Identification of the F1-binding Surface on the δ-Subunit of ATP Synthase*

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The stator function in ATP synthase was studied by a combined mutagenesis and fluorescence approach. Specifically, binding of δ-subunit to δ-depleted F1 was studied. A plausible binding surface on δ-subunit was identified from conservation of amino acid sequence and the high resolution NMR structure. Specific mutations aimed at modulating binding were introduced onto this surface. Affinity of binding of wild-type and mutant δ-subunits to δ-depleted F1 was determined quantitatively using the fluorescence signals of natural δ-Trp-28, inserted δ-Trp-11, or inserted δ-Trp-79. The results demonstrate that helices 1 and δ in the N-terminal domain of the δ-subunit provide the F1-binding surface of δ. Unexpectedly, mutations that impaired binding between F1 and δ were found to not necessarily impair ATP synthase activity. Further investigation revealed that inclusion of the soluble cytoplasmic domain of the b subunit substantially enhanced affinity of binding of δ-subunit to F1. The new data show that the stator is “overengineered” to resist rotor torque during catalysis.

ATP synthase is the membrane enzyme responsible for ATP synthesis in oxidative and photophosphorylation of prokaryotes and eukaryotes, and also for ATP-driven proton pumping to generate the transmembrane proton gradient in bacterial membranes. In Escherichia coli, ATP synthase consists of a complex of eight subunits, αβγδεσσαα(2). It was defined in earlier times in terms of a membrane-peripheral F1 sector (αβγδεσσ), containing three catalytic sites, and a membrane-embedded F0 sector (abαααα), which carries out transmembrane proton transport. Recent work has demonstrated that the enzyme functions as a rotary motor. The centrally located “rotor” consists of subunits γεσσ. At the top, it rotates inside the αβγδ hexagon, and thus modifies the activities of the catalytic sites; at the base, it rotates against subunit α, thereby facilitating proton movement. In this way, the energy of the proton gradient is transduced into the energy of ATP synthesis/hydration. Understanding the mechanism by which this occurs is currently of major interest. To ensure that subunits α and αβββ remain firmly fixed in relation to each other they are connected by a peripheral structure, the “stator” stalk, consisting of subunits βδ and δ. For recent reviews of the structure and function of ATP synthase, see Refs. 1–3.

The stator must be able to resist strain resulting from rotor torque, thus its construction is of considerable importance. The dimer of δ-subunits forms an elongated helical connection between subunit α and the C-terminal domain of the δ-subunit, it lies at one side of the αβδ hexagon, and its functional domains have been well characterized (4–6). There may exist functional interactions between βδ and the αβγδ hexagon (7). Currently only partial high-resolution structure has been reported for the b subunit (8, 9). The δ-subunit has been shown by electron microscopy to bind to the very top (“crown”) of the αβδ hexagon (10), and the homologous mitochondrial OSCP(1) subunit also binds at the top of the molecule, with its C-terminal domain at the side (11). The N-terminal region of α-subunit (residues α1–15 or α1–19) was shown to be necessary for δ binding by proteolysis experiments (12), and cross-linking between an inserted Cys at residue α2 and natural Cys residue(α5) in δ (13) provided further evidence that the N terminus of α, which is not seen in high-resolution x-ray structures, binds to δ. In our laboratory, quantitative determination of the affinity of binding between δ-subunit and the αβδγε complex (also called “δ-depleted F”) using a novel fluorescence assay dependent upon the fluorescence of the single natural Trp in δ, revealed a $K_d$ of 1.4 nM in the E. coli enzyme (14). This is equivalent to a binding energy of ~50 kJ/mol, approximately equivalent to rotor torque (15, 16). A similar $K_d$ value was reported for the chloroplast enzyme (17) using a fluorescent probe attached to δ.

We were able to show that a fragment of δ containing only N-terminal residues 1–134δ (called δ') bound with the same affinity as intact δ, demonstrating that all the determinants of binding lie in the N-terminal region of δ (14). The structure of the N-terminal domain of δ (residues 2–106 in the fragment δ') has been determined to high-resolution by NMR (18). Examination of this structure in combination with homology searches of the sequences of other species of δ and OSCP suggested possible residues that might be involved in binding interaction at the F1/δ interface. Using the quantitative binding assay that we had developed, in combination with mutagenesis, we were able here to test these ideas. From the results, this paper presents the identification of the surface on the δ-subunit that provides interactions with F1. In addition we show that inclusion of the soluble cytoplasmic domain of the b subunit substantially enhanced the affinity of binding of δ-subunit to F1.

EXPERIMENTAL PROCEDURES

Purification of F1, Purification of δ-Subunit, Preparation of δ-depleted F0, Fluorescence Binding Assays, Assay of ATP-driven Proton Pumping in Reconstituted Membrane Vesicles, Routine Procedures—These were all as described previously (14). For measurement of binding of purified δ-subunit to δ-depleted F0, the δ-depleted F0 was the “6W107 F0,” from strain pSWM86/DK8 as previously described (14). βW107 F1 contains only the Trp at residue β-107, it has normal func-

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1 The abbreviations used are: OSCP, oligomycin-sensitivity conferral protein (the mitochondrial homolog of E. coli δ); βW107, soluble cytoplasmic domain of b subunit (20).

2 E. coli residue numbers are used throughout.
Fig. 1. High resolution (NMR) structure of the N-terminal domain of δ-subunit. The protein backbone from residue δ-Thr-106 (helix 1, dark blue) to residue δ-Thr-106 (helix 6, red) is shown. Helices are numbered 1–6. Residue δ-Trp-28, located in helix 2 (light blue), is depicted in “spacefill” representation. Adapted from Ref. 18 and displayed using PyMOL (31).

Fig. 2. Sequence conservation on a proposed binding surface of δ-subunit. A, the surface of the N-terminal domain of δ is shown in the same orientation as in Fig. 1. Strongly conserved or conservatively replaced residues are colored in red; partially conserved residues are colored in pink. The remaining residues of δ (gray) show a high degree of variability. Residues 11 and 14 in helix 1 and 75 and 79 in helix 5 were mutated in this work. Residue 85 was mutated in previous work and is discussed in the text. B, series of views obtained by rotating the molecule in 90° steps. The central depiction shows the molecule from the same angle as in A above and in Fig. 1.

Results

A Potential F1-binding Site on the δ-Subunit—Fig. 1 shows the NMR structure of the N-terminal domain of the δ-subunit of E. coli ATP synthase (18) as determined using the proteolytic fragment (δ) consisting of residues 1–134 (intact δ has 177 residues). As noted in the Introduction, because proteolytic removal of the 43 C-terminal residues has no effect on the affinity for binding to δ-depleted F1 (14), it is highly likely that the domain shown in Fig. 1 contains most, if not all, of the amino acid residues involved in binding of δ to F1. Comparison of the sequences of δ or OSCP from 90 species by BLAST search (21) showed that the highly conserved residues are clustered on a relatively small portion of the δ surface, as demonstrated in Fig. 2. Because δ sequences are in general not highly conserved, for example, in comparison with those of α- or β-subunits (22), it appeared probable that this conserved part of δ, formed by helices 1 and 5 (colored dark blue and yellow, respectively, in Fig. 1), constituted the F1-binding site. We decided to test this hypothesis by mutagenesis of four conserved residues, δ-Tyr-11, δ-Ala-14, δ-Asn-75, and δ-Val-79. From the BLAST search (above) we found the following degrees of conservation of these residues. At position δ-11, Tyr occurred in 95% of species and Phe in 5%; at δ-14, Ala occurred in 90%; at δ-75 Asn occurred in 78% and Asp in 6%; and at δ-79 a hydrophobic residue (Val, Leu, or Ile) occurred in 92% of species. Fig. 2A shows the location of these residues on the hypothesized binding surface.

Mutagenesis and Purification of the δ-Subunit—Two mutations were introduced in each of the four conserved positions, namely: δY11A, δY11W; δA14D, δA14L; δN75A, δN75E, δV79A, δV79W. The Ala mutations were designed to give an estimate of the contribution of the natural residue to subunit-
subunit binding energy. The introduced Trp residues could give new probes for assaying the interaction of δ with F1, and in both cases they were combined with the ΔW28L mutation to remove the only naturally occurring Trp in δ. The remaining mutations were designed to perturb binding of δ to F1. The mutation ΔG150D was included as a control. This mutation is known to completely impair ATP synthase function (23), but because it occurs in the C-terminal domain of the δ-subunit it is expected to interfere with binding of δ to the β-subunit (24) and not with binding to F1.

For each mutant, we purified δ-subunit following previously published procedures (14, 20), except in the cases of ΔA14D and ΔG150D, where the concentration of saturated ammonium sulfate necessary to precipitate δ was 50% rather than 32%. Yields ranged from 0.5 to 3 mg/liter culture. Purity of the mutant was checked by SDS gels, and in each case was the same as with wild-type as previously shown in Ref. 14. Each purified mutant δ was assayed to determine the affinity of binding to F1 using the fluorescence assay developed previously (14). This assay depends on the fact that binding of δ to F1 that has been depleted of δ produces a large enhancement of fluorescence of the natural δ-Trp-28 residue. As noted above, for the ΔY11W and ΔV79W mutants, we removed the natural δ-Trp-28 (by combining with ΔW28L) and used the new Trp as the probe. For each mutation we also assayed the effect on function in vivo, by measuring growth of mutant cells on succinate plates or in limiting glucose medium, and we measured effects on ATP-driven proton pumping in vitro by reconstitution with membrane vesicles and δ-depleted F1 using purified mutant δ.

**Fluorescence Spectra of Purified Mutant δ**—Fig. 3, A–E, shows the Trp fluorescence spectra of the purified mutant δ-subunits in the absence of F1. Except for the ΔY11W/W28L and ΔV79W/W28L mutants (dashed curves in Fig. 3, A and D, respectively), the fluorophor is the natural Trp in position δ-28. Several of the spectra of mutant δ (ΔA14L, ΔV79A, and ΔG150D) resemble closely that of wild-type δ (dotted lines in Fig. 3, A–E) with a maximum at 326 nm, indicating a relatively unpolar environment for the tryptophan side chain. The wavelength position of the spectra for ΔN75A and ΔN75E (Fig. 3C) is also very similar; however, the fluorescence intensity is 20–30% higher, suggesting that the mutations caused minor changes in the environment of δ-Trp-28. In the ΔA14D mutant, the spectrum of δ-Trp-28 was red-shifted by 2 nm, in the ΔY11A mutant by 5 nm, indicative of an increased polarity experienced by the fluorophor. In general, however, the mutations appeared not to perturb the tertiary structure to any large extent, by this criterion.

A tryptophan inserted in position δ-11, in the ΔY11W/W28L mutant, has an emission maximum of 337 nm (Fig. 3A), suggesting clearly a more polar environment than for δ-Trp-28 in wild-type. A tryptophan in position δ-79 (Fig. 3D) has nearly aqueous surroundings, as indicated by fluorescence maximum at 350 nm of the ΔV79W/W28L mutant. These data are consistent with the predicted location from the structure (Figs. 1 and 2).

**Effect of Addition of δ-depleted F1 on the Fluorescence Spectra of Trp in δ-Subunits**—Wild-type and most of the mutant δ preparations contained δ-Trp-28 as the sole Trp. The response of the δ-Trp-28 fluorescence upon binding of δ-depleted F1 to wild-type δ is shown in Fig. 4A. As described previously (14), there was an increase in fluorescence intensity of about 50%, combined with a slight blue-shift (−4 nm), indicative of a change to a more unpolar environment upon binding. The same change was seen in the ΔV79A and ΔG150D mutants (data not shown). In the ΔN75A and ΔN75E mutants the fluorescence signal of unbound δ was higher to begin with (Fig. 3C), but the final signal after binding to δ-depleted F1 was very similar to wild-type δ, as in Fig. 4A (data not shown). In the ΔY11A mutant, which had a lower intensity and a more red-shifted spectrum before addition of F1, the fluorescence increase upon F1 binding was about 40%, and the blue-shift was by 6 nm. Mutants ΔA14D and ΔA14L did not show any change in fluorescence under the experimental conditions used (discussed below).

The engineered δ-Trp-11, in the ΔY11W/W28L mutant, experiences a very pronounced fluorescence increase upon F1 binding, by 80–90% at 330 nm, combined with a blue-shift of 4 nm (Fig. 4B). In contrast, the fluorescence of the engineered δ-Trp-79, in the ΔV79W/W28L mutant, is quenched upon F1 binding, by about 50% at 350 nm, accompanied by a blue-shift of 14 nm (Fig. 4C). The blue-shifts of all three Trp residues (δ-Trp-11, δ-Trp-28, and δ-Trp-79) reflect a more unpolar environment of the tryptophan side chains upon F1 binding, suggesting that the fluorophors are better shielded from the medium in this state. This, in turn, provides good evidence that all three fluorophors are actually located at, or close to the F1-binding surface on the δ-subunit. These data indicated that for all except the ΔA14D and ΔA14L mutants a direct fluorescence...
Fig. 4. Tryptophan fluorescence spectra of purified wild-type and mutant δ-subunits after addition of Δ-depleted F1. Spectra are shown before (dotted lines) and after (solid lines) addition of 2–3-fold excess of Δ-depleted F1. All spectra are corrected for the contribution by F1 and unbound A, wild-type B, ΔY11W/W28L, C, ΔY79W/W28L.

Fig. 5. Fluorescence titrations to assess binding of Δ-Tyr-11 and Δ-Ala-14 mutant δ to Δ-depleted F1. Pure δ-subunit was mixed with Δ-depleted F1, and the resulting fluorescence enhancement at 325 nm (after subtraction of the contribution of δ alone and of F1 alone) was plotted against the concentration of δ. δF1 concentrations are given in the figure. A, open circles/dotted line, wild-type δ; inverted triangles/solid line, ΔY11A; triangles/dashed line, ΔY11A; triangles/dashed line, ΔY11W/W28L, B, inverted triangles/solid line, ΔY11A; triangles/dashed line, ΔY11W/W28L; squares, ΔA14D; diamonds, ΔA14L.

assay was available for binding of δ-subunit to Δ-depleted F1, and that in the cases of A14D and A14L a competition assay would be required.

Fluorescence Titration of Binding of Δ-Subunit to Δ-depleted F1 and Determination of Binding Affinities of Mutant Δ-Subunits—Binding of mutant δ to Δ-depleted F1 was measured by monitoring the tryptophan fluorescence of δ, either because of the natural δ-Trp-28 or because of the engineered δ-Trp-11 or δ-Trp-79. For each mutant, initially an F1 concentration of 0.05 μM was chosen. If this concentration was too high to obtain a reliable Kd value (because the resulting titration curve was “stoichiometric,” for example, the curve for wild-type δ in Fig. 5A), the titrations were repeated using 0.01 μM F1. If the initial F1 concentration was too low (i.e., the titration curves did not reach saturation), the experiments were repeated using 0.5 μM F1. Typical titration curves are shown in Figs. 5 and 6 and the resultant calculated Kd values are given in Table I. In all cases where a binding stoichiometry could be determined reliably (i.e., with stoichiometric or close-to-stoichiometric binding curves), it was very close to 1 (0.9 to 1.2) mol of δ per mol of F1.

Both mutations in position δ-11 resulted in a significantly decreased binding affinity. Based on the results obtained with the ΔY11A mutant, the δ-Tyr-11 side chain in wild-type δ contributes about 12 kJ/mol of binding energy (Table I). In this case, tryptophan is not a good substitute for tyrosine, because the ΔY11W mutation also causes a loss of close to 12 kJ/mol in binding energy (calculated by comparing ΔY11W/W28L to ΔW28L mutant).

Titrations with the ΔA14D and ΔA14L mutants, even using 0.5 μM F1 and up to 2.5 μM δ, did not result in significant changes in the δ-Trp-28 fluorescence (Fig. 5B). One possible explanation is that the binding affinity is so low that even at the highest concentrations used, binding is negligible. In this case, Kd would be >5 μM (and the loss in binding energy >20 kJ/mol). Alternatively, it is possible that binding occurs, but that the mutations affect the interaction between δ and F1 in such a way that the fluorescence of δ-Trp-28 does not respond to binding. To address this question, we performed competition titration experiments, which showed that a 6–10-fold excess of ΔA14D or ΔA14L mutant δ-subunit did not significantly reduce binding of wild-type δ. On the basis of these results, we can calculate that the Kd for ΔA14D or ΔA14L mutant is at least 0.1 μM. Thus, from either calculation, both mutations at residue δ-14 perturb interaction between δ and F1 considerably.

In position δ-75, neither mutation ΔN75A nor ΔN75E has a significant effect on the F1 binding affinity (Fig. 6A and Table I). Specifically the results obtained with the ΔN75A mutant suggest that the natural asparagine side chain does not contribute binding energy. In contrast, the valine side chain of residue δ-Val-79 makes a moderate contribution of about 6 kJ/mol (Table I). A tryptophan in this position strongly interferes with binding to F1, with a loss of binding energy of more than 11 kJ/mol (comparing ΔV79W/W28L to ΔW28L). As expected, the mutation ΔG150D in the C-terminal region of δ, used as a control here, had no effect on F1 binding affinity.

Effect of the δ Mutations on ATP Synthase in Vivo—Plasmids containing mutant uncH (δ-subunit) genes were transformed into strain AN2015, which contains the chromosomal mutation δ-Trp-28 → stop and is therefore unable to grow by oxidative phosphorylation. All plasmids carrying mutations in positions 11, 14, 75, and 79 of δ were able to restore oxidative phosphorylation, as demonstrated by their growth yields on limiting glucose (Table II) and their ability to grow on plates containing succinate as the sole carbon source (not shown). In contrast, the mutation ΔG150D prevented ATP synthesis by the ATP synthase in vivo (Table II).

Effect of δ Mutations on ATP-driven H+ Pumping in Vitro—KSCN-stripped membranes were reconstituted with wild-type or mutant δ together with Δ-depleted F1 and proton pumping was initiated by addition of ATP. It was found that none of the mutants at positions 11, 14, 75, or 79 of δ caused significant impairment of ATP-driven H+ pumping (Table II). These data indicate that, in the presence of intact F1o, functional binding of the mutant δ-subunits to F1o did occur. From the considerations presented in the Introduction, one likely mechanism for such
an effect would be through involvement of the $b$ subunit dimer, and specifically the cytoplasmic domain of $b$ that interacts with $\delta$. In contrast, the $\delta$G150D mutant prevented formation of a proton gradient upon ATP hydrolysis (Table II) as expected from previous work (23). As already noted, this mutation occurs in the C-terminal region of $\delta$ and is expected to interrupt interaction of $\delta$ with $b$.

**Effect of the Soluble Cytoplasmic Domain of the $b$ Subunit on $F_1$-binding Site on ATP Synthase $\delta$-Subunit**

**FIG. 6.** Fluorescence titrations to assess binding of $\delta$-Asn-75 and $\delta$-Val-79 mutant $\delta$ to $\delta$-depleted $F_1$. Pure $\delta$-subunit was mixed with $\delta$-depleted $F_1$, and the resulting fluorescence changes at 325 nm (A and B) or 360 nm (C) were plotted (after subtraction of the contribution of $\delta$ alone and of $F_1$ alone) against the concentration of $\delta$ $F_1$ concentrations are given in the figure. A, open circles/dotted line, wild-type $\delta$; inverted triangles/solid line, $\delta$N75A; triangles/dashed line, $\delta$N75E. B, $\delta$V79A. C, $\delta$V79W/W28L.

**TABLE I**

Effect of mutations in $\delta$-subunit on affinity of binding of $\delta$ to $\delta$-depleted $F_1$

| $\delta$ Mutation | $K_d$ (nM) | Loss of binding energy$^a$ | $\Delta G^o$, kJ/mol |
|------------------|------------|----------------------------|---------------------|
| Wild-type        | 0.0014     |                           |                     |
| $\delta$Δ–134 ($\delta$) | 0.0014     | 0                         |                     |
| $\delta$W28L$^B$ | 0.0046     | 2.9                       |                     |
| $\delta$Y11A     | 0.21       | 12.3                      |                     |
| $\delta$Y11W/W28L| 0.50       | 11.6                      |                     |
| $\delta$A14D     | >0.1       | >11.6                     |                     |
| $\delta$A14L     | >0.1       | >11.6                     |                     |
| $\delta$N75A     | 0.0010     | −0.8                      |                     |
| $\delta$N75E     | 0.0020     | 0.8                       |                     |
| $\delta$V79A     | 0.017      | 6.1                       |                     |
| $\delta$V79W/W28L| 0.47       | 11.4                      |                     |
| $\delta$G150D    | 0.0011     | −0.6                      |                     |

$^a$ $\Delta G^o$ calculated as difference from wild-type except in the case of $\delta$Y11W/W28L and $\delta$V79W/W28L, where difference from $\delta$W28L is calculated.

$^b$ Data are from Ref. 14.

**FIG. 7.** Effect of $b_{ST34–156}$ on binding of $\delta$Y11W/W28L mutant $\delta$ to $\delta$-depleted $F_1$. The signal used was the fluorescence increase of residue $\delta$-Trp-11 upon binding of $\delta$Y11W/W28L mutant $\delta$ to $\delta$-depleted $F_1$. Contributions of mutant $\delta$ alone, $\delta$-depleted $F_1$, and $b_{ST34–156}$ were subtracted. A, titration of $\delta$-depleted $F_1$ with $\delta$Y11W/W28L mutant $\delta$ in the presence of 10 nM $b_{ST34–156}$; B, titration of equimolar (100 nM) concentrations of $\delta$-depleted $F_1$ and $\delta$Y11W/W28L mutant $\delta$ with $b_{ST34–156}$ (plotted as $b_{ST34–156}$ dimer).

**TABLE II**

Effect of mutations in $\delta$-subunit on growth of cells in vivo and ATP-driven proton pumping in vitro

| Mutation | Growth yield | % of wild-type | ATP-driven $H^+$-pumping |
|----------|--------------|----------------|-------------------------|
| Wild-type | 100          | 84 (85°)       |                         |
| unc$^−$ (pUC118) | 52          |                |                         |
| $\delta$W28L | 101        | 82             |                         |
| $\delta$Y11A | 100        | 79             |                         |
| $\delta$Y11W/W28L | 101      | 74             |                         |
| $\delta$A14D | 98          | 72             |                         |
| $\delta$A14L | 93          | 74             |                         |
| $\delta$N75A | 99          | 80             |                         |
| $\delta$N75E | 99          | 77             |                         |
| $\delta$V79A | 99          | 77             |                         |
| $\delta$V79W/W28L | 98      | 77             |                         |
| $\delta$G150D | 50          | 0              |                         |

$^a$ Membranes reconstituted directly with wild-type $F_1$, not $\delta$-depleted.
diamonds concentration and the amount of added dotted line/wild-type H9254/H9262 between F0 and added measured. Inhibition comes about as a result of competition on the Trp fluorescence of the signal and a relatively low affinity for binding to F1, namely H9254 concentration given in the figure) in the presence of 4 μM bST34–156. Fig. 7Kd preparation, from the fluorescence spectrum in 6M guanidine hydrochloride, revealed a Trp content of 0.1 mol/mol. The fluorescence because of Trp contamination in bST34–156 was corrected routinely.

We demonstrated that addition of bST34–156 to wild-type or any of the mutant δ-subunits in the absence of F1 had no effect on the Trp fluorescence of the δ-subunit. In initial experiments with F1 present, we chose to use a mutant δ with a high Trp signal and a relatively low affinity for binding to F1, namely δY11W/W28L. Fig. 7A demonstrates an experiment in which bST34–156 and δ-depleted F1 were added at a constant concentration and the amount of added δY11W/W28L subunit was varied. This fluorescence titration showed that addition of bST34–156 to the binding assay had a very large effect, reducing Kd for binding of δY11W/W28L to F1 from 0.5 μM to < 5 nM. In Fig. 7B we determined the apparent Kd for bST34–156. Here, with constant concentrations of δ-depleted F1 and δY11W/W28L subunit present, the concentration of bST34–156 was varied. The apparent Kd for the bST34–156 dimer (the physiological form, Refs. 4 and 5) was 150 nM, with two independent experiments showing excellent agreement.

![Fig. 8. Effect of bST34–156 on binding of mutant δ-subunits to δ-depleted F1](image)

**Fig. 8.** Effect of bST34–156 on binding of mutant δ-subunits to δ-depleted F1. Mutant δ-subunit was added to δ-depleted F1 (concentration given in the figure) in the presence of 4 μM bST34–156. Trp fluorescence intensity changes at 325 nm (A–C, squares) or 360 nm (C, diamonds) were plotted against the concentration of δ. A, open circles/dotted line, wild-type δ; inverted triangles/solid line, δY11A. B, δY11W/W28L. C, squares/solid line, δV79A; diamonds/dashed line, δV79W/W28L.

dependent proton pumping by F1 in stripped membranes is measured. Inhibition comes about as a result of competition between F0 and added b subunit cytoplasmic domain for a limited number of F1 molecules. We found that reconstitution was inhibited by bST34–156 in a dose-dependent manner. The ratio of bST34–156/F1, giving 50% inhibition of reconstitution was 2 μg/μg, the same as in Ref. 25. Theoretically bST34–156 should contain no Trp residue. Calculation of the Trp content of our preparation, from the fluorescence spectrum in 6 M guanidine hydrochloride, revealed a Trp content of 0.1 mol/mol. The fluorescence because of Trp contamination in bST34–156 was corrected routinely.

With regard to wild-type δ, the above mentioned technical constraints prevented us from determining whether the binding affinity for F1 was increased by inclusion of bST34–156 (the Kd in the absence of bST34–156 was already 1.4 nM, Table I). If we assume that a 500-fold increase in affinity occurs for wild-type, as it did for δY11W/W28L, this would give a Kd for wild-type δ in the presence of bST34–156 of ≤ 3 pM.

In contrast, with mutants δA14D and δA14L the titration curves (not shown) looked the same as those in Fig. 5B (squares and diamonds) even when bST34–156 was present. From this we can conclude that either these mutant δ-subunits do not bind significantly to F1, even in the presence of bST34–156, or that if they do bind, the binding does not engender a change in the fluorescence signal of δ-Trp-28.

**Fig. 9.** ATP-driven proton pumping in stripped membranes reconstituted with mutant δ-subunit and δ-depleted F1; titration with mutant δ. Membrane vesicles were stripped of F1 by KSCN treatment, then reconstituted with δ-depleted F1 and varying amounts of mutant or wild-type δ-subunit. ATP-driven proton pumping was monitored by quenching of acridine orange fluorescence, and the maximal percent quench was plotted as a function of δ concentration in the cuvettes. Open circles/dotted line, wild-type δ; inverted triangles/solid line, δY11A; open squares/solid line, δA14D; open diamonds/dashed line, δA14L; filled diamonds/dashed line, δV79W/W28L.

**Table I.**

| Mutant          | Kd (nM) |
|-----------------|---------|
| δA14D           | 1.4     |
| δV79A           | 1.4     |
| δV79W/W28L      | > 500   |
| δY11A           | > 500   |
| δY11W/W28L      | > 500   |

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In contrast, with mutants δA14D and δA14L the titration curves (not shown) looked the same as those in Fig. 5B (squares and diamonds) even when bST34–156 was present. From this we can conclude that either these mutant δ-subunits do not bind significantly to F1, even in the presence of bST34–156, or that if they do bind, the binding does not engender a change in the fluorescence signal of δ-Trp-28.

**Titrination of Mutant δ-subunits with KSCN-stripped Membranes and δ-depleted F1 in the Reconstituted Proton-pumping Assay—**Table I demonstrated that the mutant δA14D and δA14L subunits did reconstitute ATP-driven proton pumping in membrane vesicles when added back under saturating conditions with δ-depleted F1 to KSCN-stripped membranes. To investigate the binding properties of these mutant δ-subunits in the presence of intact F0 further we titrated them in this assay with constant amounts of stripped membranes and δ-depleted F1. Whereas we had shown before (14) that such a titration cannot be used to determine absolute values of Kd for binding of δ to F1, nevertheless, we expected that by comparison of the mutants with wild-type we could get qualitative
information regarding the binding of the δA14D and δA14L proteins. Fig. 9 indicates that the titration curves obtained with these (and other) mutants are not very dissimilar from wild-type. We therefore conclude that the failure to see enhancement of fluorescence when these mutant subunits are added to δ-depleted F₁ in the presence of bST34–156 (above) is most likely because of the fact that the mutations affect the environment of residue δ-Trp-28 and prevent enhancement of its fluorescence signal upon binding.

**DISCUSSION**

The goal of this study was to investigate in detail the interaction between the δ-subunit and F₁ in ATP synthase, and specifically to identify the F₁-binding surface on the δ-subunit. Based on the location of conserved or conservatively replaced residues in one particular region of the high resolution NMR structure of δ, we hypothesized that the F₁-binding surface might be formed by helices 1 and 5 (see Figs. 1 and 2). Our results confirmed the hypothesis. However, several mutations in δ that clearly disrupted binding of δ to F₁ did not lead to impaired function, an unexpected finding. Additional studies showed that inclusion of the soluble cytoplasmic domain of the b subunit substantially enhanced the binding affinity between δ and F₁ and compensated for loss of binding affinity caused by δ mutations.

As target residues for mutational analysis we selected δ-Tyr-11 and δ-Ala-14 in helix 1 and δ-Asn-75 and δ-Val-79 in helix 5. The first evidence that these residues are at or close to the F₁-binding site came from tryptophan substitutions in positions 11 and 79, whose fluorescence signals responded strongly to binding of F₁ (Fig. 4, B and C). Further evidence came from the titrations in Figs. 5 and 6, and from competition binding experiments. Calculated binding affinity measurements (Kᵣᵥ values, see Table I) indicated that three of the four residues are directly involved in binding. The tyrosine side chain of residue δ-Tyr-11 contributes about 12 kJ/mol binding energy, possibly because of π-π or π-cation interactions. Increasing the size of the side chain in position δ-Ala-14, either by itself (δA14L mutant) or in combination with introduction of a negative charge (δA14D mutant) reduces the affinity for F₁ significantly, probably by affecting interprotein surface complementarity. The valine side chain in position δ-Val-79 contributes about 6 kJ/mol binding energy, very likely because of hydrophobic interactions. The lack of binding energy contribution of the side chain of residue δ-Asn-75 suggests this residue is not directly involved in binding. However, it might not necessarily be taken as an argument against its location at the F₁-binding surface; in an analysis of the interaction between the human growth hormone and its receptor (26) it was found that only one-quarter of the residues at the protein-protein interface had a significant impact on the binding energy. Whereas this might be an extreme case, at many protein-protein interface amino acid side chains can be found that should have the potential to participate in binding, but in fact play no or only a very minor role (see examples summarized in Ref. 27).

Surprisingly, despite losses in F₁-binding energy of up to 15 kJ/mol, all mutations of residues in helices 1 and 5 were still fully or nearly fully functional in *vivo* and *in vitro*. In a previous study (14) we determined that the a292D mutation reduces the binding affinity between δ and F₁, moderately, corresponding to a loss in binding energy of about 7 kJ/mol. We ascribed the strong functional impairment of this mutant ATP synthase to the interruption of binding of δ to F₁. This led us to conclude that the stator resistance function was finely balanced. However, the results of the present study showed that much larger losses of binding energy between δ and F₁ were well tolerated. Thus, we must now conclude that not all of the binding energy necessary to affix the stator stalk to F₁, to resist the elastic strain generated by rotational catalysis, must necessarily be derived from δF₁ interactions. In all likelihood, interactions between the b subunits and δ and/or F₁ also contribute.

To explore this possibility we included the soluble cytoplasmic domain (bST34–156 Ref. 20) in δ binding assays and found that it substantially decreased the Kᵣᵥ of binding of δ to F₁. Due to technical limitations of the fluorescence assays, absolute values for this Kᵣᵥ in the presence of bST34–156 could not be obtained, but an enhancement of ≈500-fold in affinity was evident from the results, equivalent to an additional binding energy of ≈15 kJ/mol. The additional binding energy could come from interactions between δ subunit and α or β, and/or from b-induced conformational changes in δ. Interestingly, the Kᵣᵥ for interaction between δ and F₁, isolated δ (in the absence of F₁) was 5–10 μM (20) but the Kᵣᵥ measured here for binding of bST34–156 in the presence of isolated δ and δ-depleted F₁ was 150 nM, indicating a considerable cooperativity between the stator subunits. Overall, the new data indicate that the wild-type stator stalk is “overengineered,” i.e. it is equipped with excess binding energy. This might explain why only very few impairing point mutations in δ have been found (23, 28).

The experiments presented here supplement and extend earlier studies on OSCP. A study using deletion mutants of bovine OSCP indicated that removal of the N-terminal 28 residues, corresponding approximately to all of helix 1 in *E. coli* δ, impaired binding of OSCP to F₁ (29). Also a study of rat OSCP showed that the strongly conserved residue δ-Arg-85 (Arg or Lys in 95% of sequences, residue Arg-94 in OSCP contributed significantly to F₁-binding energy (30). This residue occurs in the loop following helix 5 (see Fig. 2). Interestingly, despite the loss of binding energy, the δR85A and δR85Q mutants were still functional in *vivo* (30), and the authors concluded, as we do here, that other interactions provide binding energy. In *E. coli*, the mutation δR85Q reduced the membrane-bound ATPase activity, by 50%, but had little effect on growth characteristics (28).

The quantitative binding assays described here for wild-type and mutant δ binding to δ-depleted F₁ in the presence or absence of the soluble cytoplasmic domain of the b subunit will allow us in future work to assess further aspects of stator subunit interactions. The influence of specific residues of the b subunits on δ binding affinity can readily be studied by mutagenesis, for example. In addition, we can now investigate, by mutagenesis and other techniques, the binding site for mutagenesis, for example. In addition, we can now investigate, by mutagenesis and other techniques, the binding site for

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Identification of the $F_1$-binding Surface on the $\delta$-Subunit of ATP Synthase
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