Structure–Function Relationships of the *Escherichia coli* ATP Synthase Probed by Trypsin Digestion†

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ABSTRACT: Trypsin cleavage has been used to probe structure–function relationships of the *Escherichia coli* ATP synthase (ECF_1F_0). Trypsin cleaved all five subunits, α, β, γ, δ, and ϵ, in isolated ECF_1. Cleavage of the α subunit involved the removal of the N-terminal 15 residues, the β subunit was cleaved near the C-terminus, the γ subunit was cleaved near Ser_{202}, and the δ and ϵ subunits appeared to be cleaved at several sites to yield small peptide fragments. Trypsin cleavage of ECF_1 enhanced the ATPase activity between 6- and 8-fold in different preparations, in a time course that followed the cleavage of the ϵ subunit. This removal of the ϵ subunit increased multisite ATPase activity but not unisite ATPase activity, showing that the inhibitory role of the ϵ subunit is due to an effect on cooperativity. The detergent lauryldimethylamine oxide was found to increase multisite catalysis and also increase unisite catalysis more than 2-fold. Prolonged trypsin cleavage left a highly active ATPase containing only the α and β subunits along with two fragments of the γ subunit. All of the subunits of ECF_1 were cleaved by trypsin in preparations of ECF_1F_0 at the same sites as in isolated ECF_1. Two subunits, the β and ϵ subunits, were cleaved at the same rate in ECF_1F_0 as in ECF_1 alone. The α, γ, and δ subunits were cleaved significantly more slowly in ECF_1F_0. Subunit b of the F_0 part was cleaved only slowly in ECF_1F_0, but the α, γ, δ, and b subunits were all cleaved rapidly when the detergent lauryldimethylamine oxide was added to disrupt the interaction between ECF_1 and the F_0 part. These results are taken to indicate that the α, γ, δ, and b subunits are located at least partly in the interface between the two major domains of the ATP synthase.

The *Escherichia coli* ATP synthase (ECF_1F_0) is a multisubunit enzyme organized into two parts. The ECF_1 part contains five different subunits, α, β, γ, δ, and ϵ, in the molar ratio 3:3:1:1:1; the F_0 part contains three different subunits, a, b, and c, in the molar ratio 1:2:10–12 (Senior & Wise, 1983; Walker et al., 1984). The ECF_1 part is extrinsic to the membrane bilayer and can be released from F_0 by mild treatments as a water-soluble protein of M_r 380000 retaining ATPase activity (Senior & Wise, 1983; Ernster et al., 1986). F_1 isolated from mitochondria also shows ATP synthesis activity under narrowly defined assay conditions (Feldman & Sigman, 1982; Sakamoto & Tonomura, 1983).

The F_0 part is intrinsic to the membrane bilayer, functioning as a proton channel that is gated in situ by the F_1 (Bragg, 1984; Hoppe & Sebald, 1984). The F_1 and F_0 parts are linked by a so-called "stalk", visualized originally in negatively stained electron micrographs (Fernandez-Moran et al., 1964; Soper et al., 1979) but seen recently in ECF_1F_0 preparations examined in a thin layer of amorphous ice (Gogol et al., 1987). This stalk, 50 Å long and 20 Å in diameter (Gogol et al., 1987), probably contains subunits contributed by both the F_1 and F_0 parts.

The ATP synthase complex contains at least two and probably three active sites on different β subunits (Grubmeyer & Penefsky, 1981). The rate of hydrolysis of ATP in any one of these sites alone is very slow (unisite catalysis) but is enhanced dramatically (10^4–10^6) by binding of substrate at a second active site (Cross et al., 1982). This cooperativity is a feature of both ATP hydrolysis and ATP synthesis (Gresser et al., 1979). The α subunits are thought to contain nucleotide binding sites with a regulatory role (Futai & Kanazawa, 1980). ATP bound in these sites is nonexchangeable during multiple turnovers at the catalytic sites.

Some α subunit mutants as well as β subunit mutants have been found to show normal unisite catalysis but reduced cooperative or multisite ATPase activity, indicating that cooperativity between active sites depends on conformational changes involving both subunits (Wise et al., 1984; Duncan & Senior, 1985). Mutants in the γ and ϵ subunits affect assembly of the complex as well as binding of ECF_1 to F_0 (Kanazawa et al., 1983; Klionsky et al., 1984; Miki et al., 1986).

In addition to genetic studies, reconstitution experiments have been used to determine the function of different subunits. It has been found that preparations of F_1 missing either the δ or ϵ subunit bind poorly or not at all to F_1-depleted membranes (Futai et al., 1974; Smith & Sternweis, 1977; Sternweis, 1978). The ϵ subunit has also been found to act as an inhibitor of ATPase activity (Smith & Sternweis, 1977; Laget & Smith, 1979).

A third general approach used to study the role of subunits in F_1 is protease digestion of the complex. It has been shown in several laboratories that protease digestion of F_1 leads to activation of ATPase activity but that the proteolytically modified enzyme no longer binds to F_0 (Nelson et al., 1974; Neiuwenhuis et al., 1974; Bragg & Hou, 1975; Laget, 1978; Dunn et al., 1980). These effects have been related to cleavage of the α, δ, and ϵ subunits. Nelson et al. (1974) have reported that prolonged trypsin cleavage of F_1 generates a complex containing only α and β subunits and that this core of the enzyme retains full activity. Protease digestion studies of the F_0 part have been taken to indicate a role for subunit b in binding ECF_1 (Hermolin et al., 1983; Hoppe et al., 1983; Perlin & Senior, 1985).

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In experiments described here, we have compared the rate of cleavage of individual subunits of ECF_1 in the isolated ECF_1 complex and in membrane-bound ECF_1F_0. These time-course experiments are interpreted in terms of the functioning of subunits. Also, they provide insight into the topology of the ATP synthase complex.

Experimental Procedures

Enzyme Purification. ECF_1 was isolated from *E. coli* strain AN1460 by a modification of the method of Senior et al. (1979) and Wise et al. (1981) described elsewhere.

ECF_1F_0 was isolated from *E. coli* strain AN1460 by the procedure of Foster and Fillingame (1979). ECF_1F_0 was reconstituted in egg phosphatidylcholine vesicles as described in Foster and Fillingame (1979).

Trypsin Digestion Studies. ECF_1 or ECF_1F_0 was incubated at a concentration of 1 mg/mL with trypsin in a ratio of 1:100 (w/w) or 1:10 (w/w where indicated) in 50 mM tris(hydroxymethyl)aminomethane (Tris)-SO_4, pH 8.0, 500 μM $MgSO_4$, and 500 μM KH_2PO_4 at room temperature. Lauryldimethylamine oxide (LDAO) at a concentration of 0.2% was included as indicated. Trypsin cleavage was stopped by addition of pancreatic trypsin inhibitor or soybean trypsin inhibitor in a ratio to trypsin of 4:1 (w/w), and aliquots were withdrawn for measurement of ATPase activity and for gel electrophoresis.

Where indicated, trypsin-treated ECF_1 was concentrated by ultrafiltration in an Amicon PM10 membrane at room temperature. The sample concentrated in 0.4 mL was loaded onto a Sephadex G-100 column (51 cm × 1.2 cm) equilibrated in 50 mM Tris-HCl, pH 7.0, 2 mM ATP, 2 mM $MgCl_2$, 0.1 mM dithiothreitol (DTE), and 10% glycerol. The column was eluted with the same buffer at 4 °C. The cleaved ECF_1 complex eluted from the column in the exclusion volume. Fractions of 1.5 mL were collected and ATPase activity was measured. Fractions with ATPase activity were pooled and concentrated through an Amicon PM10 membrane.

[^{14}C]-N-Ethylmaleimide (NEM) Labeling. ECF_1, treated with trypsin in the presence of 0.2% LDAO for 2 h, was incubated with [^{14}C]NEM as described by Satre et al. (1982). The treatment with trypsin in the presence of LDAO accelerates the digestion of ECF_1 but gives the same cleavage pattern. Samples labeled with [^{14}C]NEM were loaded on 10–15% polyacrylamide gels, and after staining and destaining, the lanes were sliced and radioactivity was measured as described by Aggeler et al. (1987).

Other Methods. Protein concentrations were determined by a modification of the Lowry procedure (Markwell et al., 1978).

Antibodies against the β or ϵ subunits were raised in rabbits. The immunoglobulin G (IgG) fraction was partially purified by ammonium sulfate fractionation and stored at −20 °C.

Unisite hydrolysis of ATP was measured at 25 °C as described by Tommasino and Capaldi (1985) by using ECF_1 (0.5 μM) in 50 mM Tris-SO_4, 1 mM KH_2PO_4, and 0.5 mM $MgSO_4$, pH 8.0, and by adding 0.15 μM [γ-^{32}P]ATP. Multisite ATPase activity was determined by using an ATP regenerating system at 37 °C as described by the same authors.

Polyacrylamide Gel Electrophoresis. Samples for Na$DodSO_4$ polyacrylamide gel electrophoresis were supplemented with 10% Na$DodSO_4$, 8 M urea, 5% mercaptoethanol, and 0.5 M Tris-SO_4, pH 6.1. Two-millimeter-thick slab gels were run as described by Laemmli (1970) with a 5% acrylamide stacking gel and 10–18% separating gel in a linear gradient. When a separation of the α and β subunits was required, 10% acrylamide was used in the separation gel and samples were electrophoresed for a longer time. Staining with Coomassie brilliant blue R and destaining were carried out according to the method of Downer et al. (1976). Staining with silver nitrate was done as described by Wray et al. (1981), with the fixation step modified according to the procedure of Oakley et al. (1980).

Table I: Amino-Terminal Sequences of the α, β, and γ Subunits after Trypsin Cleavage

	16				20													30			
α'	I	A	Q	F	N	V	V	S	E	A	H	N	E	G	T	I	V	S	V	S	
	1				5					10					15						
β'	A	T	G	K	I	V	Q	V	I	G	A	V	V	D	V	E	F	P	Q		
	1				5																
γ'	A	G	A	K	E	I	R	S													
	202								209												
γ''	S	W	D	Y	L	Y	E	P													

Immunoblotting was conducted according to the procedure of Towbin et al. (1979) as follows: Samples of ECF_1 and ECF_1F_0 were applied in duplicate in the same gel; half of the gel was stained as indicated above, and the other half was treated to bind β or ϵ antibodies. Densitometric traces of gels were done by using a scanning densitometer Zeidel Model SL-50h-XL soft laser. The areas of the peaks were integrated by an Apple II computer.

Electroblotting onto Activated Glass. ECF_1 samples for sequencing of α or β subunits were subjected to Na$DodSO_4$ polyacrylamide gel electrophoresis using 10% polyacrylamide gels. One hundred picomoles of total protein was applied, and the gels were run until the smaller subunits had electrophoresed from the bottom of the gel. The γ subunit fragments were sequenced from 10 to 18% gradient polyacrylamide gels with 300 pmol of enzyme applied.

Samples were transferred electrophoretically to activated glass fiber paper as described by Aebersold et al. (1986) with modifications according to the procedure of Yuen et al. (1986).

Trifluoroacetic acid etched glass filter paper (Whatman GF/F) was derivatized by immersion into a silane solution containing 97 mL of acetonitrile, 2 mL of *N*-[(trimethoxysilyl)propyl]-*N*,*N*,*N*-trimethylammonium chloride (Petrarc Systems) and 1 mL of water. The electrophoresis buffer was freshly made 20 mM ethylmorpholine (Pierce Chemical Co.) titrated to pH 8.3 with 20% formic acid. Electroblotting was performed for 1–3 h at 250–350 mA and 15 °C, depending on the efficiency of transfer of the polypeptide(s) of interest.

Amino acid sequencing was performed by using an Applied Biosystems Model 470A protein sequenator.

Results

Trypsin Digestion Cleaves All of the Subunits of the Isolated F_1 ATPase. Incubation of ECF_1 with trypsin at a ratio of 100:1 by weight was found to cleave all five subunits of the enzyme within 5 h at room temperature (Figure 1). The α subunit was cleaved rapidly to a major fragment α' as shown most clearly in the 10% polyacrylamide gels in Figure 1B. This fragment was transferred electrophoretically onto activated glass and its N-terminal sequence determined. It began IAQ as shown in Table I, confirming the finding of Dunn et al. (1980) that trypsin cleaves away the N-terminal 15 residues of the α subunit. The β subunit was cleaved more slowly than the α subunit. The major cleavage product migrated with a larger molecular weight than the unmodified polypeptide, as shown by immunoblotting with a β-subunit-specific antibody (Figure 1C). Protein sequencing of the β subunit fragment gave the same N-terminal sequence as for unmodified subunit, indicating that the protease cleavage has occurred at the

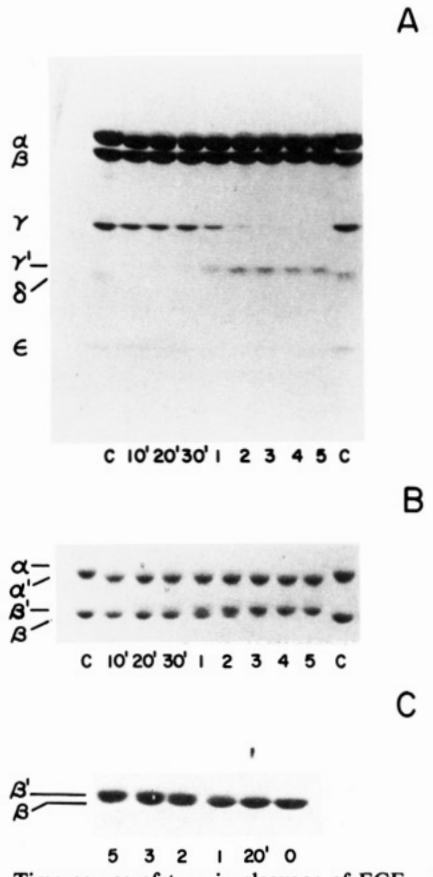

FIGURE 1: Time course of trypsin cleavage of ECF_1. ECF_1 was incubated with trypsin for 0, 10, 20, and 30 min and for 1–5 h before the reaction was stopped by addition of trypsin inhibitor. (A) Samples (15 μg) were run on a 10–18% $NaDodSO_4$ polyacrylamide gel. (B) Same experiment with sample (3 μg) electrophoresed on a 10% gel to resolve α and β subunits. (C) Immunoblot with antibody against the β subunit.

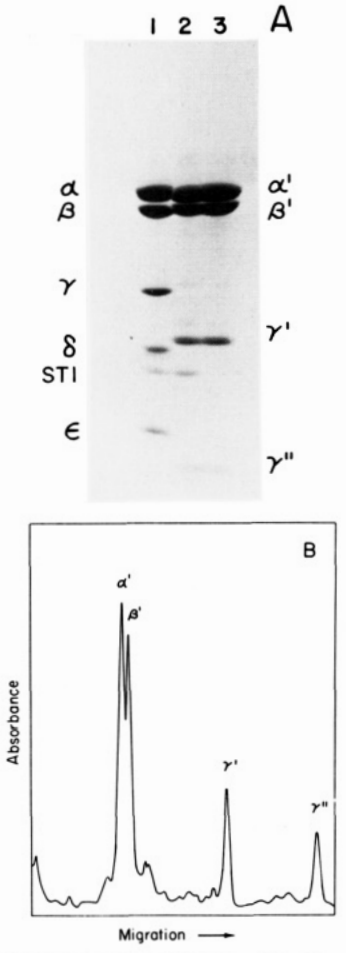

FIGURE 2: Subunit composition of ECF_1 after cleavage with a high concentration of trypsin (10:1 w/w) for 2 h in the presence of 0.2% LDAO. The reaction was stopped by adding trypsin inhibitor. Samples were concentrated and subjected to gel filtration on Sephadex G-100 to separate the core complex from unbound fragments. (A) Fifteen micrograms of protein was electrophoresed on a 10–18% polyacrylamide gel. (Lane 1) ECF_1 control. (Lane 2) ECF_1 after trypsin cleavage for 2 h without separation of the core enzyme from trypsin fragments. (Lane 3) Enzyme complex after gel filtration . (B) Densitometric scan of the sample in lane 3 after it was stained with silver.

C-terminus, probably at residue 456 or 458.

The time course of cleavage of the γ, δ, and ε subunits was monitored on the 10–18% polyacrylamide gradient gel (Figure 1A). In the case of the ε subunit, the cleavage was followed by Coomassie blue staining and by Western blotting with a subunit-specific antibody. The δ subunit was cleaved very rapidly, followed by the ε subunit, with the γ subunit being the most slowly modified by the trypsin treatment. The cleavage of the δ and ε subunits did not produce any major fragments that could be detected in the staining profile or by the ε-subunit-specific antibody, implying that both subunits were digested to small peptides lost from the gel during the staining and destaining procedures. Trypsin cleavage of the γ subunit gave two fragments, γ′ and γ″, of apparent M_r 22000 and 9000, respectively. The γ′ fragment is clearly seen in Figure 1 (part A) but the γ″ band, which stains poorly with Coomassie blue, is not. Prolonged incubation of ECF_1 with trypsin at a ratio of 10:1 (w/w) caused no additional cleavage of subunits above that seen in Figure 1. The modified enzyme, isolated by gel filtration on Sephadex G-100 after this prolonged cleavage time, retained high ATPase activity and contained α′, β′, γ′, and γ″. This is evident from polyacrylamide gels stained with Coomassie brilliant blue (Figure 2A) but is more clearly revealed in gels that were silver stained (Figure 2B), where the γ″ fragments appears in approximately equimolar amounts as γ′. In one set of experiments, trypsin-treated and a control ECF_1 sample were labeled with [^{14}C]-*N*-ethylmaleimide (NEM) after denaturation in $NaDodSO_4$. The molar ratio of sulfydryl reagent incorporated into the subunits of unmodified enzyme (determined after the subunits were separated on $NaDodSO_4$ polyacrylamide gels) was 4:1:2:2 (α/β/γ/δ) (Figure 3), the same ratio reported by Stan Lotter and Bragg (1984). The α′, β′, γ′, γ″ fragment obtained by trypsin cleavage contained [^{14}C]NEM in a ratio of 4:1:2:0 (Figure 3), showing that the cleavage site(s) of trypsin in the γ subunit is (are) C-terminal to Cys_{113}. Protein sequencing of the γ subunit fragments after electrophoretic transfer to activated glass showed that γ′ contains the N-terminus and γ″ begins at residue Ser_{202} (Table I).

Trypsin Cleavage Increases the Latent ATPase Activity of ECF_1. The ECF_1 preparation used in this study was latent, having an ATPase activity of 6–8 μmol of ATP hydrolyzed/min·mg when assayed at high protein concentration (10 μg/mL), compared with values of 20–110 μmol of ATP hydrolyzed min^{-1} mg^{-1} reported in other laboratories [e.g., Futai et al. (1974), Bragg and Hou (1975) and Wise et al. (1984)]. This multisite ATPase activity could be increased 3-fold by dilution. Table II shows the effect of trypsin digestion on both unisite ATPase activity and multisite ATPase activity. Protease digestion increased multisite ATPase activity 6–8-fold with no effect on unisite ATPase activity. The highest multisite ATPase activities were obtained by adding the detergent

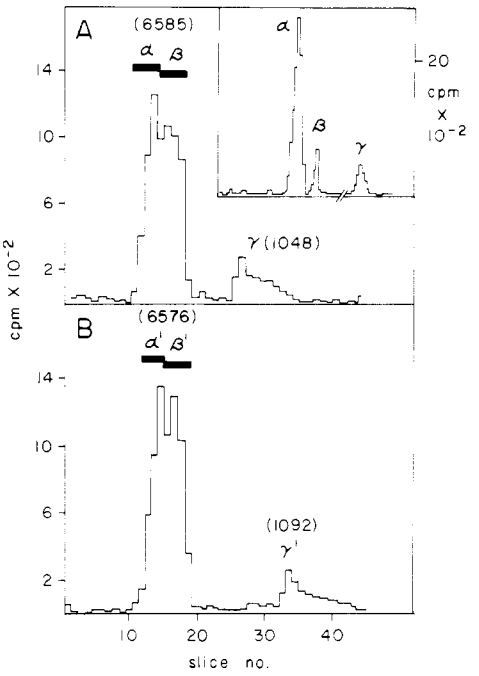

FIGURE 3: Sulfydryl group labeling of trypsin-treated ECF_1 with [^{14}C]NEM. Control enzyme (A) and trypsin-treated ECF_1 (B) were denatured in 1% $NaDodSO_4$ and then reacted with [^{14}C]NEM as described under Experimental Procedures. Samples (25 μg) were run on 10–18% polyacrylamide gels. (Inset) Control ECF_1 labeled with [^{14}C]NEM run on a 10% gel to resolve α and β subunits. Numbers in parentheses are the total number of cpm in each peak.

Table II: Effect of Trypsin Treatment and LDAO Activation on Unisite and Multisite ATPase Activity of ECF_1

preparation	unsite act. [mol of ATP hydrolyzed (mol of enzyme)$^{-1}$ s^{-1}]		activation by LDAO	
	−LDAO	+LDAO	unisite	multisite
ECF_1 control	0.005	0.013	2.5	20
ECF_1 trypsin treated	0.005	0.011	2.4	2.3
uncA 401 ECF_1	0.004	0.008	2.0	2.3

LDAO to ECF_1 preparations, i.e., 150–180 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$. Table II shows that LDAO (0.2% in the assay) increased the single-site turnover site (unisite catalysis) by a factor of 2.5.

The effect of LDAO on the catalytic sites, as monitored by unisite activity, and on cooperativity between active sites, as measured by multisite ATPase, was further examined in experiments with the *E. coli* mutant uncA401 (kindly provided by Dr. A. E. Senior, University of Rochester). Wise et al. (1984) have shown that this mutant, with an altered α subunit, has normal unisite catalytic activity but shows essentially no cooperativity between active sites, so that the multisite ATPase activity is very low. LDAO was found to increase unisite catalysis of this mutant by a factor of 2 and multisite activity by a factor of 2.3, cf. 20-fold in the wild-type enzyme. Thus, the actions of LDAO are multiple and include alterations of the active site as well as modification of the interactions between these sites.

Figure 4 summarizes the time course of cleavage of the subunits of ECF_1 with trypsin at a ratio of 1:100 (w/w), while Figure 5 shows the rate of activation of the ATPase activity of the enzyme. The rate of activation of multisite ATPase activity is much slower than cleavage of the α or δ subunits, much faster than the cleavage of the β subunits, and it most closely follows the rate of cleavage of the ε and γ subunits.

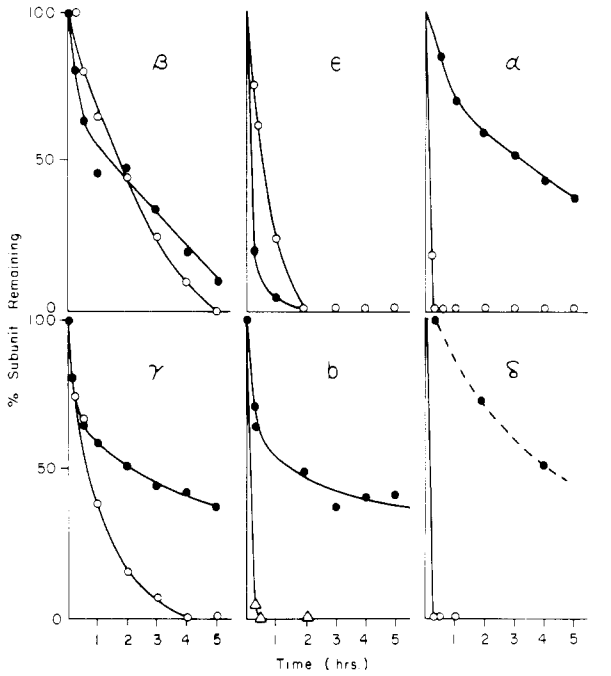

FIGURE 4: Kinetics of trypsin cleavage of subunits in ECF_1 and ECF_1F_0. Enzyme preparations were reacted with trypsin for varying lengths of time. Samples were electrophoresed on polyacrylamide gels as in Figure 1 and the gels densitometered to determine the disappearance of each subunit due to trypsin cleavage as a function of time. (Open circles) Cleavage of subunits in ECF_1. (Solid circles) Cleavage in ECF_1F_0. (Open triangles) Cleavage of the b subunit in the presence of 0.2% LDAO.

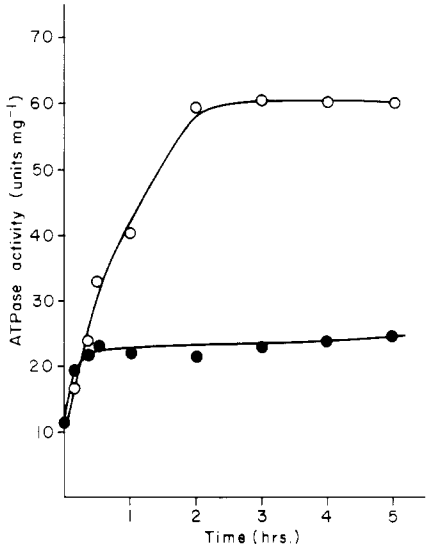

FIGURE 5: Effect of trypsin cleavage of the ATPase activity of ECF_1 and ECF_1F_0. Trypsin cleavage was conducted as described under Experimental Procedures. Samples (2 μg) were taken to measure ATPase activity at the indicated times. (Open circles) ECF_1. (Closed circles) ECF_1F_0.

Trypsin Cleavage of ECF_1F_0. The time course of trypsin cleavage of ECF_1F_0 reconstituted into vesicles of egg phosphatidylcholine was conducted in parallel with the trypsin cleavage of ECF_1, so that the rates of cleavage of each subunit in ECF_1 alone and in ECF_1F_0 could be compared. As an additional control, the cleavage of ECF_1F_0 was done in the presence and absence of LDAO, as this detergent has been shown to disrupt the interaction between ECF_1 and F_0 (Lotscher et al., 1984).

Figure 6 shows these time courses of cleavage with and without LDAO present, as examined on a 10–18% poly-

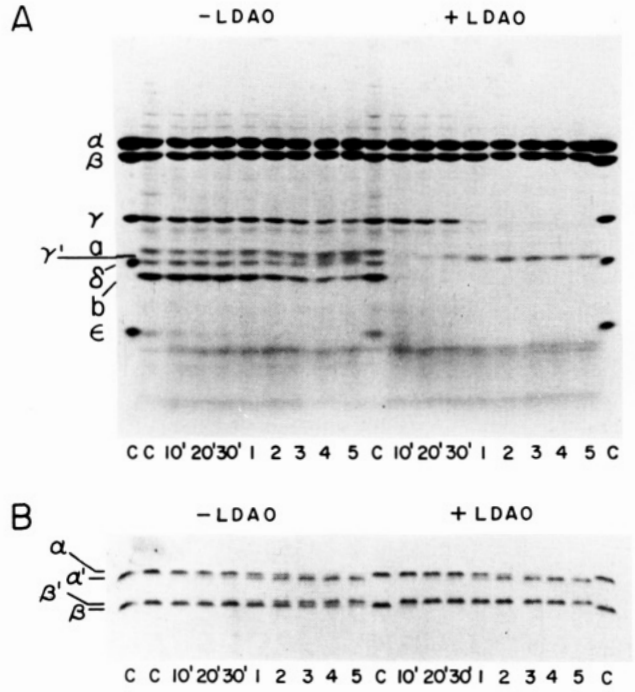

FIGURE 6: Time course of trypsin cleavage of subunits in ECF_1F_0 in the presence and absence of 0.2% LDAO. (A) Twenty micrograms of protein per lane on a 10–18% gradient gel. (B) Five micrograms of protein on a 10% polyacrylamide gel.

acrylamide gradient gel (part A), and on a 10% gel (part B) for clearer analysis of the α and β subunits. All of the subunits of ECF_1 became accessible to trypsin digestion in the intact ATP synthase (ECF_1F_0) in the 5-h time course of the experiment. The cleavage products of the α and β subunits in ECF_1F_0 were sequenced and found to be the same as in ECF_1 alone. The cleavage products of the γ subunit were the same in the two preparations on the basis of size (i.e., by the migration on the gel). Also, the cleavage of the ϵ subunit appeared to follow the same pattern in ECF_1F_0 as in ECF_1 in that the cleavage product failed to react with subunit-specific antibody.

The effect of trypsin cleavage on the ATPase activity of the ECF_1F_0 complex is shown in Figure 5. Enzyme reconstituted into vesicles of egg phosphatidylcholine had a (multisite) ATPase activity of 11–16 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$ and was 90–95% inhibited by dicyclohexylcarbodiimide (DCCD). Trypsin cleavage caused a relatively rapid 2-fold increase in activity that was completed by the time all of the ϵ subunit had been digested from the complex. Thereafter, the ATPase activity remained constant but at a level less than half that expected if trypsin cleavage had released the ECF_1 part from the membrane (compare the activation of ECF_1 and ECF_1F_0 in Figure 5). These activity data imply that protease digestion does not release ECF_1 from the F_0 part, and yet the sensitivity of ATPase activity to DCCD dropped from 90–95% initially to around 50% within 2 h, in a time course that followed the cleavage of the α, γ, δ, and b subunits.

Figure 4 summarizes the rates of cleavage of individual subunits in the intact ECF_1F_0 complex and compares these time courses with those obtained in isolated ECF_1. The rate of cleavage of the b subunit is shown in the presence and absence of LDAO. There was an initial cleavage of 20–25% of each subunit in ECF_1F_0 at rates comparable to that in isolated ECF_1. This may represent a population of partly denatured, inactive enzyme. (The rapidly cleaved material is unlikely to be free ECF_1 because of the high sensitivity of the ATPase to DCCD, as described already.) The cleavage of subunits in the second phase of proteolysis followed two general time courses. Cleavage of the β and ϵ subunits was as fast or slightly faster in ECF_1F_0 than in isolated ECF_1. The cleavage of α, γ, and δ was much slower in ECF_1F_0 than in ECF_1. The rates of cleavage of α, γ, δ, and subunit b of the F_0 part were increased dramatically when LDAO was added to disrupt the connection between the F_1 and F_0 parts (Lotscher et al., 1984). In Figure 4 the kinetics of the δ subunit are only approximate because the disappearance of this subunit was difficult to quantitate. One of the cleavage products of the γ subunit (γ') migrates very close to the δ subunit on gels, and the two peaks were not resolved completely by the laser scanner. Nevertheless, it can be seen clearly in Figure 6 that significant amounts of δ subunit were present after 5 h of digestion, while this subunit is cleaved completely within 5 min in isolated ECF_1.

Discussion

Trypsin cleavage of F_1 has been used before to examine the topology as well as the importance of individual subunits for the functioning of this ATPase [e.g., Nelson et al. (1974), Neiuwenhuis et al. (1974), Bragg and Hou (1975), Laget (1978), and Dunn et al. (1980)]. Our results confirm much of the previous work and extend the protease digestion method to an analysis of the topology of the intact ATP synthase complex. Several new observations were made.

It has been reported that cleavage of ECF_1 with low levels of trypsin enhances the ATPase activity of the enzyme (Bragg & Hou, 1975; Laget, 1978), and this effect has been linked to the removal of the ϵ subunit. A role for the ϵ subunit as an inhibitor has also been demonstrated in reconstitution studies (Smith & Sternweis, 1977; Sternweis, 1978). Our data suggest that the ϵ subunit modulates cooperativity between catalytic sites in ECF_1 rather than acting at these sites directly. Thus, we observed a 3-fold increase in multisite ATPase activity that correlated well with the cleavage of the ϵ subunit, but no change in unisite ATPase activity was observed. Cleavage of ECF_1F_0 by trypsin increased ATP hydrolysis by a factor of 2 in a time course that followed the cleavage of ϵ subunit. However, the activation obtained in ECF_1F_0 was never as high as in isolated ECF_1, suggesting that the cooperativity between active sites in ECF_1F_0 depends on interactions between ECF_1 and F_0 subunits as well as on the binding of the ϵ subunit.

Prolonged digestion of ECF_1 with trypsin has been used by Nelson et al. (1974) to obtain the minimum unit retaining ATPase activity. These workers claimed to obtain an active complex containing only the α and β subunits by this procedure. Smith and Sternweis (1982) repeated these experiments and found that the protease-resistant core of ECF_1 contained α and β subunits along with a small fragment tentatively identified as a part of the γ subunit. We subjected ECF_1 to incubation with trypsin at a 100:1 ratio (w/w). This treatment gave an active complex retaining the α and β subunits along with two fragments of γ, both of which were retained with the core complex through gel filtration on Sephadex G-100. One fragment of γ included the N-terminus and both cysteines and had an apparent M_r on gels of 22 000. The second fragment was C-terminal to residue Ser_{202} with an apparent M_r of 9000. This C-terminal fragment stained poorly with Coomassie blue, explaining why it was not detected in previous studies (Nelson et al., 1974; Smith & Sternweis, 1982). It could be visualized clearly by silver-staining of gels and appeared to be present in the complex after gel filtration in close to equimolar amounts with the N-terminal fragment. Preliminary cross-linking

studies of the α', β', γ', γ'' complex show the γ' fragment linked to β and the γ'' fragment linked to γ' (M. Gavilanes-Ruiz, unpublished results). Both the N- and C-terminal segments of the γ subunit are highly conserved in plants, animals, and bacteria (Walker & Cozens, 1986; Cozens & Walker, 1987), indicating an important role of both parts of the subunit in enzyme functioning [see also the genetic studies of Miki et al. (1986)]. There are major differences in sequence only in the middle part of the γ subunit, and this is around the trypsin cleavage site (Walker & Cozens, 1986).

One additional difference in our trypsin digestion studies from those reported previously is the cleavage of the β subunit. We found that trypsin modified the β subunit to give a product with a slightly slower migration, but this was only seen under highly resolving gel conditions and hence missed in earlier studies (Nelson et al., 1974; Dunn et al., 1980; Smith & Sternweis, 1982). This cleavage must be from the C-terminus, and the likely sites are Lys_{456} and Lys_{458}.

A comparison of trypsin cleavage of ECF_1 and ECF_1F_0 shows that the cleavage sites of α, β, and γ (and probably δ and ϵ) are the same in the two preparations. No additional cleavage sites were available in the membrane-bound ECF_1 to indicate major conformational changes in the F_0-associated versus free ECF_1 part.

Analysis of the rates of cleavage of ECF_1 subunits in ECF_1 alone and in intact ECF_1F_0 separates subunits into two classes. The β and ϵ subunits were equally accessible to trypsin in the two preparations, while the α, γ, δ, and b subunits were shielded from cleavage by the protease. The shielding of the δ and b subunits is consistent with other experiments, including genetic studies, that place these polypeptides at the interface between ECF_1 and F_0 [e.g., Futai et al. (1974), Sternweis and Smith (1977), Schneider and Altendorf (1984) and Hermolin et al. (1983)]. A role for the N-terminus of the α subunit in anchoring ECF_1 to F_0 has also been suggested before (Bragg & Hou, 1975; Dunn et al., 1980), and we have found that this part of the α subunit is labeled by amphipathic probes designed to label subunits of the ATP synthase in the lipid bilayer head-group region (Aggeler et al., 1987).

The shielding of α, γ, δ, and b subunits, while pronounced, was not complete. There was slow cleavage of all of these subunits at a similar rate during the 5-h time course of proteolysis (after an initial rapid cleavage of a fraction of possibly denatured protein). The slow cleavage of these subunits could result from disruption of the interaction between ECF_1 and F_0 without release of ECF_1 and/or to denaturation of the ECF_1F_0 complex during the experiment. Alternatively, there could be accessibility of trypsin into the interface between ECF_1 and F_0, and this might be expected if the two domains are separated by the narrow stalk 50 Å long, seen recently in electron micrographs of the ECF_1F_0 complex embedded in a thin layer of amorphous ice (Gogol et al., 1987). Our data do not allow us to distinguish between these possibilities unambiguously. However, it has been reported recently that antibodies to the δ subunit are able to bind to ECF_1F_0 (Dunn & Tozer, 1987). Given the strong evidence that the δ subunit links ECF_1 to F_0, the antibody results argue for the stalk structure, and the shielding of α, γ, δ, and b subunits from trypsin would then be due to subunit–subunit interactions in the stalk or to conformational changes such as altered breathing motions of polypeptides involved in stalk formation.

The observation that the ϵ subunit is cleaved as rapidly in ECF_1F_0 as in ECF_1 alone is surprising in that this subunit has been implicated in binding the two domains together (Sternweis, 1978; Klionsky et al., 1984). The ϵ subunit could be in the interface between ECF_1 and F_0 but exposed, as evidenced not only by our protease digestion data but by binding of ϵ-subunit-specific monoclonal antibodies to ECF_1F_0 (Dunn & Tozer, 1987).

One other interesting result of the studies described here relates to the effect of the amphipathic detergent LDAO. We have previously shown that LDAO activates ECF_1F_0 significantly and have ascribed this effect to the release of the ϵ subunit. The results in Table I show that LDAO has multiple effects, increasing unisite catalysis as well as multisite ATPase activity. Whether these effects are due to single or multiple interactions on the ECF_1 molecule is not clear at present.

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Interactions among Red Cell Membrane Proteins

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ABSTRACT: Interactions between human red cell band 2.1 with spectrin and depleted inside-out vesicles were studied by fluorescence resonance energy transfer and batch microcalorimetry. The band 2.1–spectrin binding isotherm is consistent with a one to one mole ratio. The association constant of 1.4×10^8 M^{-1} corresponds to the association free energy of −11.1 kcal/mol. Under our experimental conditions, the enthalpy of interaction of band 2.1–spectrin was found to be −10.8 kcal/mol and is independent of the protein mole ratio. The calculated entropic factor ($-T\Delta S$ = 0.3 kcal/mol) strongly suggests a predominantly enthalpic character of the reaction. In addition, we investigated the role of band 2.1 on the binding of band 4.1 to spectrin [Podgorski, A., & Elbaum, D. (1985) *Biochemistry 24*, 7871–7876] and concluded that only small, if any, alterations of binding of band 4.1 to spectrin have taken place in the presence or absence of band 2.1. This suggests thermodynamic independence of the binding sites. Although the attachment of the cytoskeletal network to the membrane takes place through, at least, two different interactions, band 2.1–band 3 and 4.1–glycophorin, the relative enthalpy values suggest that band 2.1 contributes significantly more than band 4.1 to the energy of the interaction. In addition, we observed that polymerization of actin is modulated by the cytoskeletons as judged by their effect on the rate of actin polymerization.

The mechanical properties of mammalian erythrocytes are defined by the organized system of proteins located on the cytosolic side of the membrane. Despite the fact that the major cytoskeletal proteins (spectrin, band 4.1, band 2.1, and actin) have been isolated, purified, and, to some extent, characterized, the thermodynamics and the kinetics of the network formation are still an open and unexplored area. Spectrin molecules are the major structural component (approximately 70% by weight) able to bind a single molecule of band 4.1. Two heterodimers of spectrin associated head to head have been postulated to be the predominant form of the protein in the red cell (Ungewickell & Gratzer, 1978). The location of the band 4.1 binding site near the ends of the proteins (Tyler et al., 1979) as judged by electron microscopy renders the tet-

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