The γ subunit in the *Escherichia coli* ATP synthase complex (ECF_1F_0) extends through the stalk and contacts the c subunits of the F_0 part

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Abstract A mutant, in which a cysteine has been site-directed into the polar loop region of the c subunit at residue 44, has been studied. Cross-linking of the c subunit to both the γ and ϵ subunits was observed with cupric 1,10-phenanthrolinate treatment. The linkage between the c and γ subunits was localized to that part of the γ subunit between residues 202–286, based on peptide analysis. Reference to the high resolution structure of F_1 [Abrahams et al. (1994) Nature 370, 621–628] appears to limit this contact site to the region including residues 202–230. This segment contains 4 tyrosines and 1 tryptophan as possible reactive residues for cross-linking with the c subunit cysteine.

Key words: F_1F_0 ATPase; c Subunit mutant; Cross linking; γ and ε Subunits

1. Introduction

The H^+ ATPase of *Escherichia coli* (ECF_1F_0) catalyzes both ATP synthesis coupled to a proton electron chemical gradient and ATP hydrolysis-driven proton translocation. The enzyme is made up of two parts, an extrinsic F_1 part, that carries out the catalytic functions, and a membrane intercalated F_0 part that contains the proton channel. The F_1 part is composed of five subunits, α, β, γ, δ and ε in the stoichiometry 3:3:1:1:1. The F_0 part contains 3 different subunits, a, b and c in the stoichiometry 1:2:10–12 [1–4].

Low resolution structural studies of the F_1 part have shown the three α and β subunits alternating in a hexagon around a cavity in which is located a part of the γ subunit [5,6]. The γ subunit extends from one end of F_1 (the end closest to the F_0 part) [7] where it interacts with the ε subunit [8]. Cryoelectron microscopy studies show the F_1 part separated from F_0 by a fairly narrow stalk of 40–45 Å in length [9]. In addition to the γ and ε subunits, this stalk region is thought to contain the δ subunit of the F_1 part and segments of the b subunits of the F_0 part [1,2].

The recent high resolution structure of bovine heart F_1[10] has provided details of the interaction between, and different conformations of, the α and β subunits. A part of the γ subunit is also revealed by the X-ray structure determination. The N-terminal 45 amino acids and the C terminal 63 residues of the γ subunit are arranged as α helices running from within the central cavity of the F_1 into the stalk region. A short α helical region involving residues 82–99 (*E. coli* numbering system) is bound to a β subunit at the bottom of the structure (nearest to the F_0 part). Unfortunately, almost half of the γ subunit, and the δ subunit which is the homologue of the ε subunit of ECF_1, are disordered in the crystal form studied by Abrahams et al. [10], and so no structural data for these key parts of the F_1 were obtained.

Our present picture of the F_0 is much less detailed than that of the F_1 part. The best studied subunit is the c subunit, which is arranged as a helical hairpin, oriented with the loop that links the two α helices facing the F_1 part [4,11]. Models of the F_0 have the c subunits organized in a ring with the a and b subunits inside, but it has also been argued that they are more peripherally placed [4,12].

One approach to determining interactions between subunits in multisubunit complexes is to introduce cysteine (Cys) residues, either singly as sites for reaction with chemical cross-linking reagents, or doubly in order to create disulfide bonds. Such experiments with ECF_1F_0 have shown that the ε subunit has contacts with the α, β and γ subunits of the F_1 part [13,14] and is linked to the c subunits of the F_0 part [15]. Here, we examine mutants in which a Cys residue has been introduced into the loop region of subunit c. We had intended to react these Cys residues with tetrafluorophenylazide maleimides [14] and look for cross links between the c subunits and the various subunits of F_1. However, in preliminary experiments, it was found that with the mutant, cD44C, covalent cross links were formed in significant yield, in the absence of added chemical cross-linker. Here, we describe this phenomenon and show that the cross links formed are between c and γ, and c and ε subunits, respectively, establishing that the γ subunit, as well as the ε subunit, spans the full length of the stalk.

2. Experimental

The two strains used here, referred to as cA39C (YZ436) and cD44C (YZ437), carry **uncE** mutations which result in Cys substitutions at either position 39 or 44 of subunit c, respectively. Strains YZ436 and YZ437 carry their respective mutations on both the chromosome and on pBR322 derived plasmids, pYZ203 (cA39C) or pYZ205 (cD44C), which encode the entire **unc** operon [15]. The mutant chromosomal background strains were generated by incorporation of mutated **uncE** gene into chromosome, and then co-transduction with ILV^+ into strain MJM63 (AN346, ΔuncE334, ilv:Tn10) as described by Miller et al. [16]

ECF_1F_0 was overexpressed by 2- to 3-fold in the strains described above and purified according to the method of Foster et al. [17]. Purified ECF_1F_0 was reconstituted into lipid vesicles according to the method of Aggeler et al. [13]. A 1 ml aliquot was passed through Sephadex G-50 column (medium 1 × 18 cm). Turbid fractions from the column were pooled and the membranes pelleted at 65,000 rpm at 4°C for 30 min in a Beckman TLA100.2 rotor. Membranes were resuspended and washed twice in 50 mM Tris pH 7.8, 10% glycerol, 2 mM $MgCl_2$, and finally resuspended at 1 mg/ml in the wash buffer and stored in liquid nitrogen.

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2.1. Cupric 1,10-phenanthrolinate treatment

Oxidation of reconstituted F_1F_0 by cupric 1,10-phenanthrolinate was adapted from Bragg and Hou [18] and Dunn and Tozer [19]. F_1F_0 at 0.7 mg/ml in 50 mM Tris pH 7.8, 10% glycerol, 2 mM $MgCl_2$ was supplemented with 2 mM ATP and incubated for 30 min at room temperature. One aliquot was removed as a control; to a second was added cupric 1,10-phenanthrolinate to 30μM from a stock solution of 3.1 mM $CuSO_4$/6.3 mM 1,10-phenanthroline. This sample was incubated for 1 h at room temperature before being divided in two, one aliquot then being retained while the second was incubated for 30 min at room temperature with 10 mM dithiothreitol (DTT). ATPase activity was then measured for the untreated control, Cu^{2+} treated and Cu^{2+}/DTT treated samples.

2.2. Other methods

The anti-c polyclonal antibody has been described before [20]. The anti-γ antibodies are described in Aggeler et al. [21]. ATPase activity was measured using the ATP regenerating system [21]. Samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared according to Aggeler et al. [21] with a final concentration of 3% SDS in the loading buffer. SDS-PAGE was performed according to Laemmli [22] using 10–22% gradient gels. Polypeptides were blotted onto poly (vinylidene difluoride) (PVDF) (Immobilon; Millipore Corp.) for sequence analysis according to the method of Matsudaira [23]. Electroblotting for Western analysis was in 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) transfer buffer [24] in a Bio-Rad Trans-Blot cell for 2 h at 100 mA. Protein bands were visualized on gels by staining with Coomassie-brilliant blue R according to Downer et al. [25]. The gel 'image' was digitized using a Relisys 4816 scanner and relative band density determined using the program NIH Image 1.53 and gel plotting macro. Protein concentrations were measured using the BCA protein assay from Pierce Chemical Co.

2.3. Proteolytic cleavage of the γ-c cross-linked product

Proteolysis of reconstituted F_1F_0 was performed by a modification of the methods of Gavilanez-Ruiz et al. [26] and Tang et al. [27]. ECF_1F_0 at 1 mg/ml was treated with 1 U/ml endoproteinase lys C in 50 mM Tris pH 7.8, 10% glycerol, 2 mM ATP, 2 mM $MgCl_2$ for 6 h at room temperature. The reaction was terminated by reducing the pH to 5.5 and by the addition of TLCK (700 μM).

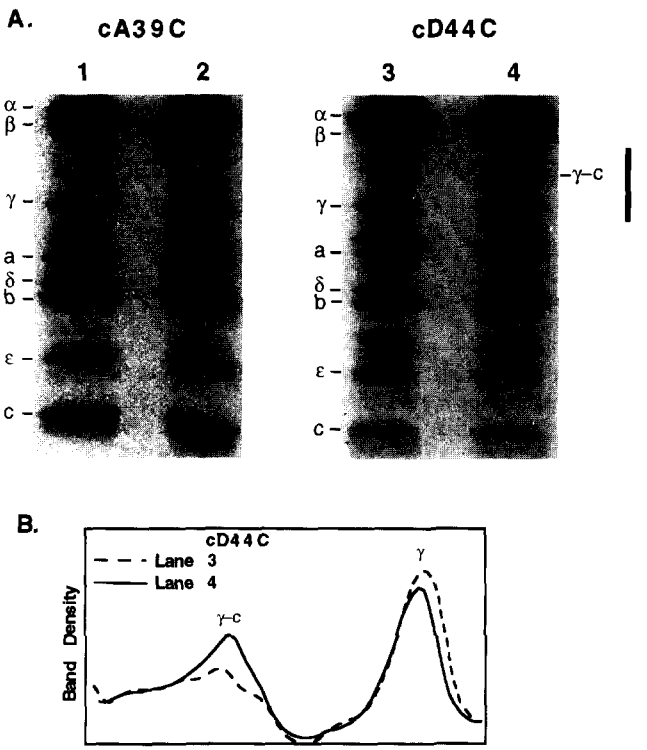

Fig. 1. SDS-PAGE of untreated and cupric 1,10-phenanthrolinate treated ECF_1F_0 from strains cA39C and cD44C. (A) 15 μg of protein are loaded in each lane. Lanes 1 and 3 are untreated controls, and lanes 2 and 4 are cupric 1,10-phenanthrolinate oxidized enzyme. The Cu^{2+} induced band is labeled as γ–c. (B) The Coomassie-blue stained gel above was digitized and relative band density determined for lanes 3 and 4 in the region indicated by the bar to the right of lane 4.

3. Results and discussion

3.1. Activity of ECF_1F_0 from c subunit mutants in the presence and absence of reducing agents

Two mutants, cA39C and cD44C, were used in this study. ECF_1F_0 was isolated from both mutants and stored in buffer containing 1 mM DTT. The activity of enzyme isolated from mutant cA39C was 19–23 μmol ATP hydrolyzed per min per mg; that from cD44C, 18–25 μmol ATP hydrolyzed per min per mg. On removing DTT during dialysis to reconstitute the cD44C enzyme into membranes, some loss of activity was observed, which preliminary SDS-PAGE of samples in the absence of reducing agents, indicated could be associated with cross-linking of c subunits to one another, and to other subunits of the complex. ECF_1F_0 from the mutant cA39C did not show the same phenomenon, indicating that the effect was due to the presence of a Cys at position 44 but not at 39 in the loop region of the c subunits.

Further studies showed that the loss of activity (and concomitant internal cross-linking) of ECF_1F_0 from the mutant cD44C could be speeded up and increased in yield by addition of cupric 1,10-phenanthrolinate to preparations after removal of the DTT. In one typical experiment, the activity of the mutant fell from 17.7 μmol ATP hydrolyzed per min per mg to 10.1 μmol ATP hydrolyzed per min per mg after incubation for 1 h with Cu^{2+}. Subsequent addition of DTT to disrupt disulfide bonds formed between c subunits (the only other exposed Cys in the complex is in the δ subunit, and this has no near neighbor Cys in ECF_1F_0) increased activity to 13.8 μmol ATP hydrolyzed per min per mg enzyme. The percentage loss of activity due to covalent linkage of c subunits to other subunits of the complex ranged from 18–30% in different experiments. Cu^{2+} treatment and subsequent reduction with DTT had no effect on the activity of ECF_1F_0 from the mutant cA39C.

3.2. Cross-linking of subunits induced by Cu^{2+} treatment

Fig. 1 shows SDS-PAGE analysis of ECF_1F_0 prepared from the two mutants. The purity of our ECF_1F_0 preparations is variable. It can be seen that the enzyme prepared from mutant cA39C is relatively clean, while the preparation of cD44C used for the studies in Fig. 1 contains impurities migrating above the α band, just above the band of subunit a and between subunits b and ε. The samples of ECF_1F_0 from cD44C also showed a band of M_r 38,000 migrating between the β and γ subunits that was not present in enzyme from the mutant cA39C. Western blotting, using a panel of monoclonal antibodies (against the α, β, γ, δ and ε subunits of F_1 and the b subunit of F_0), as well as polyclonal antibodies (to subunits a and c of F_0) allowed identification of the 38,000 Da band as a cross-linked product of subunits γ and c. (Fig. 2). This product was found in small amount in enzyme without Cu^{2+} treatment, but was in much higher yield in samples treated with Cu^{2+}. A second cross-linked product could be identified by Western blotting that migrated

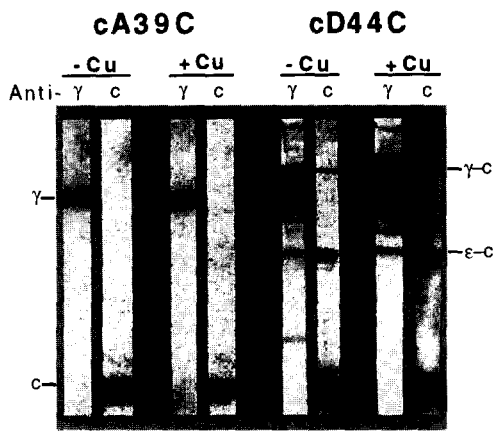

Fig. 2. Western analysis of untreated and cupric 1,10-phenanthrolinate treated reconstituted ECF_1F_0 from strains cA39C and cD44C. 1 μg total protein of ECF_1F_0 from samples identical to those of Fig. 1, were separated on 10–20% SDS-PAGE and transferred to PVDF in CAPS buffer and each lane cut into two strips. Proteins were detected by incubating strips for each lane with either anti-γ mAbs or anti-c pAbs. Blots using an anti ε mAb (not shown) identified ε subunit in the gels.

close to the δ subunit band. This product contained the ε subunit along with c.

3.3. Further analysis of the γ–c subunit product

Samples of Cu^{2+}-treated cD44C were subjected to SDS-PAGE and then electro-transferred to PVDF membrane for N-terminal amino acid sequencing. A sequence of the N terminal part of the γ subunit, A-G-A-K-E, was obtained. The N terminus of the c subunit is blocked [28]. To further characterize the site of the cross-linking of γ to the Cys44 in subunit c, ECF_1F_0, which had been treated with Cu^{2+}, was subjected to protease digestion.

It has been shown that cleavage of the intact ECF_1 with endoproteinase lys C under mild conditions cleaves the γ subunit at residue 201. The C-terminal fragment obtained (residues 202–286), can be followed in polyacrylamide gels with the monoclonal antibody γ_{III} [21,27]. As shown by the Western blotting data in Fig. 3, prolonged incubation of ECF_1F_0 from either mutant cA39C or cD44C with endoproteinase lys C, as described in section 2, led to partial cleavage of the γ subunit in the complex. For cA39C, used as a control, the C terminal part of the γ subunit generated migrated just above the band of (monomer) c subunits.

In the mutant cD44C, there was proteolysis of both cross-linked and uncross-linked γ subunit. The C terminal cleavage product migrated in two positions, one at the same position as the control, the second as a 16,000 Da product which, in addition to the C terminus of the γ, also contains c subunit based on the Western blotting data (Fig. 3).

3.4. Chemistry of the covalent linkage between the γ and c subunits

The antibody blotting and protease cleavage data above show the formation of a zero length cross link between the γ subunit and c subunit dependent on the presence of the Cys at position 44 in the c subunit. The possibility that this product and the ε–c cross-linked product are generated by disulfide bond formation can be ruled out. Neither is disrupted by reducing agents. Moreover, the region of the γ subunit involved, i.e. the C-terminal 82 residues, does not include a Cys [29] while the ε subunit is totally devoid of Cys residues [29].

A search of the literature shows that cross-linking between a Cys and other amino acids is possible, and occurs in several well-studied proteins where it can have functional significance. For example, in galactose oxidase, there is a covalent linkage between Cys228 near the active site of this enzyme and tyrosine 272 [30,31], while in the enzyme tyrosinase, there is cross-linking of a Cys and a histidine [32]. Such covalent cross links between Cys residues and Tyr, His or Trp residues may be common in proteins, but have not been looked for systematically.

We can narrow down the interaction site of the γ subunit with the polar loop of subunit c by reference to the high resolution structural data for beef heart F_1 [10]. Residues from around 240 to the C terminus 286 (*E. coli* numbering) form an α-helix within the α-β barrel and are, therefore, unlikely to contact the c subunits. The likely binding site of γ for subunit c, then, is the region between 202 and 230. As shown by Fig. 4, this segment contains 5 candidate residues for interaction with Cys, i.e. 4 Tyr and 1 Trp. Attempts to fragment the 16,000 Da γ subunit (C terminus)–c subunit product have proved unsuccessful. Therefore, to define the site of interaction further, we are now conducting mutagenesis experiments in which the Tyr and Trp residues highlighted in Fig. 4 are being converted to Cys. This should allow disulfide bond formation between the most closely placed candidate residue(s) and the Cys in subunit c, not only providing the structure identification, but allowing functional analysis of the role of contacts between the γ and c subunits in ECF_1F_0 function. Such an approach has led to identification of a contact site between the polar loop of subunit c and residue 31 of subunit ε [15].

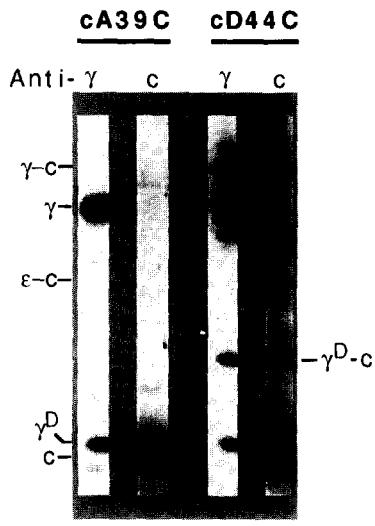

Fig. 3. Western analysis of proteolytic fragments from endoproteinase lys C digests of cupric 1,10-phenanthrolinate treated cA39C and cD44C. Cupric 1,10-phenanthrolinate samples of cA39C and cD44C as in Fig. 1 lanes 2 and 4, were subjected to proteolysis at 1 mg/ml ECF_1F_0, 2 mM ATP and 1U/ml endoproteinase lys C in 50 mM Tris pH 7.8, 2 mM $MgCl_2$, 10% glycerol and incubated at room temperature for 6 h. Protein was separated on 10–22% gels under reducing conditions at 1 μg protein/lane, and then blotted to PVDF in CAPS buffer as described in the Experimental section. The C terminal fragment of the γ subunit was detected with anti-γ_{III} mAb.

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SWDYLYEPD PKALLDTLLR RYVESQVYQ
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202       211        221
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Fig. 4. Sequence of the γ subunit between residues 202 to 229 [29]. Aromatic residues that are candidates for cross-linking to Cys44 of the c subunit are highlighted.

In summary, the study described here provides the first evidence of direct contact between the γ subunit and the c subunits of the F_0 part of the ECF_1F_0 complex. Confirmation of the contact of the ε subunit with c subunits was also obtained[15]. Therefore, both the γ and ε subunits extend for the full length of the stalk, with the γ subunit having close contact with the catalytic sites at one end and with the proton channel at its other end.

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