for determination of $K_{f}$ values.

Typical titration curves for binding of $b_{ST34}$ to wild-type, αL55W, αF406W, βF269W, and βF410W F1 in the presence of Mg2+ are shown in Fig. 1, A–E, filled symbols. Calculated $K_{f}$ values are shown in Table I. There was good agreement among $K_{f}$ calculated for all five F1 enzymes, with the values being 100–150 nM. One would not expect that the introduced Trp residues in the mutant F1 would themselves perturb $K_{f}$ significantly since all of the enzymes used show normal oxidative phosphorylation in vivo as judged by growth tests.

Effect of δ Subunit on Binding of $b_{ST34}$ to F1—Two approaches were used to investigate the effect of δ subunit on binding of $b_{ST34}$ to F1. First, we prepared δ-depleted F1 following the procedure in Ref. 21. This was done for several of the enzymes including αL55W, αF406W, βF269W, βF410W, and wild type. In all cases, the absence of δ was checked on SDS gels. We then titrated the δ-depleted F1 with $b_{ST34}$ and found that the titration curves were essentially the same as for the corresponding intact F1, yielding similar $K_{f}$ values. Typical titration curves are shown in Fig. 2, A and B, for the βF410W and βF269W enzymes, respectively. In a second approach, we added pure δ subunit containing the δW28L mutation at 500 nM concentration (i.e. 5-fold excess over F1) to either intact F1 or δ-depleted F1 and then carried out the $b_{ST34}$ titrations. Again, no change in the titration curve was seen (data not shown). Therefore, the presence or absence of δ did not affect the titration curves or the $K_{f}$ values.

Effect of EDTA on Binding of $b_{ST34}$ to F1—When 0.5 mM EDTA was included in the buffer instead of Mg2+, there was a clear effect upon the titration curves, as shown in Fig. 1, open symbols. For all of the enzymes, the degree of fluorescence enhancement was much reduced in the presence of EDTA, although there was indication of residual binding, and it appeared that the $K_{f}$ value was increased to ≥1 μM. Of the five enzymes shown in Fig. 1, open symbols, only the βF410W enzyme had a signal enhancement in EDTA that was large enough (∼5%, Fig. 1E) to enable us to attempt to determine a
determination of $K_d$ in EDTA-containing buffer. The enhancement of fluorescence upon the addition of $b_{156}$. The same effect was seen with other weakly binding mutants from 0.5 M to 5 nM in the absence to 5 nM in the presence of $b_{156}$ was reduced to $b_{156}$ due to technical limitations of procedures. We had previously shown that $b_{156}$ strongly promotes the binding of isolated $b$ subunit to $b$-depleted $F_1$ in the presence of Mg$^{2+}$ (22). In that work, we had used a mutant $\delta$ subunit preparation ($\delta$Y11W/W28L) that binds relatively weakly in the absence of added $b_{156}$ to see the effect. (With wild-type $\delta$, the $K_d$ is already so low without added $b_{156}$ that little effect is seen upon the addition of $b_{156}$ due to technical limitations of the assay.) The $K_d$ for binding of $\delta$Y11W/W28L $\delta$ was reduced from 0.5 M in the absence to $\approx$ 5 nM in the presence of $b_{156}$. The same effect was seen with other weakly binding mutant $\delta$ preparations (22). Here we repeated those experiments in EDTA-containing buffer. The enhancement of fluorescence of the $\delta$-Trp-11 residue upon binding of $\delta$Y11W/W28L subunit to $\delta$-depleted $\beta$W107 $F_1$ was measured (see “Experimental Procedures”). It was first confirmed that the addition of $b_{156}$ to the $\delta$Y11W/W28L subunit had no effect on $\delta$-Trp-11 fluorescence in the absence of $F_1$. It is apparent (Fig. 4) that in the presence of EDTA, the Y11W/W28L mutant $\delta$ preparation showed essentially no binding to $F_1$ in the absence of $b_{156}$; but in the presence of $b_{156}$ there was measurable binding, and the calculated $K_d$ value for binding of $\delta$ was 745 nM (mean of five experiments). We confirmed that $b_{156}$ had the same effect to increase the affinity of other weakly binding mutant $\delta$ preparations ($\delta$Y11A, $\delta$V79W/W28L, $\delta$V79A; data not shown).

To calculate the apparent $K_d$ for the effect of $b_{156}$ to increase the affinity of $\delta$-binding in EDTA, we carried out titrations in which $b_{156}$ was added incrementally to $\delta$-depleted $F_1$ (500 nM) in the presence of constant $Y11W/W28L$ mutant $\delta$ (1 M). Control experiments showed that under these conditions, there was essentially no change in the fluorescence of $F_1$ due to the addition of $b_{156}$ when added $\delta$ was absent. Fig. 5 shows a typical experiment. From three such experiments, an average $K_d$ (apparent) for $b_{156}$ of 1.36 M was calculated. We also varied the concentration of added $\delta$ from 1 to 5 M; this did not significantly affect the result. Previously, in Mg$^{2+}$-containing buffer, $K_d$ (apparent) of 150 nM for $b_{156}$ was reported (22). The effect of EDTA is therefore substantial, and we may conclude that binding of $b_{156}$ is much tighter in the presence of divalent cation.

**Insertion of Trp at Positions 92 and 109 of $b$ Subunit**—Previous work has shown that Cys residues, inserted at positions Ala-92 and Ile-109 of the $b$ subunit, may be cross-linked

**TABLE I**

$K_d$ values for binding of the soluble cytoplasmic portion of the $b$ subunit ($b_{156}$) to soluble $F_1$

| $F_1$          | $K_d$ (nM) |
|---------------|------------|
| Wild type     | 93 ± 27 (6) |
| $aL55W$       | 157 ± 43 (6) |
| $aF406W$      | 152 ± 63 (6) |
| $aF410W$      | 63 ± 46 (14) |
| $aF410W$      | 91 ± 21 (12) |

*Values given are ± standard deviation with number of experiments in parentheses. All values are calculated for $b_d$ dimer.

$K_d$ value. By using higher concentrations of $F_1$ and $b_{156}$, we determined a $K_d$ value of 1.25 M (average of four experiments using 250–500 nM $F_1$, $K_d$ value range was 870 nM to 1.71 M). A typical experiment is shown in Fig. 3.

**Effect of $b_{156}$ to Increase Affinity of Binding of Isolated $\delta$ Subunit to $\delta$-depleted $F_1$ in Presence of EDTA**—We had previously shown that $b_{156}$ strongly promotes the binding of isolated $\delta$ subunit to $\delta$-depleted $F_1$ in the presence of Mg$^{2+}$ (22). In that work, we had used a mutant $\delta$ subunit preparation ($\delta$Y11W/W28L) that binds relatively weakly in the absence of added $b_{156}$ to see the effect. (With wild-type $\delta$, the $K_d$ is already so low without added $b_{156}$ that little effect is seen upon the addition of $b_{156}$ due to technical limitations of the assay.) The $K_d$ for binding of $\delta$Y11W/W28L $\delta$ was reduced from 0.5 M in the absence to $\approx$ 5 nM in the presence of $b_{156}$. The same effect was seen with other weakly binding mutant $\delta$ preparations (22). Here we repeated those experiments in EDTA-containing buffer. The enhancement of fluorescence

**FIG. 1.** Fluorescence titration of wild-type, $aL55W$, $aF406W$, $aF410W$, and $aF410W$ $F_1$, with $b_{156}$. Experiments using 100 nM $F_1$ are shown; higher concentrations were also used. Excitation was at 295 nm, and emission was measured at 335 nm. The solid lines are fits to the data by nonlinear regression analysis. Calculated $K_d$ values for binding are given in Table I. A, wild type; B, $aL55W$; C, $aF406W$; D, $aF410W$; E, $aF410W$ $F_1$. Filled symbols, with 5 mM MgSO$_4$; open symbols, with 0.5 mM EDTA. Note that when the fluorescence response was small (<5%), no fits for determination of $K_d$ were attempted (A–D, open symbols).
by a photoactivated cross-linking reagent to residues in the α and/or β subunits of F₁ (27), suggesting that these residues of β subunit, or the region around them, might normally be close to or directly interact with F₁. Here we inserted Trp at these residues in βST34–156 to generate βA92WST34–156 and βI109WST34–156. Both variant forms of βST34–156 could be purified in the same yield and purity as for parent βST34–156. Fig. 6 shows the results of competition assays in which binding of wild-type F₁ to stripped membranes containing F₀ was measured in the presence of increasing concentrations of mutant βST34–156. It is seen that although βA92WST34–156 competed as well as wild-type βST34–156, βI109WST34–156 did not compete, demonstrating that the substitution of Trp at residue Ile-109 abrogates binding to F₁, whereas a Trp at residue Ala-92 had no effect. It was also confirmed, using the same assay, that βA92WST34–156 and βST34–156 bound equally well to Trp-free F₁ (data not shown). As we discussed earlier (21), this assay cannot give absolute values of $K_d$ for βST34–156 binding; hence no fits to the curves were attempted in Fig. 6.

Both βA92WST34–156 and βI109WST34–156 had substantial fluorescence signals, with emission maxima at 347 and 342 nm, respectively. However, when βA92WST34–156 was added to Trp-free F₁, only a small enhancement of the Trp-92 fluorescence signal was seen (maximally +3%), which was too small for determination of $K_d$.

**DISCUSSION**

The first goal of this work was to determine whether and with what affinity the β subunit binds directly to F₁ in *E. coli* F₁F₀-ATP synthase. To do this, we used the soluble cytoplasmic portion of the β subunit named βST34–156, which had previously been constructed by Dunn and Chandler (34). Using fluorimet-
contain Mg^{2+}. The binding of b_{ST34-156} to F1 was strongly dependent on Mg^{2+}, and in the presence of excess EDTA, binding was much reduced. Using the F1 with the largest signal (βF410W), a K_d of 1.25 μM for binding of b_{ST34-156} in EDTA was calculated. In previous work, we showed that binding of δ subunit to δ-depleted F1 was also promoted by the presence of Mg^{2+}. X-ray crystallography of F1, has so far visualized Mg^{2+} cation bound only in nucleotide binding sites. Our work predicts that bound "structural Mg^{2+}" may be present in the stator and that it contributes in an important way to the ability of the stator to resist rotor strain. Methods in current use to release F1 from membranes in soluble form invariably utilize conditions in which free divalent cation concentration is minimized, and it is well known that reconstitution of F1 with F0 is strongly favored by Mg^{2+} ions.

The size of the fluorescence responses of inserted Trp residues that occurred upon binding of b_{ST34-156} to F1 should not be taken as evidence of direct interaction (or not) of that specific residue with b_{ST34-156}; nevertheless, by comparing the location of these Trp residues with the fluorescence responses seen, a general correlation is obvious. Those residues that gave zero or small fluorescence response lie buried inside the F1 molecule (e.g. αF291W, αF346W, βF297W, βF148W, βF331W, βR398W) or are cryptically sited close to γ (αF090W). On the other hand, those that gave strong signals are located on the external surface of the α_δβ_3 hexagon (αL55W, αF406W, βF26W, βF410W). One apparent exception could be βF17W, which lies on the outside and gave no signal, but it is actually located in the N-terminal β-barrel domain right at the top of the molecule, probably well away from where the b subunit binds (see e.g. Ref. 44). As noted under “Results,” the signal seen in wild-type F1 is to a significant extent referable to the naturally occurring β-Trp-107, which is also located on the exterior surface. Therefore, the residues that reported binding of b_{ST34-156} by fluorescence enhancement are all located on the exterior surface of the α_δβ_3 hexagon. It may be noted that no attempt was made here to specifically design Trp-containing enzymes for the purpose of measuring b subunit binding; rather, we tested a group of enzymes that had been previously constructed for a variety of purposes in our laboratory. Our work shows that in principle, it should be possible, with the advent of high resolution structure information, to design probes with better signals that could report conformational changes at the F1/b subunit interface during rotation.

The functionally active form of the b subunit, and of cytoplasmic portions of the b subunit such as b_{ST34-156} has previously been shown to be a dimer (32–37). In recognition of this fact, we plotted all our titration data as a function of b concentration, and the K_d values for b_{ST34-156} listed in Table I are those calculated for the b_{ST34-156} dimer. Still, there is one puzzling point, which is that the K_d for b dimer formation is reported to be in the 1 μM range (36), which seems rather high (equivalent to 17 μg/ml b subunit in cells). We found K_d values of 100–150 nM for binding of b_{ST34-156} to F1. Our titration curves were well fitted by a hyperbolic binding curve with n = 1 (for b_{2} dimer) over the range of b concentration from zero to 3.0 μM, or higher in some instances; thus, we saw no concentration dependence of binding that would indicate a dependence on monomer-dimer equilibrium. Most previous experiments in which dimerization of b or cytoplasmic portions of b was studied were done in buffers devoid of Mg^{2+}, and the actual concentration dependence (K_d) for dimer formation in Mg^{2+} has not been studied carefully. This might be one explanatory factor. In Ref. 35, the possibility was also raised that the presence of F1 may provide a surface on which dimerization of

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**Fig. 5.** Determination of apparent K_d(b_{ST34-156}) for promotion of binding of δY11W/W28L δ subunit to δ-depleted F1 in presence of EDTA. δ subunit (1 μM) and δ-depleted F1 (500 nM) were present at fixed concentrations, and b_{ST34-156} was added incrementally as shown. Binding of δ subunit to δ-depleted F1 was measured by following the enhancement of δ-Trp-11 fluorescence at 325 nm. The buffer contained 0.5 mM EDTA.

**Fig. 6.** Investigation of binding of b{A92W}_{ST34-156} and b{I109W}_{ST34-156} to wild-type F1. The competition assay of Dunn (32) was used. Briefly, stripped E. coli membranes containing F0 (500 μg) were preincubated with wild-type F1, in an amount sufficient to almost saturate the F0 sites (40 μg, determined beforehand by titration). The degree of F0 binding was determined by the percentage of quench of acridine orange in the ATP-driven proton pumping assay. Increasing amounts of b_{ST34-156} b{A92W}_{ST34-156} or b{I109W}_{ST34-156} were included in the preincubation as noted. O, wild-type b{ST34-156}; C, b{A92W}_{ST34-156}; □, b{I109W}_{ST34-156}.
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b might be facilitated in cells, and this appears to provide the best current explanation of our results.

In previous work, we had shown that bST34–156 greatly promoted binding of δ subunit to δ-depleted F₁ in the presence of Mg2+ ions, with a Kd apparent of 150 nM for the δ dimer. It is interesting that this is close to the Kd values measured here for direct binding of bST34–156 to F₁ in the presence of Mg2+. We extended the previous work by measuring the binding of δ subunit binding to δ-depleted F₁ in the presence of EDTA. The new studies showed that the bST34–156 did still promote δ-binding but with a higher Kd apparent for the δ dimer of 1.36 μM. Interestingly, this is quite similar to the Kd value determined for the direct binding of bST34–156 to F₁ in the presence of EDTA (1.25 μM). Together the data reinforce the two conclusions evident above, that divalent cation (Mg2+) is important in stator structure and that direct binding of the cytoplasmic portion of δ subunit to F₁ provides an important contribution to stator stability.

Finally, we found that insertion of a novel Trp at position b-Ile-109 in bST34–156 prevented binding to F₁. Although this could be due to prevention of dimerization of bST34–156 or to disruption of normal folding, it is consistent with the idea that this is a specific site of interaction of b with F₁ as adumbrated by cross-linking studies (27). However, substitution of residue b-Ala-92 by Trp did not affect F₁ binding. In neither case could the fluorescence signal of the introduced Trp be used to assay binding to F₁ or to isolated δ subunit.

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Quantitative Determination of Direct Binding of b Subunit to F₁ in *Escherichia coli* F₁F₀-ATP Synthase

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