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Structure–function relationships of domains of the δ subunit in *Escherichia coli* adenosine triphosphatase

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The topology of the and subunit of the *Escherichia coli* adenosinetriphosphatase (ECF_1) has been explored by proteinase digestion and chemical labeling methods. The δ subunit of ECF_1 could be cleaved selectively by reaction of the enzyme complex with very low amounts of trypsin (1:5000, w/w). Cleavage of the δ subunit occurred serially from the C-terminus. The N-terminal fragments of the δ subunit remained bound to the core ECF_1 complex through sucrose gradient centrifugation, indicating that part of the binding of this subunit involves the N-terminal segment. ECF_1, in which around 20 amino acids had been removed from the C-terminus of δ, still bound to ECF_0 but DCCD sensitivity of the ATPase activity was lost. When ECF_1 was reacted with *N*-ethyl[^{14}C]maleimide ([^{14}C]NEM) in the native state, only one of the two Cys residues on the δ subunit was modified. This residue, Cys-140, was also labeled in ECF_1F_0. Cys-140 was shown to be involved in the disulfide bridge between α and δ subunits that is generated when ECF_1 is treated with $CuCl_2$. Thus, the C-terminal part of the δ subunit around Cys-140 can interact with the core ECF_1 complex. These results suggest a model for the δ subunit in which the central part of polypeptide is a part of the stalk, with both N- and C-termini associated with ECF_1.

Introduction

An F_1F_0-type ATPase is responsible for ATP synthesis coupled to respiration in the bacterial plasma membrane, the mitochondrial inner membrane and the chloroplast thylakoid membrane. The best characterized F_1F_0-type ATPase is the enzyme from *Escherichia coli*. The F_1 part of this enzyme (ECF_1) is made up of five different subunits (α, β, γ, δ and ε) in the ratio 3:3:1:1:1 and the F_0 part (ECF_0) is composed of three different subunits a, b and c in the ratio 1:2:12–15 (reviewed in Refs. 1–3).

Electron microscopy experiments have shown that ECF_1 is linked to ECF_0 by a stalk approx. 40 Å long and 25–30 Å in diameter [4,5]. Several subunits have been implicated as components of the stalk in that they are required for binding of ECF_1 to ECF_0; including a [6,7], and [8,9] and and [10] of ECF_1 and subunit b [11,12] of ECF_0. Additional evidence that some of these four subunits are in the interface between ECF_1 and ECF_0 has come from chemical labeling and also from crosslinking experiments [13–15].

The work reported here focuses on the δ subunit. This subunit is highly sensitive to proteinase digestion in isolated ECF_1 [1516,1516], but is protected from cleavage in ECF_1F_0 [15]. We have found conditions for limited proteolytic cleavage of the δ subunit in intact ECF_1. The sites of cleavage have been localized and the effect of removing segments of the δ subunit on the ability of ECF_1 to bind to ECF_0 has been examined. Also, a reactive Cys-residue in the δ subunit has been identified. Modification of this Cys-under different conditions is used in conjunction with the proteoly-

Abbreviations: ECF_1, water-soluble, membrane-extrinsic ATPase sector of the F_1F_0 complex of *E. coli*; CF_1, water-soluble, membrane-extrinsic ATPase sector of the F_1F_0 complex of chloroplasts; DTT, DL-dithiothreitol; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; PVDF, poly(vinylidene difluoride); SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; TPCK, tosylphenylalanine chloromethyl ketone; εACA, ε amino hexanoic acid; NEM, *N*-ethylmaleimide; OSCP, oligomycin sensitivity conferring protein from the mitochondrial F_1F_0 complex; mAb, monoclonal antibody; LDAO, *N*,*N*-dimethyldodecyl *N*-oxide; PAB, *p*-aminobenzamidine; PAGE, polyacrylamide gelelectrophoresis.

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sis experiments to explore the topology of the δ subunit in ECF_1 and ECF_1F_0.

Experimental procedures

ECF_1 was isolated from *E. coli* strain AN1460 by a modification of the method of Wise et al. [17] to be described elsewhere. ECF_1F_0 was purified according to Foster et al. [18]. The purification and characterization of monoclonal antibodies used here is reported in Gogol et al. [4] and Aggeler et al. [19].

Trypsin cleavage of ECF_1

ECF_1 (1–5 mg/ml) was incubated in trypsin (ratio of 1:5000, w/w proteinase to enzyme) in a buffer of 50 mM Tris-HCl (pH 8.0), 5 mM $MgSO_4$ and 5 mM ATP. At times indicated in the figure legends, aliquots were withdrawn, inhibited by 1 mM phenylmethanesulfonyl fluoride (PMSF) and soybean trypsin inhibitor (ratio of 4:1, w/w inhibitor to proteinase), and analyzed for ATPase activity, activation by *N,N*-dimethyldodecyl *N*-oxide (LDAO) [20] and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Tryptic fragments of the δ subunit were identified by immunodetection, after electroblotting duplicate gels to those stained for protein with Coomassie brilliant blue onto nitrocellulose membranes as described by Towbin et al. [21].

Sequence analysis of tryptic fragments, resolved by polyacrylamide gel electrophoresis, was performed after transferring onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore) following the procedure of Matsudaira [22]. After minimal staining with Coomassie brilliant blue, the bands were excised and sequencing performed by a gas-phase protein sequencer (Applied Biosystems Model 470A) equipped with an online PTH analyzer (Applied Biosystems Model 120A).

Reconstitution experiments with ECF_1 and δ-truncated ECF_1

ECF_1F_0 was reconstituted into egg phosphatylcholine vesicles by the dialysis technique described by Foster and Fillingame [18]. Membranes were collected by centrifugation at $100\,000 \times g$ for 1 h and then suspended in 2 mM Tris-HCl (pH 7.0), 5 mM EDTA, 0.1% LDAO, 1 mM dithiothreitol (DTT) and 5% glycerol to remove the F_1 part. After 1 h of incubation at room temperature, the vesicles were collected by centrifugation as above, and the extraction procedure then repeated a second time. The pellet was washed by suspension in 50 mM Tris-HCl (pH 8.0), 1 mM DTT and 5% glycerol, followed by centrifugation and final suspension in 50 mM Tris-HCl (pH 8.0), 5 mM $MgSO_4$, 6 mM *p*-aminobenzamidine (PAB) and 10% glycerol. Stripped membranes were analyzed for ATPase activity, and for subunit composition using SDS-PAGE.

In rebinding experiments, stripped membranes (40 μg) were incubated with 150 μg ECF_1 or δ-truncated ECF_1 in 50 mM Tris-HCl (pH 8.0), 5 mM $MgSO_4$, 6 mM PAB and 10% glycerol for 5 h at room temperature. Reconstituted ECF_1F_0 vesicles were separated from unbound ECF_1 by centrifugation at $100\,000 \times g$ for 1 h in a Beckman Airfuge, and both supernatant and pellet assayed for ATPase activity, LDAO activation and inhibition by DCCD using 40 μM of the carbodiimide [23].

The δ-truncated ECF_1 used in rebinding experiments was obtained by incubating ECF_1 with trypsin as described above for 40 min at room temperature. After inhibiting proteolysis with 1 mM PMSF and soybean trypsin inhibitor, the sample was passed through a Sephacryl 5300 column (28 × 1.5 cm) equilibrated in 50 mM Tris-HCl (pH 8.0), 5 mM $MgSO_4$, 6 mM PAB and 10% glycerol.

Labeling of cysteines in ECF_1 and ECF_1F_0

For labeling of Cys-residues, ECF_1 (1–4 mg/ml) eluted from a Sephadex G50 centrifuge column (1 ml volume) in 50 mM Tris-HCl (pH 7.0), 5 mM $MgSO_4$, 10% glycerol was reacted with 200 μM [^{14}C]NEM (60 μCi/ml) without other additions and in the presence of 0.2% LDAO or 2% SDS, respectively. After 1 h at room temperature, unbound NEM was quenched with excess L-cysteine and aliquots were subjected to SDS-PAGE. Subunit labeling was quantitated by counting radioactivity in 2 mm slices of the gel as described by Aggeler et al. [14].

Characterization of the α–δ crosslinking

ECF_1 precipitated with $(NH_4)2SO_4$ was resuspended in 50 mM sodium phosphate (pH 7.4) and desalted through two consecutive centrifuge columns of Sephadex G50-fine equilibrated in the same buffer. The sample was divided into three aliquots. Copper sulfate (20 μM) was added to an aliquot previously modified by NEM, and to a second aliquot as a control, and the protein was centrifuged through Sephadex G50-fine in 50 mM sodium phosphate (pH 7.4), 20 μM $CuCl_2$ as described by Tozer and Dunn [24]. The third aliquot was applied to the centrifuge column without $CuCl_2$ present. Once collected from the column, EDTA (2 mM) was added to samples followed by 100 μM [^{14}C]NEM and the mixture was incubated for 1 h at room temperature. The distribution of radioactivity was determined after SDS-PAGE as described above.

Other methods

Protein concentrations were determined according to Markwell et al. [25]. Samples for SDS-PAGE were dissolved in 2% SDS, 5% glycerol and 0.12 M Tris-HCl

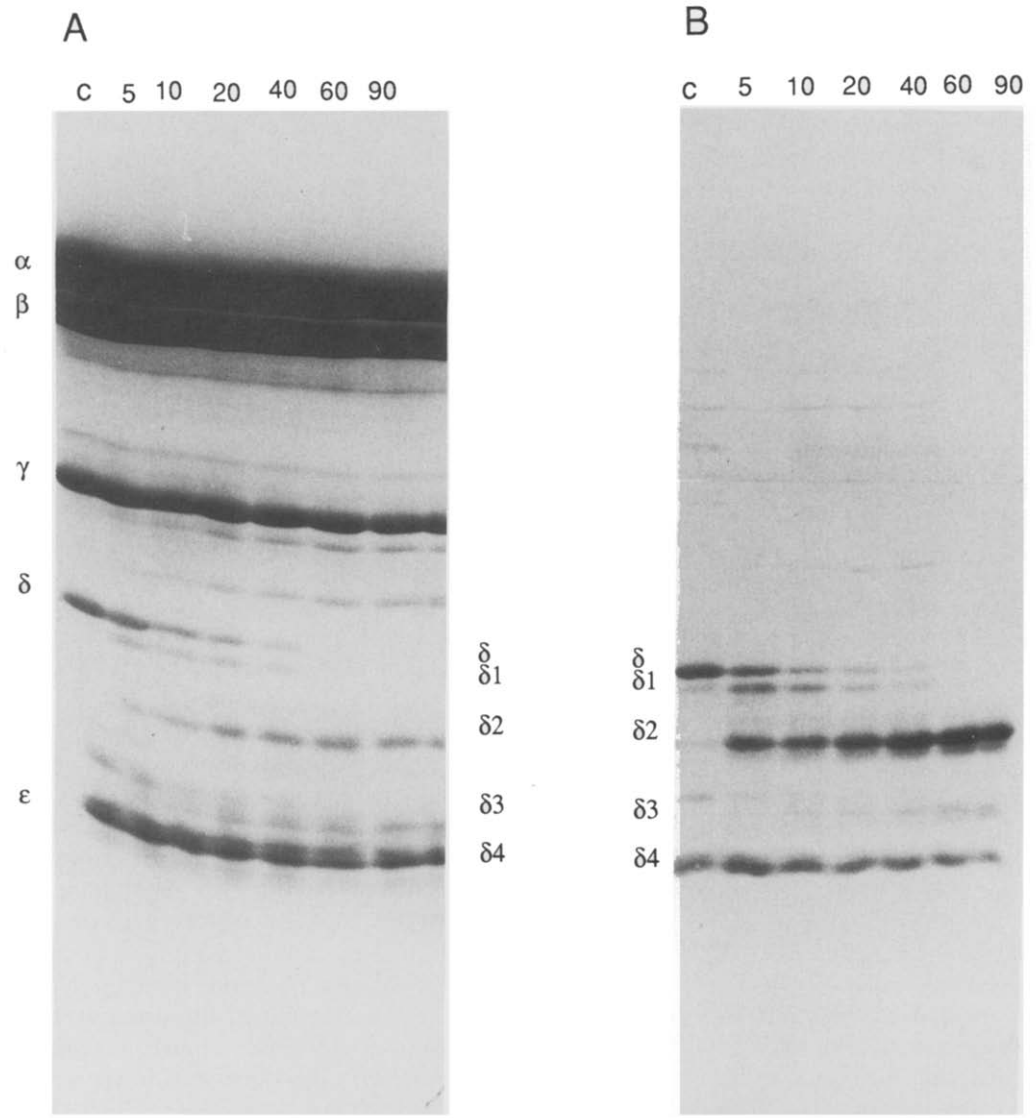

Fig. 1. Time course of trypsin cleavage of ECF_1. ECF_1 (3 mg/ml) was cleaved with trypsin at a ratio of 1:5000 (proteinase to protein, w/w). Before (control) and after indicated times of proteolysis (min), 40-μl samples were removed and inhibited by 1 mM PMSF and soybean trypsin inhibitor (1:4, trypsin to inhibitor, w/w). Each sample was then applied in two aliquots to identical 10–18% SDS-polyacrylamide gels to be (A) stained by Coomassie brilliant blue and (B) transferred to nitrocellulose for immunoblotting with δ subunit mAbs.

(pH 6.8), 1.5 mm thick slab gels were run as described by Laemmli [26] using 3% acrylamide gel as stacker and a 10–18% gradient of acrylamide in the separating gel. Staining with Coomassie brilliant blue and destaining were carried out according to Downer et al. [27]. Immunoblotting was conducted according to Towbin et al. [21] using a buffer containing 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) pH 11.0 and 10% methanol. Alkaline phosphatase-conjugated goat anti-mouse antibodies were used for color development [28].

ATPase activity was assayed using the ATP regenerating system described previously [29].

Results

Selective proteolysis of the δ subunit in ECF_1

All five subunits of ECF_1 are sensitive to trypsin (50:1, w/w) with the δ and ϵ subunits cleaved most rapidly followed by the α and γ and finally the β subunit [15,16]. The cleavage of the ϵ subunit by trypsin has been found recently to depend on which nucleotides are present in the buffer [30]. There is a rapid cleavage of the ϵ subunit in the presence of ADP + Mg^{2+} but slow cleavage of this subunit when ATP + Mg^{2+} are added. Fig. 1 follows the time-course of cleavage of ECF_1 by trypsin at a ratio of 1:5000 (w/w)

with respect to enzyme in the presence of 5 mM ATP + 5 mM Mg^{2+}. Under these conditions the δ subunit alone is cleaved: the lack of cleavage of α, β, γ or ϵ subunits being confirmed by Western blotting with monoclonal antibodies (mAbs) specific to each of these subunits (results not shown). Trypsin cleavage of the δ subunit was followed by Western blotting using a subunit-specific mAb (Fig. 1B). Lane 1 shows the reaction of the anti-δ mAb with untreated enzyme. Fragments of the δ subunit are present in all preparations, presumably generated by endogenous proteinase impurities. The first product of trypsin cleavage, $\delta 1$, is only slightly smaller than intact δ. With time, intact δ and $\delta 1$ disappear and $\delta 2$, $\delta 3$ and $\delta 4$ are formed with M_r of 16000, 12500 and 9800, respectively. Prolonged incubation with trypsin leads to disappearance of $\delta 1$, $\delta 2$, $\delta 3$ and $\delta 4$, with smaller products being formed which are not recognized by the mAb.

The major cleavage products of the δ subunit were collected by electrotransfer onto Immobilon from gels such as in Fig.1 and the N-terminal sequences of each were determined. All four fragments had the same N-terminus (Ser-Glu-Phe-Ile-Thr-Val-Ala-Arg), which is the N-terminal sequence of the mature polypeptide. Therefore, cleavage of the δ subunit in intact F_1 must occur stepwise from the C-terminus. Likely, sites of cleavage, based on molecular weight considerations, are shown in Fig. 2.

Binding of δ fragments to the ECF_1 core

Binding of truncated forms of the δ subunit to the ECF_1 core complex was monitored by sucrose gradient centrifugation. Enzyme was incubated in trypsin as above, and then the protein was centrifuged for 12 h through a sucrose gradient of 10% to 40% sucrose. The $\delta 1$, $\delta 2$, $\delta 3$ and $\delta 4$ fragments all co-sedimented with the core ECF_1 complex. In contrast, when ECF_1 was treated with 0.2% LDAO, the δ subunit could be separated from the ECF_1 core by sucrose gradient centrifugation [29]. Trypsin cleavage of the N-terminus

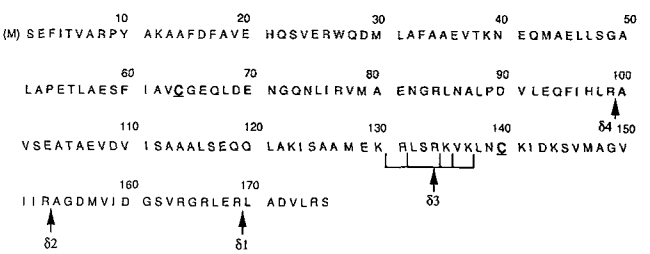

Fig. 2. Putative sites of trypsin cleavage of the δ subunit of ECF_1. Trypsin sensitive sites in the δ subunit corresponding to the estimated molecular weights of each cleavage product are indicated in the amino acid sequence of this subunit. The two cysteine residues occur at positions 64 and 140, as indicated.

of the a subunit also prevented binding of the intact δ in sucrose gradient centrifugation experiments [7]. The tight binding of the several δ subunit fragments to ECF_1, each with different lengths of the C-terminus removed, indicates that binding of the δ subunit involves the N-terminal half of the polypeptide.

Binding of ECF_1 containing a truncated δ subunit to ECF_0

The interaction of ECF_1 containing truncated forms of the δ subunit with ECF_0 was monitored in reconstitution experiments. ECF_1 was reacted with trypsin until the δ subunit was predominantly in the $\delta 2$ form as shown by the Coomassie brilliant blue stained gel and by Western blotting of a companion gel (Fig. 3A). This ECF_1 preparation, and untreated enzyme as a control, were each reacted with stripped membranes that had been made by mixing ECF_1F_0 and lipids and then removing most of the ECF_1 by low salt-EDTA treatment (see Experimental procedures). Fig. 3B shows the reduction of δ subunit caused by the stripping procedure.

Rebinding of intact ECF_1 and ECF_1 containing a truncated δ subunit to stripped membranes was confirmed by Western blotting (Fig. 3C, lanes 1 and 2, respectively). Also shown is a control in which stripped membranes were reacted with intact ECF_1 and subsequently reacted with ECF_1 containing the cleaved form of δ (lane 3). (These blots were overloaded with protein to detect small amounts of cleaved δ, and hence the smearing.) The prior binding of the intact enzyme blocked interaction of the proteolyzed enzyme (which remains in the supernatant), indicating that both are reacting with the same sites in the vesicles.

The ATPase activity of ECF_1F_0 in stripped membranes was measured in the presence and absence of LDAO, and with and without DCCD added (Table I). Preparations always contained a small amount of ECF_1 left by the stripping procedure, which was activated by as much as 6-fold by LDAO, cf. 15–20-fold activation observed for free ECF_1. The residual, bound, ECF_1 was sensitive to DCCD, and inhibited 90% by 40 μM DCCD. The maximal activity of stripped membranes to which native ECF_1 had been bound was 6–8-fold higher than that of stripped membranes alone. This reconstituted ECF_1F_0 was activated 4–5-fold by LDAO and inhibited 90% by DCCD modification of the F_0 part. The binding of ECF_1 containing a truncated δ subunit to stripped membranes also increased the ATPase activity 6-fold; there was a 4–5-fold activation by LDAO but with enzyme in which the δ subunit was altered, DCCD sensitivity was less than 5%. These findings indicate that ECF_1 that is missing the C-terminal part of the δ subunit still binds to ECF_0 but DCCD sensitivity is lost by a disruption of the interaction between the F_1 and F_0 parts.

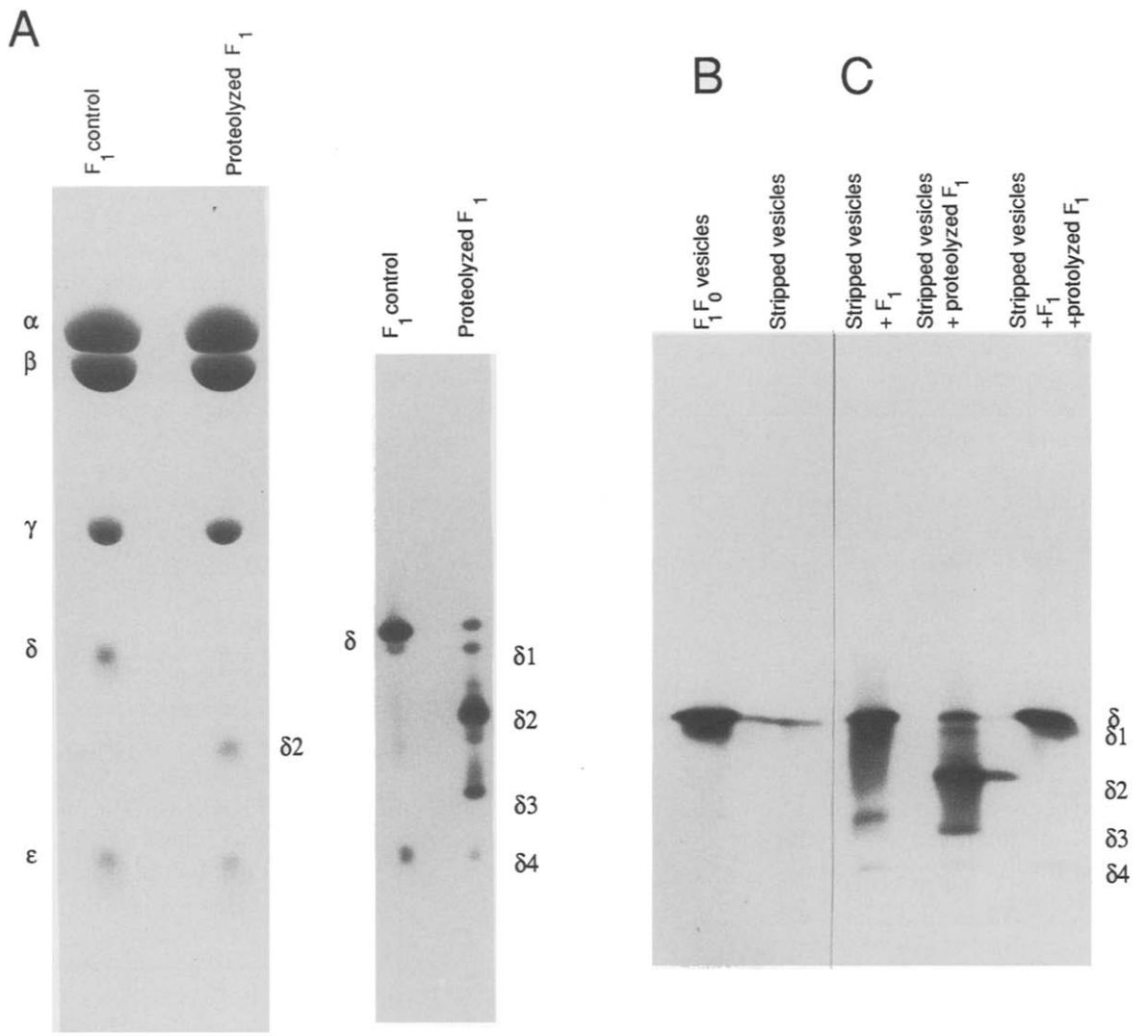

Fig. 3. Binding of δ-truncated ECF_1 to ECF_0 vesicles. (A) shows SDS polyacrylamide gels of ECF_1 samples before and after the proteolysis/column isolation procedure as stained with Coomassie brilliant blue (left part) and by Western blotting with anti-δ mAbs (right part). (B) shows Western blotting of reconstituted membranes and stripped vesicles with the anti-δ mAb. (C) shows rebinding of ECF_1 and δ-truncated ECF_1 with the stripped membranes analyzed by Western blotting with the anti-δ mAb. The protein bands are more smeared in C because of heavy overloading of samples.

TABLE I

ATPase activity (units / ml) [a] *of reconstituted vesicles*

Incubated samples	Basal activity		Activity following inhibition with 40 μM DCCD		% Activation by LDAO	% Inhibition by DCCD [b]
	–	plus 0.5% LDAO	–	plus 0.5% LDAO		
(A) Stripped vesicles (plus buffer)	2.1	13.4	0.2	12.1	730	90.5
(B) Stripped vesicles plus intact ECF_1	14.2	64.3	1.4	63.9	450	90.1
(C) Stripped vesicles plus δ cleaved ECF_1	10.4	46.9	8.3	50.6	450	2.4

[a] Values are representative of those obtained in four different experiments; measurements in all cases made in duplicate.

[b] The residual F_1F_0 content of the stripped membranes was accounted for in calculating the DCCD inhibition of (B) and (C).

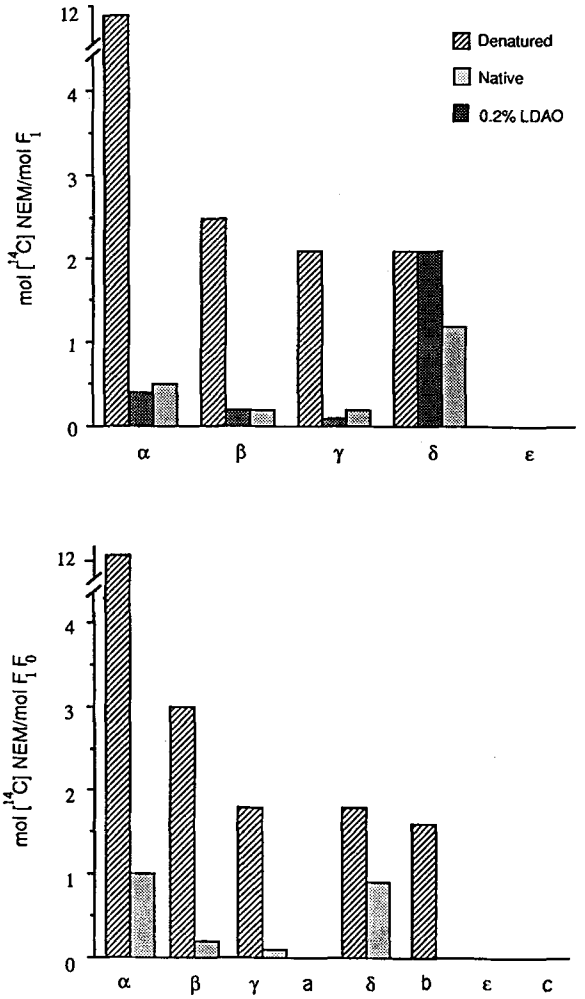

Fig. 4. Thiol accessibility in ECF_1 and ECF_1F_0. ECF_1 (1–4 mg/ml) was reacted with 100 μM [^{14}C]NEM in 50 mM Tris-HCl (pH 7.0), 5 mM $MgSO_4$ and 10% glycerol without additions, in the presence of 0.2% LDAO and after denaturation in 2% SDS (upper panel). ECF_1F_0 reconstituted into phospholipid vesicles, was reacted in the same buffer without additions and in the presence of 2% SDS (lower panel). Labeling of subunits, resolved by gel electrophoresis, was determined by quantitating radioactivity in 2 mm slices of the gel.

Labeling of Cys-residues in the δ subunit by [^{14}C]NEM

The δ subunit of ECF_1 contains two Cys-residues, one in the N-terminal part (Cys-64), the other in the C-terminal part (Cys-140). The labeling of these residues was explored, both to examine their reactivity in ECF_1 and ECF_1F_0, and as an aid to localizing proteolytic cleavage sites (see later). Fig. 4 summarizes the results of a typical labeling experiment. ECF_1 was reacted with [^{14}C]NEM before and after incubation with LDAO (to selectively release the δ subunit in a reconstitutively active form) and after denaturation in SDS. In native ECF_1, the δ subunit incorporated maximally 1.2 mol NEM per mol of ECF_1, suggesting that only one of the two Cys-residues was reacted. There was also labeling of the a subunit (0.3–0.5 mol [^{14}C]NEM per mol ECF_1 in different experiments) but very little incorporation of radioactivity into β or γ subunits. In LDAO treated enzyme, labeling of the δ subunit increased to 2 mol of reagent per mol of subunit there was more labeling of the α subunit (to 1 mol per mol of F_1) but no labeling of other subunits (i.e., β or γ). The stoichiometry of labeling of the δ subunit in SDS-denatured ECF_1 was the same as for the LDAO treated enzyme, but in this case there was labeling of α, β and γ subunits in the stoichiometry 4:1:2 on a molar basis.

Convincing evidence that [^{14}C]NEM reacts with only one of the two Cys-residues in the δ subunit in native

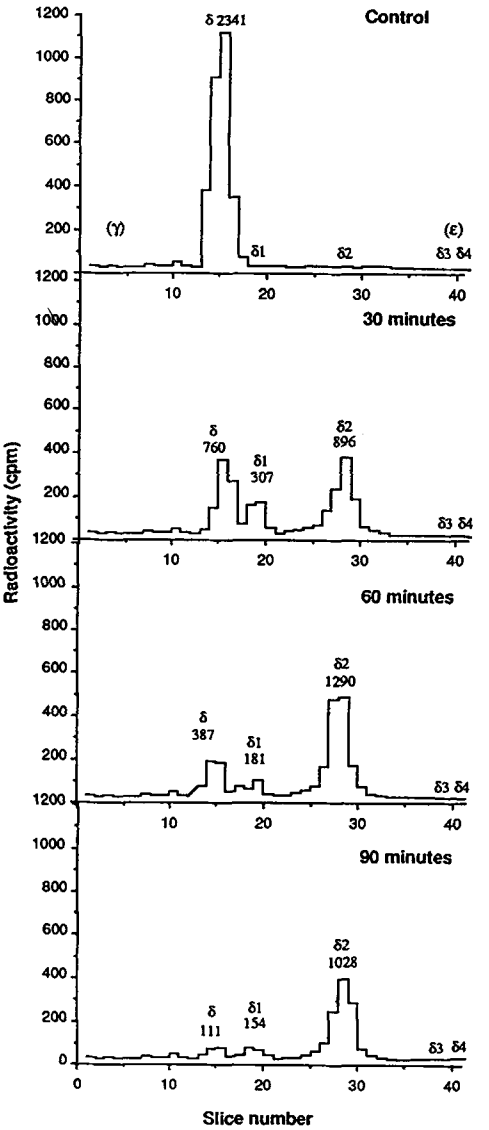

Fig. 5. Time-course of trypsin cleavage of the δ subunit in [^{14}C]NEM-labeled ECF_1. ECF_1 was reacted with [^{14}C]NEM in the native conformation and unreacted reagent was quenched, as described in Experimental procedures. The [^{14}C]NEM-labeled ECF_1 was then cleaved with trypsin (1:5000 proteinase to protein, w/w) applied to an SDS polyacrylamide gel to separate subunits and the radioactivity incorporated into the δ subunit and its fragments quantitated by slicing and counting.

ECF_1 was obtained in a joint labeling-proteolysis experiment. Enzyme was reacted first with [^{14}C]NEM in the native state, unreacted reagent was quenched and removed, and then the enzyme was reacted with trypsin (1:5000, w/w), using conditions described earlier. Fig. 5 shows the distribution of ^{14}C label in fragments of the δ subunit at three different lengths of time of proteolysis. The [^{14}C]NEM incorporated into the δ subunit was found in δ1 and δ2 but lost on generation of the δ3 or δ4 fragment. These results indicate that in native ECF_1, [^{14}C]NEM is incorporated only into the C-terminal Cys residue, Cys-140. Also, cleavage of the δ subunit to generate δ3 and δ4 must occur N-terminal of this Cys residue.

It was of interest to determine if Cys-140 was buried in ECF_1F_0 (see Discussion). Fig. 4B summarizes our experiments in which ECF_1F_0 was labeled with [^{14}C]NEM. Reaction of the native ECF_1F_0 with [^{14}C]NEM gave similar labeling to that found with ECF_1, i.e., incorporation of around 1 mol of reagent per mol δ and 1 mol of reagent per three mol of the a subunit. Thus, Cys-140 must be exposed in the intact ATP synthase. Reaction of SDS-denatured ECF_1F_0 with [^{14}C]NEM gave labeling of α, β, γ, δ and b subunits in the approximate ratio 4:1:2:2:2, as expected from sequence data.

Fig. 6 shows the sequence of the δ subunit around Cys-140 displayed as a helical wheel diagram. This cysteine residue is not a part of the face of the α-helix that contains several lysines shielded from proteolysis [15] and therefore likely buried in the stalk structure.

Identification of the Cys of the δ subunit involved in an α–δ crosslinked product

Tozer and Dunn [24], and Bragg and Hou [31] have both reported that the δ subunit crosslinks with an α subunit in high yield under conditions which favor disulfide bond formation. This crosslinking did not affect ATPase activity, but the crosslinked ECF_1 failed to couple ATP hydrolysis to proton translocation when rebound to stripped membranes. The data in Fig. 7 confirm that an α–δ crosslinked product is obtained when $CuCl_2$ is used to catalyze disulfide bond formation. The extent of crosslinking of the δ subunit was almost quantitative based on Western analysis with mAbs to the δ subunit (Fig. 7, part B). Prior reaction of ECF_1 with NEM to modify Cys-140 as described above, prevented the crosslinking of δ to α (result not shown). When enzyme that had been crosslinked by $CuCl_2$ reaction was subsequently modified with [^{14}C]NEM, there was no labeling of the α–δ product (Fig. 8, lane 1). It can be seen that incubation in NEM led to some dissociation of the crosslinked product and the small amount of free and so generated was labeled by the sulfhydryl reagent. Fig. 8, lane 2, shows enzyme crosslinked by the $CuCl_2$ treatment and then denatured in 2% SDS in the absence of sulfhydryl reagents before being labeled by [^{14}C]NEM. In this case there was labeling of the α–δ crosslinked product by incorporation of [^{14}C]NEM into the three Cys residues on α and one Cys-on δ that are not involved in crosslinking. Again, some free δ was generated and subsequently modified by the NEM treatment.

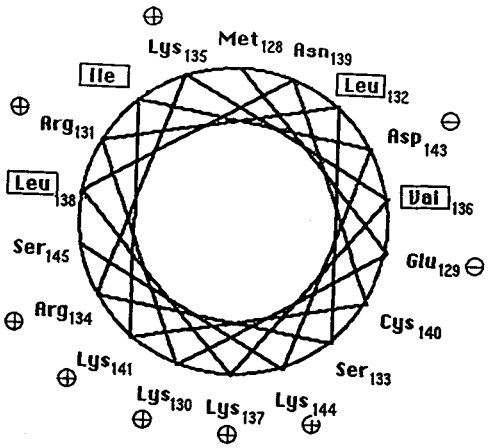

Fig. 6. Helical wheel diagram of the δ subunit between amino acids 128–145. Secondary structure predictions indicate continuous α-helical structure from residue 90 to 144 in the δ subunit sequence [41]. Five of eight lysine residues and the accessible cysteine-140 are included in the segment shown.

Discussion

Clear evidence that the δ subunit is important in linking ECF_1 to ECF_0 comes from reconstitution experiments [10,32,33]. ECF_1 depleted of the δ subunit only binds to ECF_0 if purified δ is added back. This subunit by itself is not sufficient to reconstitute a functional ATP synthase from the $\alpha\beta\gamma$ subunit complex and ECF_0: the ϵ subunit must also be added, indicating a role of ϵ in stabilizing the link between the two enzyme domains [10].

The elongated and high α-helical content of the δ subunit [8,34,35], has led to speculation that all or part of this polypeptide contributes to the stalk that links the two domains. For example, Cox et al. [36] have proposed that the C-terminal part of the δ subunit is an extended α-helix which interacts with the b subunit.

Studies described here are focused on the topology of the δ subunit, and in particular on the sites of interaction of this polypeptide with the core ECF_1 complex. Trypsin digestion was found to remove the C-terminal part of the δ subunit without dissociating this polypeptide from the $\alpha_3\beta_3\gamma\epsilon$ complex. This result implies that the N-terminal segment of the δ contacts the F_1 part and forms one site of binding. The C-terminal part of the δ subunit may also bind to ECF_1. It has been shown by Bragg and Hou [31] and Tozer and Dunn [24] and confirmed here, that the δ subunit

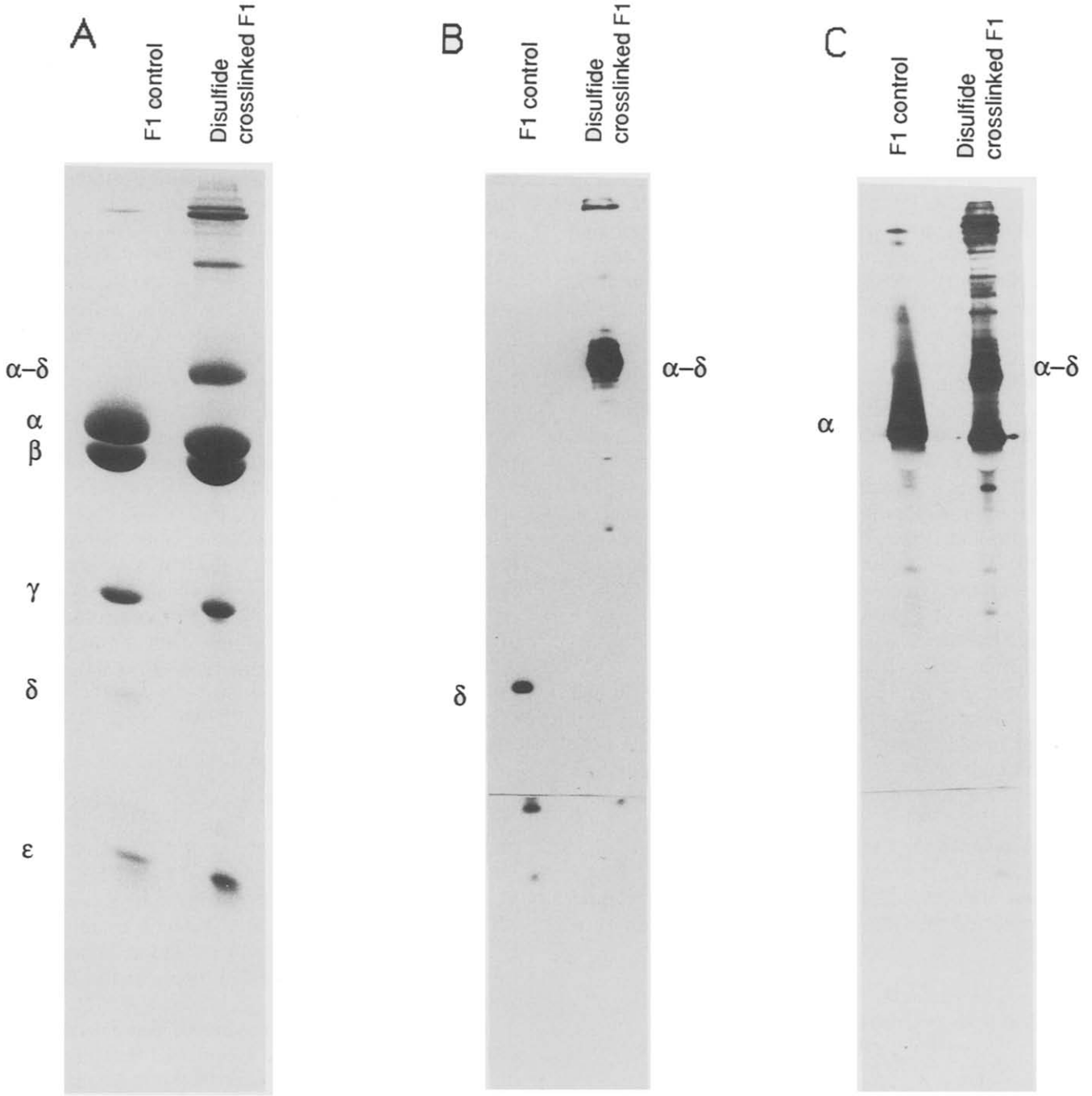

Fig. 7. Characterization of the $\alpha-\delta$ disulfide crosslink in ECF_1. Crosslinked samples were applied to a 10–18% SDS polyacrylamide gel in triplicate to be analyzed by staining with Coomassie brilliant blue (A) and immunoblotting with monoclonal antibodies to the δ subunit (B) and α subunit (C).

forms a disulfide bridge with the α subunit under mild oxidizing conditions. We have identified the Cys-involved in this linkage as Cys-140 of the δ subunit.

Taken together our results suggest a model in which the δ subunit is linked to ECF_1 by both the N- and C-termini, probably by interaction with an α subunit [24,31] and a β subunit (see the electron microscopy studies of Gogol et al. [4]). The part of the δ subunit involved in the stalk would then be the central part, which could form an extended structure as a two α-helix hairpin. Other evidence that the N- and C-termini of the δ subunit are in close proximity to one another comes from crosslinking with cupric 1,10-phenanthrolinate which can catalyze an internal disulfide bridge between Cys-64 and Cys-140 in intact ECF_1 [31].

It has been suggested that the δ subunit is mostly buried in the interface of the F_1 and F_0 parts, both in

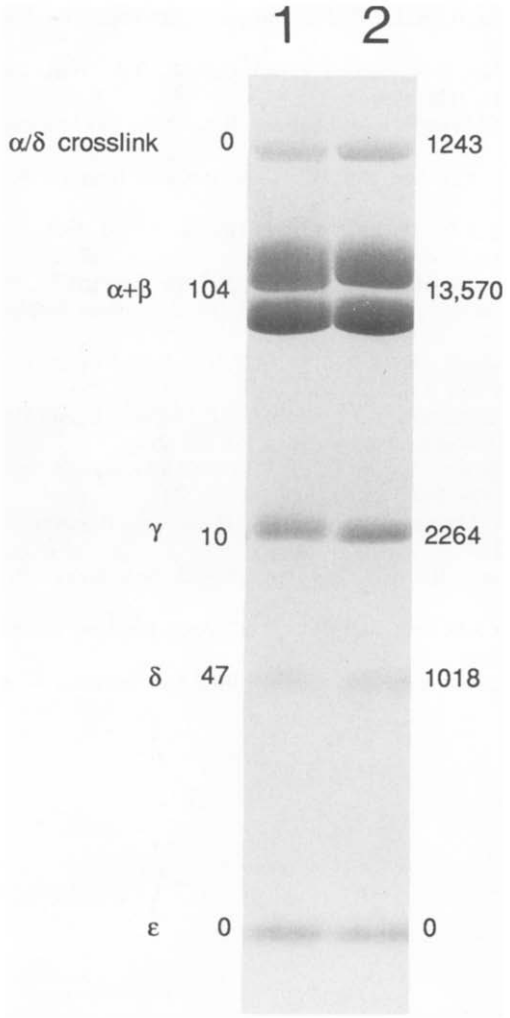

Fig. 8. [^{14}C]NEM labeling of $\alpha-\delta$ disulfide crosslinked ECF_1. Lane 1: enzyme reacted with [^{14}C]NEM while in the native state. Lane 2: ECF_1 reacted with [^{14}C]NEM after first denaturing in SDS but without disulfide breaking reagents present. Values listed are cpm in each band.

the *Escherichia coli* and chloroplast enzyme (reviewed in Ref. 37). Several antibodies that reacted with the δ subunit in ECF_1 or CF_1 have failed to react with the intact F_1F_0 complex (e.g., Refs. 19, 38, 39). Also, sites of proteinase cleavage in the δ subunit are buried [15,39]. However, the δ subunit cannot be shielded fully by other polypeptides in ECF_1F_0 because some antibodies to this subunit can bind to the intact ATP synthase [40]. Also, we find that Cys-140 is reactive to sulfhydryl reagents both in ECF_1 and in ECF_1F_0.

It is interesting that Cys-140 is in the same region of the δ subunit as several lysine residues which react with amino labeling reagents in ECF_1, but are protected from modification in ECF_1F_0 [14]. An α-helical arrangement of this region of the δ subunit places the Cys-on one face and the lysine residues all on a different side. In the model of Cox et al. [36], it is this lysine-rich region of the δ subunit that reacts with the b subunit. Cox et al. [36] suggest that the δ subunit is oriented to form Mg^{2+} ion bridges between a carboxyl-rich face of this subunit (which also contains Cys-140) and a set of carboxyls on the b subunit. An alternative is that the carboxyls on subunit b interact with the lysines on the δ subunit. Such an orientation buries the lysines but exposes the Cys to solvent.

Both Bragg and Hou [31] and Tozer and Dunn [24] have shown that disulfide bond formation between α and δ subunits has no effect on the ATPase activity of isolated ECF_1, but interferes with the interaction of the ECF_1 part with ECF_0. Tozer and Dunn [24] found that crosslinked enzyme no longer binds to ECF_0. Bragg and Hou [31], in contrast, obtain binding of the crosslinked ECF_1 with ECF_0, with retention of DCCD inhibition but no coupling of ATPase activity to proton translocation across the membrane. Our reconstitution experiments also focus attention on the C-terminal part of the δ subunit in coupling the ECF_1 and ECF_0 parts. Trypsin cleaved δ subunit, missing around 20 amino acids from the C-terminus, bound to ECF_0 but had lost DCCD sensitivity. It should be informative to use molecular biological approaches to engineer amino acid replacements in the C-terminus and then examine more precisely the role of this segment of the δ subunit, both in binding ECF_1 to ECF_0 and in the energy coupling mechanism.

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