Analysis of Sequence Determinants of $F_{1}F_{0}$-ATP Synthase in the N-terminal Region of $\alpha$ Subunit for Binding of $\delta$ Subunit*

Received for publication, March 10, 2004, and in revised form, April 2, 2004
Published, JBC Papers in Press, April 6, 2004, DOI 10.1074/jbc.M402738200

Joachim Weber‡, Alma Muharemagic, Susan Wilke-Mounts, and Alan E. Senior§

From the Department of Biochemistry and Biophysics, University of Rochester Medical Center, Rochester, New York 14622

The stator in $F_{1}F_{0}$-ATP synthase resists strain generated by rotor torque. In *Escherichia coli*, the $b_{2}\delta$ subunit complex comprises the stator, bound to subunit $\alpha$ in $F_{0}$, and to the $\alpha_{2}\beta_{2}$ hexagon of $F_{1}$. Previous work has shown that N-terminal residues of $\alpha$ subunit are involved in binding $\delta$. A synthetic peptide consisting of the first 22 residues of an $\alpha$N1–22) binds specifically to isolated wild-type $\delta$ subunit with 1:1 stoichiometry and high affinity, accounting for a major portion of the binding energy between $\delta$ and $F_{1}$. Residues $\delta$6–18 are predicted by secondary structure algorithms and helical wheel to be $\alpha$-helical and amphipathic, and a potential helix capping box occurs at residues $\delta$3–8. We introduced truncations, deletions, and mutations into $\alpha$N1–22 peptide and examined their effects on binding to the $\delta$ subunit. The deletions and mutations were introduced also into the N-terminal region of the unca (a subunit) gene to determine effects on cell growth in vivo and membrane ATP synthase activity in vitro. Effects seen in the peptides were well correlated with those seen in the unca gene. The results show that, with the possible exception of residues close to the initial Met, all of the $\alpha$N1–22 sequence is required for binding of $\delta$ to $\alpha$. Within this sequence, an amphipathic helix seems important. Hydrophobic residues on the predicted nonpolar surface are important for $\delta$ binding, namely $\alpha$le-8, $\alpha$Leu-11, $\alpha$Le-12, $\alpha$Ile-16, and $\alpha$Phe-19. Several or all of these residues probably make direct interaction with helices 1 and 5 of $\delta$. The potential capping box sequence per se appeared less important. Impairment of $\alpha\delta$ binding brings about functional impairment due to reduced level of assembly of ATP synthase in cells.

ATP synthesis by oxidative phosphorylation occurs on the enzyme $F_{1}F_{0}$-ATP synthase. In *Escherichia coli*, the enzyme consists of eight different subunit types, in stoichiometry $\alpha_{2}\beta_{2}\gamma\delta\varepsilon\eta\zeta$. Proton movement through the membrane sector of the complex, mediated by subunits $\alpha$ and $\epsilon$, is believed to generate rotation of subunits $\epsilon$, $\gamma$, and $\zeta$, which collectively form the “rotor.” In turn, this rotation acts on the catalytic sites, three in number, at $\alpha/\beta$ interfaces of the alternating $\alpha_{2}\beta_{2}$ hexagon, to generate ATP (1, 2). A “stator,” consisting of subunits $b_{2}\delta$, is necessary to resist the rotor strain. In the reverse direction, ATP hydrolysis in the catalytic sites drives rotation of the rotor (3), which then generates uphill transport of protons across the bacterial plasma membrane to form the electrochemical gradient essential for nutrient uptake, locomotion, and other functions. Again, rotor strain must be resisted by the stator for efficient function. The mechanisms by which catalysis, proton gradient formation, and subunit rotation are functionally integrated are subjects of active investigation (4–7).

The structure and function of the stator have been reviewed recently (8, 9). The stator ($b_{2}\delta$) interacts with the $\alpha_{2}\beta_{2}$ catalytic unit via $\delta/F_{1}$ interactions and with the proton-translocating machinery via $b_{2}/\alpha$ interactions. $\delta$ and $b_{2}$ interact together via their C-terminal regions. There is also interaction between $b_{2}$ and $\alpha$ or $\beta$ subunits (10–13). In recent papers, we have studied the binding of the $\delta$ subunit to $F_{1}$. Using novel tryphtophan fluorescence assays, we established quantitative parameters for $\delta$ binding to $F_{1}$ (14) and demonstrated that helices 1 and 5 of the N-terminal domain of the $\delta$ subunit form the $F_{1}$-binding surface (15). An earlier report had used NMR to establish the structure of the N-terminal domain of $\delta$ subunit, which is composed of a six-helix bundle, and had shown that helices 1 and 5 form a hydrophobic groove (16).

In Ref. 17, we also studied the $\delta$ binding surface on $F_{1}$, $\delta$ subunit (and its mitochondrial homolog oligomycin-sensitivity conferral protein) was known from electron microscopy studies to bind at the “top” of $F_{1}$, $F_{1}$ binding (18, 19). Proteolysis (20) and cross-linking (21) experiments had suggested that the extreme N-terminal residues of the $\alpha$ subunit were involved in binding of $\delta$. For example, removal of the first 15 residues of $\alpha$ by trypsin or of the first 19 residues by chymotrypsin was sufficient to greatly reduce $\delta$ binding to $F_{1}$ (20). X-ray crystallography studies have not yet been able to determine the structure of these $\alpha$ subunit residues (7, 22, 23). In Ref. 17, we showed that a 22-residue synthetic peptide corresponding in sequence to the N-terminal residues of $\alpha$ subunit with free N and C termini (an $\alpha$N1–22) was able to bind to wild-type $\delta$ subunit with high affinity and specificity ($K_{d}$ = 130 nm) and with 1:1 stoichiometry, effectively mimicking the binding of intact $F_{1}$ to $\delta$. Mutations on the $F_{1}$-binding surface of $\delta$, which impaired binding of $F_{1}$, were seen to impair binding of an $\alpha$N1–22, providing further evidence for the specificity of binding of the peptide. The data provided clear evidence that the N terminus of $\alpha$ subunit provides the major fraction of total binding energy between the stator component $\delta$ and $F_{1}$. We noted in Ref. 17 that secondary structure algorithms consistently predict residues $\delta$6–18 to be $\alpha$-helical and that a potential helix capping box sequence (24) occurs at residues $\delta$3–8. A helical wheel diagram reveals that the predicted helix would be amphipathic. These features are

*This work was supported by National Institutes of Health Grant GM25349 (to A. E. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX 79430.

§ To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, University of Rochester Medical Center, Box 712, Rochester, NY 14642. Tel.: 585-275-2777; Fax: 585-271-2683; E-mail: alan_senior@urmc.rochester.edu

1 The abbreviations used are: $\alpha$N1–22, synthetic peptide consisting of residues 1–22 of $F_{1}F_{0}$-ATP synthase $\alpha$ subunit; DCCD, dicyclohexylcarbodiimide.

2 E. coli residue numbers are used throughout.
shown in Fig. 1, A and C. Circular dichroism studies of the αN1–22 peptide supported the view that it assumed a helical structure (17). NMR studies of αN1–22 bound to δ subunit in a 1:1 complex have now shown that the peptide adopts a helical conformation when bound to δ. These considerations lead to a general model of how αN1–22 fits into the hydrophobic groove between helices 1 and 5 of δ, presented in Fig. 1B.

Here we have introduced truncations, point mutations, and deletions into αN1–22 peptide in order to examine the importance of specific regions of its sequence for binding to δ. Deletions and mutations were also made in the unca (α subunit) gene, to examine effects on cell growth in vivo and membrane ATP synthase function in vitro. The results were well correlated and allow conclusions regarding specific sequence determinants of δ binding and overall ATP synthase function in the N-terminal region of α.

EXPERIMENTAL PROCEDURES

**Binding of ATP Synthase δ to N-terminal Region of α**

**A**

| 1  | 10  | 20  |
|----|-----|-----|
| MQLNSTEI | SELI | KQRI AQFNVV |
| CCCCC | CCCCCCCC |

**B**

| 1  | 10  |
|----|-----|
| MQLNSTEI | S |
| N N N N N N |
| φ X X X E φ |

**C**

Fig. 1. The N-terminal residues of α subunit. A, α, the sequence of the first 22 residues of α corresponding to the peptide αN1–22 is shown, with the predicted helix at residues α6–18. H, indicates helix; C, random coil. b, the potential helix capping box at residues α3–8. Terminology of capping box residues is from Ref. 24. In the capping box signature motif Φ is a hydrophobic residue, and X is variable. B, model for binding of N-terminal region of α to δ subunit. The NMR structure of the N-terminal domain of δ is from Ref. 16. It consists of a six-helix bundle, with helices 1–6 shown in dark blue, light blue, teal, green, yellow, and red, respectively. The N-terminal region of α comprising the αN1–22 peptide is shown in silver, with residues 6–18 forming an α-helix, and fitted into the hydrophobic groove formed by helices 1 (dark blue) and 5 (yellow) of δ. The actual orientation of αN1–22 (i.e. N → C) is not specified here. C, helical wheel diagram of residues α6–18. The helix predicted for residues α6–18 is displayed as a helical wheel (with the addition of α19). The amphipathic nature of the predicted helix is obvious. Residues on the nonpolar surface are shown in italic type; residues on the polar surface are in normal Roman type.

**EXPERIMENTAL PROCEDURES**

**Purification of δ Subunit, Purification of F1, Preparation of Membrane Vesicles, Assay of ATPase Activity, Assay of ATP-driven Proton Pumping in Membrane Vesicles, and Growth Yield Assays**—These were as described previously (14, 15). For assay of membrane ATPase activity, 50 μg of membranes were assayed at 30 °C in 1 ml of assay medium (50 mM Tris–SO4, pH 8.5, 10 mM NaATP, 4 mM MgCl2) for appropriate times (1–2 min for wild type, 10–20 min for mutants with low activity). To assess sensitivity of ATPase to diethylthiocarbazimide (DCCD) inhibition, membranes (1 mg of protein/ml) were preincubated at 30 °C in 10 mM HEPES-KOH, pH 7.5, 100 mM KCl, 5 mM MgCl2, 0.1 mM DCCD for 30 min. Growth yields on limiting glucose were measured as in Ref. 25.

**E. coli Strains and unca Gene Mutagenesis**—For generation of deletions plasmid pBWU13.4 (26) expressing the unca operon (ATP synthase) structural genes digested with PstI; then 200-bp fragments containing the first part of the unca (α subunit) gene and its upstream region were amplified by PCR. The reverse primer corresponded to bp +216–235 of unca. Forward primers began at bp –11 and incorporated the codon deletions required. Amplified fragments were phosphorylated at 3’-ends and cloned into pUC118 pretreated with alkaline phosphatase and cut with SmaI. pUC118 derivatives with inserts containing unca sequence were identified by digestion with XhoI and ApaLI. Deletions could be readily recognized by fragment size and/or loss of a natural EcoRI site and/or gain of a new restriction site introduced by the PCR primer and were confirmed routinely by DNA sequencing. It was also routinely confirmed that no PCR-generated errors were present between SphI and XhoI sites. The deletions were transferred to pBWU13.4 on SphI-XhoI fragments, and the resultant plasmids were transformed into E. coli strain DK8 (27) for functional studies. Point mutations in the unca gene were generated by oligonucleotide-directed mutagenesis following previous methods (14) except that the template was M13mp18 containing the SphI-Sall fragment from pBWU13.4. After mutagenesis the point mutations were moved into plasmid pBWU13.4 as above and expressed in strain DK8.

**Synthetic Peptides**—Peptides were purchased from United States Biological (immunological grade). All peptides with the single exception of αN1–22Cam (see “Results”) had free N and C termini. In intact ATP synthase, the N terminus of α subunit is free Met.) For fluorescence binding assays, the peptides were dissolved at 10 mg/ml in dry MeSO and used for 1 day only. MeSO was rendered anhydrous by use of Molecular Sieves (catalog no. M-6141; Sigma). Concentration of MeSO in the fluorescence binding assays did not exceed 0.75% (v/v).

**Fluorescence Binding Assays**—Tryptophan fluorescence titrations were carried out as described in Ref. 17, with individual conditions given in the figure and table legends. Excitation was at 295 nm, and emission was measured at 325 nm. The fluorophore was the single natural Trp in the δ subunit at residue 28. The buffer was 50 mM HEPES/NaOH, 5 mM MgSO4, pH 7.0, at room temperature. A fixed concentration of δ subunit (0.6–3.2 μM) was titrated with peptide, and several concentrations of δ were used with each peptide. A control titration of buffer alone with peptide was always conducted and sub-

---

3 S. Wilkens, J. Weber, and A. E. Senior, manuscript in preparation.
Binding of ATP Synthase δ to N-terminal Region of α

truncated, although usually negligible. Binding-induced changes in Trp fluorescence were plotted versus peptide concentration, and from the resulting curves, \( K_d \) values were calculated by nonlinear regression following the methods of Eftink (28), using the equation,

\[
F = m \times \left( \frac{(E_0/2 + [L_{total}]/n/2 + K_d/n/2)^2}{(E_0 + [L_{total}]/n/2 + K_d/n/2)^2} \right) - \left( \frac{\left( [L_{total}]/n/2 + K_d/n/2 \right)^2}{[L_{total}]/n^3} \right) \tag{1}
\]

where \( F \) is the measured fluorescence change at fixed δ concentration \( E_0 \), and added peptide concentration \( L_{total} \). \( n \) is the stoichiometry of binding, and \( m \) is a factor that converts fluorescence data to ligand bound (equals \( F \) at saturation divided by \( [E_0] \)). Values of \( n \) (stoichiometry of binding of peptide to δ subunit) were consistent with one peptide molecule binding per δ subunit molecule in all cases.

RESULTS

Use of Me2SO to Dissolve Peptides—The assay that we use in this work to determine quantitatively the binding of peptides to δ subunit is a fluorescence assay that measures the strong (up to 50%) enhancement of fluorescence of the single natural Trp-28 residue in δ that occurs upon binding of wild-type δ to F1, or to synthetic peptides (14, 15, 17). We had previously encountered difficulty due to insolubility of the synthetic peptides in aqueous media at millimolar concentration, as needed to perform fluorescence titrations. For instance, peptide αN1–22Cam (the analog of αN1–22 but with an amidated C terminus) could not be studied (17). Here the same problem arose with several of the deletion or mutation peptides. We found, however, that the majority of peptides dissolved at up to 8 mM in dry Me2SO, and we confirmed that the addition of associated small amounts of Me2SO (≤0.75% v/v) had no effect on the fluorescence titration of αN1–22 with δ. Thus, all peptides were dissolved in Me2SO for fluorescence studies.

In fact, it turned out that αN1–22Cam behaved identically to αN1–22 in fluorescence assays and bound to δ with a similar \( K_d \) value of 150 nM; thus, the presence of a free or blocked carboxyl at the C terminus of the peptide had no effect.

Finding the Optimal Length of Peptide for δ Binding by Incrementally Truncating the C Terminus—Originally (17), we identified αN1–22 as a suitable peptide that might bind to purified δ, because secondary structure predictions suggested a continuous helix for residues 88–18, proteolysis experiments showed that removal of residues 1–19 prevented δ binding to F1 (20), and in x-ray structures the β-barrel domain at the crown of F1 is seen to commence in α subunits after residue α23 (22, 23). In the first part of this work, we tested binding of a series of peptides αN1–24, αN1–22, αN1–20, and so on in increments of two-residue truncations, down to αN1–12. Fig. 2 shows the results of typical fluorescence titrations measuring binding of the peptides to purified wild-type δ subunit. Fig. 3 shows similar titrations, but using δV79A mutant purified δ subunit instead of wild type. The mutation δV79A, occurring at the highly conserved hydrophobic residue 879, is known to impair binding of purified δ subunit to F1 (15) and to αN1–22 (17). It is located on the surface of helix 5 of the F1-binding face of δ. Therefore, it provides additional evidence as to the specificity of binding. \( K_d \) values for truncated peptides were calculated from several titration curves carried out as in Figs. 2 and 3, as described under “Experimental Procedures,” and are presented in Table I.

It is apparent that the original choice of αN1–22 was optimal. Here we found a \( K_d \) for αN1–22 binding to wild-type δ of 113 nM, slightly lower than the value of 130 nM reported in our previous work (17), and for binding of αN1–22 to V79A δ we found a \( K_d \) of 1.95 μM compared with 2.4 μM in the previous report. Increasing the peptide length by two residues in αN1–24 reduced affinity of binding to wild-type δ by 43-fold. Reduction in length by two residues (αN1–20) also decreased affinity in wild-type δ, by 11-fold. Further reduction in length of the peptide yielded greater decreases in binding affinity, such that neither αN1–14 nor αN1–12 peptides showed measurable binding to wild-type δ subunit. The same general pattern of reduced affinity was seen for the series of peptides binding to V79A mutant δ. It may be noted that binding of the parent αN1–22 peptide to V79A δ is already 17-fold weaker than to wild-type δ. Overall, the data show that for optimal binding to δ, a peptide with a length of 22 residues is required, suggesting that residues located along the entire sequence make interactions with δ. Possibly, the two additional residues in αN1–24 cause formation of novel structure in the peptide or simply interfere with binding to δ by folding back on the rest of the peptide.

Testing the Idea That the N-terminal Region of the α Subunit Forms an Amphipathic Helix and That Hydrophobic Residues on One Surface Make Interactions with δ Subunit—Fig. 1C shows a helical wheel diagram of residues α6–α19. A series of hydrophobic residues shown in italic type (Leu-11, Ile-8, Phe-19, Ile-12, and Ile-16) clusters on one surface of the helical
wheel. (Arg-15 on this surface may also be viewed as potentially hydrophobic, depending on its orientation). Conversely, the other surface shows mainly polar residues (Ser-9, Lys-13, Thr-6, Gln-10, Glu-14, Glu-7, Gln-18). Given that we had identified helices 1 and 5 of α as forming the F1-binding surface (15) and that the NMR structure of α shows these helices forming a hydrophobic groove (16), it seemed likely that some or all of the hydrophobic residues listed above might make direct interaction with δ. Furthermore, an analysis of sequences of α-subunit from multiple species revealed that hydrophobic residues are strongly favored at positions α8, α11, α12, α16, and α19 (data not shown).

Initially, we tested binding of peptides containing point mutations at position α8-8 to purified wild-type and V79A mutant δ subunits (wild type, 1.6 μM; V79A, 3.2 μM) was titrated with peptide (added incrementally as a 0.5–4 μM solution in MeSO), and fractional enhancement of fluorescence of residue δTrp-28 was measured at 325 nm. For details, see “Experimental Procedures.” αN1-22(1E) with wild-type δ; αN1-22(I8E) with V79A mutant δ; αN1-22(I8K) with wild-type δ; αN1-22(I12E), αN1-22(I16E), and αN1-22(F19E)). Fig. 5 shows fluorescence titrations with these peptides using wild-type δ. The calculated $K_d$ values are given in Table I. Substitution of Glu at any one of these positions impaired binding impressively. Binding affinity was decreased at least 10-fold in all cases. Interestingly, the αN1-22(I16E) peptide brought about a quench of the fluorescence with wild-type δ rather than the usual fluorescence enhancement. Of 23 peptides tested in this and other work, this was the only peptide with this property, suggesting that residue α16-16 may come very close to residue δTrp-28 when α is bound to δ.

We also tested binding of the same series of point mutant peptides to V79A mutant δ (titrations not shown), and resultant calculated $K_d$ values are listed in Table I. A similar pattern of reduction of binding affinity was apparent. It may be noted that a fluorescence quench was again seen when αN1-22(116E) was titrated with V79A mutant δ; however, the fluorescence changes were too small to permit confident calculation of $K_d$ values.

Overall, this section of work supports the concept that the nonpolar surface of an amphipathic helix at the N terminus of α-subunit is important for α/δ binding. It also suggests that residues α8-8, αLeu-11, αlle-12, αlle-16, and αPhe-19 on this surface make direct contact with δ. Testing the Idea That a Postulated Helix Capping Box at Residues α3–8 Is Important for Binding of α Subunit to δ Subunit—Fig. 1A showed that within the amino acid sequence at the N terminus of α subunit, residues α3–8 conform to a consensus helix capping box motif (24). To test whether the postulated capping box plays a critical role in binding of α to δ, we introduced point mutations and deletions in this region, both in synthetic peptides and in the uncA gene in cells. Residue α8-8 corresponds to “N6” of the capping box motif, where hydrophobic residues are preferred (24). Evidence described above showed that hydrophobic residues are favored at this position, with the charged Glu and Lys substitutions impairing both peptide/δ binding and ATP synthase function.

![Table I](image)

| Peptide   | $K_d$ for wild-type δ (μM) | $K_d$ for mutant V79A δ (μM) |
|-----------|----------------------------|-----------------------------|
| αN1-22    | 0.113                      | 1.95                        |
| Truncated peptides |
| αN1-24    | 4.9 (43×)                  | 14.6 (7.5×)                 |
| αN1-20    | 1.2 (11×)                  | 6.5 (3.4×)                  |
| αN1-18    | 6.1 (54×)                  | 16.9 (8.7×)                 |
| αN1-16    | 13.5 (119×)                | 117 (60×)                   |
| αN1-14    | NS                         | NS                          |
| αN1-12    | NS                         | NS                          |
| Point mutations |
| αN1-22(I8K) | 0.86 (7.6×)              | 7.6 (6.7×)                  |
| αN1-22(I8E) | 0.62 (5.5×)              | 7.6 (6.7×)                  |
| αN1-22(I11E) | 2.0 (13×)               | 19.9 (10.2×)                |
| αN1-22(I12E) | NS                      | NS                          |
| αN1-22(I16E) | 1.3 (12×)               | 10.2 (5.2×)                 |
| Internal deletions |
| αN1-22(3–6) | 0.34 (3.0×)             | 9.7 (5.0×)                  |
| αN1-22(3–7) | NS                       | NS                          |
| αN1-22(3–8) | 12.9 (114×)              | 76.5 (30×)                  |
| αN1-22(4–9) | NS                       | NS                          |
| αN1-22(4–10) | NS                     | NS                          |
| αN1-22(6–9) | 7.9 (70×)                 | NS                          |
| αN1-22(10–13) | NS                    | NS                          |

*NS, no significant change in fluorescence.

* A small quench of fluorescence was seen, of insufficient magnitude to permit confident calculation of $K_d$ value.
residues in \(\alpha N1-22(\Delta 3-6)\), had a small effect, reducing affinity by 3-fold in wild-type and 5-fold in \(V79A\) \(\delta\). The five-residue deletion in \(\alpha N1-22(\Delta 3-7)\), which removes the conserved \(\alpha Glu-7\) residue referred to above, abolished binding. However, the next larger deletion, of six residues in \(\alpha N1-22(\Delta 3-8)\), which removes additionally the \(\alpha Ile-8\) residue, did show some, albeit weak, binding. The two next larger deletions (\(\alpha N1-22(\Delta 3-9)\) and \(\alpha N1-22(\Delta 2-10)\)) were both inactive in binding. Of course, in these cases, the length of the helix will be considerably reduced.

Effects of these deletions on growth of cells and function of ATP synthase in membranes were also studied (Table III). It is seen that the \(\alpha \Delta 3-6\) deletion reduced growth yield, showing that ATP synthesis by oxidative phosphorylation was partly impaired \(i n\ do\). In contrast, the other deletions in this series appeared to completely prevent ATP synthesis, because they gave growth yields the same as the null control. Growth yield data in Table III were corroborated by analysis of growth on succinate plates, with \(\alpha \Delta 3-6\) showing partial growth and the rest showing no growth. Also shown in Table III are ATPase activities of membrane vesicles prepared from the deletion strains. The \(\alpha \Delta 3-6\) strain showed partial activity (13.5% of wild-type), and the \(\alpha \Delta 3-8\) showed a small residual activity (1% of wild-type, see footnote c of Table III for absolute activity), whereas \(\alpha \Delta 3-7\), \(\alpha \Delta 3-9\), and \(\alpha \Delta 2-10\) showed less. The ATPase activity in \(\alpha \Delta 3-6\) had somewhat reduced sensitivity to inhibition by DCCD (Table III); for the other strains, the activity was low, so DCCD sensitivity was not tested. ATP-driven proton pumping in membrane vesicles was measured by the acridine orange fluorescence quenching technique. The deletion strain \(\alpha \Delta 3-6\) showed only partial quench with ATP, consistent with its reduced ATPase activity, whereas all the other strains showed zero quench with ATP. These data corroborate the peptide binding data in Table I. It may be noted that NADH-induced proton gradient formation in membrane vesicles from all of the deletion strains was the same as for wild type (data not shown).

Two further deletions were studied, namely \(\alpha \Delta 6-9\), which removed the later part of the helix capping box sequence, and \(\alpha \Delta 10-13\), which served as a control involving a four-residue deletion located away from the capping box. Fluorescence titrations with peptides containing these deletions were carried out using wild-type and mutant \(V79A\) \(\delta\) subunit, and the \(K_f\) values are listed in Table I. The deletion in \(\alpha N1-22(\Delta 6-9)\) was detrimental, reducing binding affinity by 70-fold in wild-type \(\delta\) and abolishing it in \(V79A\) mutant \(\delta\). The deletion in \(\alpha N1-22(\Delta 10-13)\) abolished binding to either \(\delta\) preparation. These deletions were also introduced into the uncA gene, and their
effects on growth yield in cells and ATP synthase function in membrane preparations are described in Table III. The αΔ6–9 deletion had a significant impairing effect on growth yield, membrane ATPase, and ATP-driven proton pumping. DCCD sensitivity of ATPase was much reduced. The αΔ10–13 deletion abolished ATP synthase function. NADH-induced proton gradient formation was normal in both strains (data not shown).

Overall, whereas the data show significant impairment caused by point mutations or deletions within the postulated helix capping box sequence, it is also notable that point mutations at residue αGlu-7 were only partly debilitating, and the results were not consistent with those expected from statistical analyses of preferred residues at this position of the capping box sequence (24). Also, deletions αΔ3–6 and αΔ6–9 were only partly debilitating, whereas αΔ10–13, away from the capping box sequence, was completely debilitating.

Deletion of Residue αGln-2—Ogilvie et al. (21) found that when the mutation αQ2C was introduced into ATP synthase, the new Cys was able to cross-link to a Cys in δ subunit, implying that residue αGln-2 might play a functional role in δ binding to α. However, as the last line of Table III shows, the deletion αΔ2 had no effect on growth yield in vivo. Membrane ATPase activity was 54% of wild type, this reduction in activity apparently not sufficient to affect growth in vivo. (Note that the ATPase activities in membranes of pBWU13.4/DK8 wild type and residues αΔ10–13, away from the capping box sequence, was extremely debilitating.)

DISCUSSION

In recent studies of the stator subunits (b3δ) of ATP synthase, we established quantitative parameters for binding of δ subunit to α subunit (14) and identified (15) the F1δ binding surface on the NMR structure (16) of the N-terminal domain of δ. We further demonstrated, using a synthetic peptide called αN1–22, that the N-terminal 22 residues of α form a major component of the δ-binding surface on F1, and that just one of the three α subunits in ATP synthase binds δ (17). As noted in the Introduction, residues α6–18 are predicted to be α-helical, and residues α3–8 appear to form a potential helix capping box (Fig. 1A). This, together with circular dichroism and NMR information that αN1–22 does form helical structure, lead to a model of how the N-terminal region of α subunit might bind to δ, shown in Fig. 1B. In the model, a continuous helix comprising most of the residues α1–22 binds to the F1-binding face presented by helices 1 and 5 of the N-terminal domain of the δ subunit.

In this paper, we have examined sequence characteristics within the N-terminal 22 residues of α, looking for determinants of δ binding. Our approach was to introduce truncations, point mutations, and internal deletions into the synthetic αN1–22 peptide and into the α subunit in ATP synthase, determining Kd values for binding of the peptide to δ and measuring effects on growth yield, membrane ATPase, and ATP-driven proton pumping activities in cells.

We can first make the general point that there was strong correlation between effects of deletions and point mutations on quantitative impairment of binding of synthetic peptides to purified δ subunit in fluorescence titrations and effects on impairment of ATP synthase function in cells and membrane preparations. This not only validates the approach used; it also has ramifications for future work, in that it will be less expensive by far to examine multiple mutations in this region by mutating unαA than by synthesizing many novel peptides. Interesting mutations identified in unαA can later be assayed for quantitative effects on δ binding using peptides.

Initially, we determined the optimal length of the peptide for binding to δ using a series of peptides of differing length. From these data, it was apparent that αN1–22 was optimal, with extension to αN1–24 or truncation to αN1–20 leading to loss of binding affinity. Further truncation to αN1–18 and αN1–16 led to further loss of binding affinity, and αN1–14 and αN1–12 showed no significant binding. One might conclude from this series that residues between positions α14 and α22 provide most of the binding interactions; however, this conclusion is negated by the facts that deletions αΔ2–10 and αΔ10–13, as well as e.g. αΔ3–7 and others, also abrogated binding. The summary picture that emerges is that (with the possible exception of residues at the extreme N terminus) the whole sequence αN1–22 is required. This region of α subunit is disordered in x-ray structures (7, 22, 23). A speculative possibility is that this region of the α subunit normally exists in an equilibrium between disordered and helical structure, and only when it collapses productively with the binding surface on δ does it assume the fully folded helical structure.

A helix formed by the αN1–22 peptide will be strongly amphipathic as shown in Fig. 1C. In the N-terminal domain of δ subunit, helices 1 and 5 form a hydrophobic groove (16). These

| Strain                | Growth yield | Membrane ATPase activity | DCCD sensitivity of ATPase activity | ATP-driven proton pumping |
|-----------------------|--------------|--------------------------|------------------------------------|--------------------------|
|                       | % of wild type | % of wild type | % inhibition | % fluorescence quench |
| Wild type (pBWU13.4/DK8) | 100          | 100          | 85            | 88                        |
| ATP synthase null (pUC118/DK8) | 43            | 0.24*           | ND           | 0                          |
| αΔ3–6                 | 79            | 13.5          | 61           | 44                        |
| αΔ3–7                 | 44            | 0.53          | ND           | 0                          |
| αΔ3–8                 | 43            | 1.02*         | ND           | 0                          |
| αΔ3–9                 | 42            | 0.42          | ND           | 0                          |
| αΔ2–10                | 42            | 0.30          | ND           | 0                          |
| αΔ6–9                 | 55            | 9.7           | 17           | 7                          |
| αΔ10–13               | 43            | 0.43          | ND           | 0                          |
| αΔ2                  | 98            | 54            | 79           | 87                        |

* Activity of wild type varied from 4.2 to 6.8 μmol/min/mg membrane protein in different experiments. A wild type was always run alongside to allow comparison with mutants.

** Assayed in membrane preparations by measuring quench of acridine orange fluorescence.

*** Absolute values (μmol/min/mg) were as follows: pUC118/DK8, 0.014 ± 0.0058 (S.D., n = 10); αΔ3–8, 0.058 ± 0.0034 (S.D., n = 10).

† ND, not determined.

** Table III Effects of deletions in the N-terminal region of α subunit on ATP synthase function in cells and membrane preparations

All data are means of at least quadruplicate determinations, except ATPase values are means of quintuplicates.
same helices form the F$_1$-binding surface on δ (15). It seemed likely, therefore, that the nonpolar side of the α11–22 helix would bind to δ, and this was made more likely by the fact that the specific residues on this surface (α1le-8, αLeu-11, αle-12, αle-16, and αPhe-19) are almost invariably hydrophobic in multiple species. Results obtained using point mutations of these residues to Glu or Lys showed that both binding of peptides to δ and ATP synthase function in cells and membranes were impaired. These data indicate that it is the nonpolar surface of the α subunit N-terminal helix that binds to δ.

The importance of the postulated helix capping box (Fig. 1A) was studied using point mutations and deletions. As noted under “Results,” point mutations at residue αGlu-7 gave ambiguous data in relation to the involvement of this residue in a capping box motif. Effects of mutation of residue αle-8 were consistent with its playing a role as part of the helix capping motif, but this residue is also part of the nonpolar surface of the helix, probably involved directly in interacting with δ. Results obtained with deletions placed in and around the capping box motif were similarly equivocal. Therefore, in regard to the potential importance of the helix capping box sequence, we lean toward the view that this sequence per se is not critical for function.

In all cases where membrane ATPase activity was reduced, the membranes were not proton-leaky as judged by NADH-induced proton pumping assay. The lowered activity was due therefore to a reduced level of assembly of the enzyme into the membranes rather than to dislocation of F$_1$ from F$_0$ during membrane isolation. A further conclusion from our work is that correct αδ interaction is necessary for membrane assembly of ATP synthase. Interestingly, with the αΔ6–9 deletion, the resultant partial membrane ATPase activity was less sensitive to inhibition by DCCD than wild type, suggesting transient dissociation/association between α and δ during turnover. In other strains, there was less indication that the mutant complexes, once formed, were “uncoupled.” This might be explained as follows. As we showed in Ref. 15, the δ subunit has a strong effect to increase the affinity of binding between α and δ, possibly reducing the $K_d$ to 3 pm in wild type. Therefore, once the intact complex is formed, the stator could well be strong enough to resist uncoupling and to hold F$_1$ firmly to F$_0$ even in strains where interaction between the N terminus of α and δ is impaired.

**Acknowledgment**—We thank Christina DeVries for excellent technical assistance.

**REFERENCES**

1. Diez, M., Zimmerman, B., Börsch, M., König, M., Schweinberger, E., Steigmiller, S., Reuter, R., Felekys, S., Kudryavtsev, V., Seidel, C. A. M., and Gruber, P. (2004) *Nat. Struct. Mol. Biol.* 11, 135–141

2. Hoh, H., Takahasahi, A., Adachi, K., Noji, H., Yasuda, R., Yoshida, M., and Kinonita, K. (2004) *Nature* 427, 465–468

3. Nishizaka, T., Oiwa, K., Noji, H., Kimura, S., Muneyuki, E., Yoshida, M., and Kinonita, K. (2004) *Nat. Struct. Mol. Biol.* 11, 142–148

4. Weber, J., and Senior, A. E. (2003) *FEBS Lett.* 545, 61–70

5. Noji, H., and Yoshida, M. (2001) *J. Biol. Chem.* 276, 1665–1668

6. Nakamoto, R. K., Ketchum, C. J., and Al-Shawi, M. K. (1999) *Annu. Rev. Biophys. Biomol. Struct.* 28, 205–234

7. Menz, R. I., Walker, J. E., and Leslie, A. G. W. (2001) *Cell* 106, 331–341

8. Dunn, S. D., McLachlin, D. T., and Revington, M. (2000) *Biochim. Biophys. Acta* 1458, 356–363

9. Cain, B. D. (2000) *J. Bioenerg. Biomembr.* 32, 365–371

10. McLachlan, D. T., Coveny, A. M., Clark, S. M., and Dunn, S. D. (2000) *J. Biol. Chem.* 275, 17571–17577

11. Kersten, M. V., Dunn, S. D., Wise, J. G., and Vogel, P. D. (2000) *Biochemistry* 39, 3856–3860

12. Weber, J., Wilke-Mounts, S., Nadanaciva, S., and Senior, A. E. (2004) *J. Biol. Chem.* 279, 11293–11298

13. Diez, M., Borsch, M., Zimmerman, B., Turina, P., Dunn, S. D., and Gruber, P. (2004) *Biochemistry* 43, 1054–1064

14. Weber, J., Wilke-Mounts, S., and Senior, A. E. (2002) *J. Biol. Chem.* 277, 18390–18396

15. Weber, J., Wilke-Mounts, S., and Senior, A. E. (2003) *J. Biol. Chem.* 278, 13409–13416

16. Wilkens, S., Dunn, S. D., Chandler, J., Dahlquist, F. W., and Capaldi, R. A. (1997) *Nat. Struct. Biol.* 4, 197–201

17. Weber, J., Muharemaccse, A., Wilke-Mounts, S., and Senior, A. E. (2003) *J. Biol. Chem.* 278, 13623–13626

18. Wilkens, S., Zhou, J., Nakayama, R., Dunn, S. D., and Capaldi, R. A. (2000) *J. Mol. Biol.* 295, 387–391

19. Rubinstein, J. L., Walker, J. E., and Henderson, R. (2003) *EMBO J.* 22, 6182–6192

20. Dunn, S. D., Heppel, L. A., and Fullmer, C. S. (1986) *J. Biol. Chem.* 255, 6991–6996

21. Ogilvie, I., Agerbo, R., and Capaldi, R. A. (1997) *J. Biol. Chem.* 272, 16652–16656

22. Gibbons, C., Montgomery, M. G., Leslie, A. G. W., and Walker, J. E. (2000) *Nat. Struct. Biol.* 7, 1055–1061

23. Blanchet, M. A., Hullihen, J., Pedersen, P. L., and Amzel, L. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 11065–11070

24. Aurora, R., and Rose, G. D. (1998) *Protein Sci.* 7, 21–38

25. Senior, A. E., Latchney, L. R., Ferguson, A. M., and Wise, J. G. (1984) *Arch. Biochem. Biophys.* 228, 49–53

26. Ketchum, C. J., Al-Shawi, M. K., and Nakamoto, R. K. (1998) *Biochem. J.* 330, 707–712

27. Klionsky, D. J., Bruslow, W. S. A., and Simoni, R. D. (1984) *J. Bacteriol.* 160, 1055–1060

28. Elrinf, M. R. (1997) *Methods Enzymol.* 278, 221–257
Analysis of Sequence Determinants of F$_1$F$_0$-ATP Synthase in the N-terminal Region of $\alpha$ Subunit for Binding of $\delta$ Subunit

Joachim Weber, Alma Muharemagic, Susan Wilke-Mounts and Alan E. Senior

J. Biol. Chem. 2004, 279:25673-25679.
doi: 10.1074/jbc.M402738200 originally published online April 6, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402738200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 28 references, 12 of which can be accessed free at http://www.jbc.org/content/279/24/25673.full.html#ref-list-1