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STRUCTURAL ASYMMETRY OF THE F_1 OF *ESCHERICHIA COLI* AS INDICATED BY REACTION WITH DICYCLOHEXYLCARBODIIMIDE

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SUMMARY: Dicyclohexylcarbodiimide (DCCD) inhibits the ATPase activity of F_1 from Escherichia coli by covalent modification of a single glutamic acid in the β subunit. 95% inhibition was obtained after incorporation of around 1 mole of DCCD per mole F_1, i.e. 1 mole of reagent per 3 β subunits; and up to 2 moles of DCCD per mole F_1 were readily incorporated into the protein. One of the 3 β subunits per F_1 can be crosslinked to the ε subunit by 1-ethyl-3-[3(dimethylamino)propyl]carbodiimide (EDC). This β subunit (β^1) is here shown to be shielded from reaction with DCCD, presumably by its association with ε and also possibly the γ subunit. Thus the three β subunits are not equivalent in the enzyme complex.

INTRODUCTION: The ATP synthetase of plants, animals and bacteria is a multi-subunit enzyme composed of a part extrinsic to the membrane bilayer (F_1) and an intrinsic transmembrane part (F_0) (1-3 for reviews). It is now generally agreed that F_1 from all sources contains five subunits α, β, γ, δ, and ε in the stoichiometry of 3:3:1:1:1 (1-3). The α and β subunits appear to be in a hexagonal structure of alternating α and β subunits (4). The γ, δ and ε subunits are thought to link the F_1 to the F_0 portion (5,6). Crosslinking experiments (7,8) and reconstitution studies with F_1 from E. coli (ECF_1) (9) place the δ subunit in contact with α and the ε subunit in contact with both the γ and a β subunit. Given the above stoichiometry, the linkage of γ, δ and ε should distinguish structurally one α and one β subunit in the monomer, a structural asymmetry which could be fixed or transient with α plus β subunit pairs alternating in their interaction with the smaller subunits.

The experiments reported here are the first to demonstrate the structural asymmetry of F_1 directly. Dicyclohexylcarbodiimide (DCCD) when reacted with ECF_1 under conditions which inhibit ATPase activity almost completely, is shown to distribute asymmetrically into the β subunits with no labeling of

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that β subunit which can be crosslinked to ε. The implications of this result for functional asymmetry of the F_1 complex are discussed.

MATERIALS AND METHODS: Materials: [^{14}C]DCCD was obtained from Research Products International (45 mCi/mmol). EDC was purchased from Sigma.

Source of Cells and Preparation of ECF_1: E. coli strain KY7485 containing a λ-transducing phage which carries the genes of the unc operon was grown on universal medium with glucose as energy source. The λ-phage production was induced by raising the temperature to 42°C for 30 min (10). ECF_1 was prepared using the procedure of Foster and Fillingame (11). Protein was determined according to Lowry et al. (12).

Reaction of ECF_1 with [^{14}C]DCCD followed by EDC: ECF_1 (1.5 mg/ml) was incubated at room temperature with 135 μM [^{14}C]DCCD (22 x 10^3 cpm/nmol) in a buffer containing 50 mM 3-(N-morpholino) propane sulfonic acid (Mops) pH 7.0 and 50 mM KCl. At the appropriate times, 90 μl aliquots were withdrawn and unreacted [^{14}C]DCCD was removed on 1 ml Sephadex G-50 columns equilibrated in 50 mM Mops pH 7.2, 50 mM KCl, 1 mM $MgCl_2$, 10% glycerol using the column-centrifugation-elution technique as described by Penefsky (13). The eluted ECF_1 was then incubated with 6.5 mM EDC for 60 min. Control samples were treated in the same way but the addition of EDC was omitted.

$NaDodSO_4$ Gel Electrophoresis: Samples were dissolved in 6% $NaDodSO_4$, 10% mercaptoethanol, 20% glycerol and 0.125 M tris(hydroxymethyl)aminomethane pH 6.2. One mm thick slab gels were run as described by Lemmli (14) with a 5% polyacrylamide stacking gel and a 12 to 18% polyacrylamide separating gel (linear gradient), both containing 0.2% $NaDodSO_4$. Staining with Coomassie brilliant blue R and destaining were carried out according to Downer et al. (15). The gels were scanned in a Gilford spectrophotometer at 560 nm and then fractionated into 1 mm slices on a Mickel gel slicer. The slices were dissolved in 0.5 ml of 15% H_2O_2 at 60°C for 10 hr, mixed with 3 ml of Ommifluor (New England Nuclear; 2.66 g/liter in 2:1 toluene: Triton X-100), and counted in a Beckman LS 7000 scintillation counter.

ATPase Activity Assay: The ATPase activity of ECF_1 was assayed in 1 ml of a solution containing 25 mM tris(hydroxymethyl)aminomethane-HCl pH 7.5, 25 mM $MgCl_2$, 5 mM KCN, 2 mM phosphoenol pyruvate, 2 mM ATP, 0.5 mM NADH, 30 units L-lactic dehydrogenase and 30 units pyruvate kinase at 37°C. The absorbance change at 340 nm was followed in a Beckman DU7 spectrophotometer. The specific activity is expressed as units/mg. 1 unit corresponds to 1 μmole ATP hydrolyzed/min.

RESULTS: DCCD has been found to be a potent inhibitor of the ATPase activity of F_1 (16-19), probably though reaction with a single carboxyl group on the β subunit of the enzyme which is in, or close to, the active site for the hydrolase reaction (18-20). Figure 1 shows the relationship between the ATPase activity of ECF_1 and the amount of [^{14}C]DCCD bound to the protein, as measured after gel filtration through Sephadex using the mini-column procedure of Penefsky (13). Maximal inhibition of activity required around 1 mole of reagent per mole of ECF_1, although more molecules of the reagent could be incorporated under certain conditions (see later).

Not all of the [^{14}C]DCCD bound to the ECF_1 complex through the gel filtration step was retained by the enzyme after denaturation of the protein

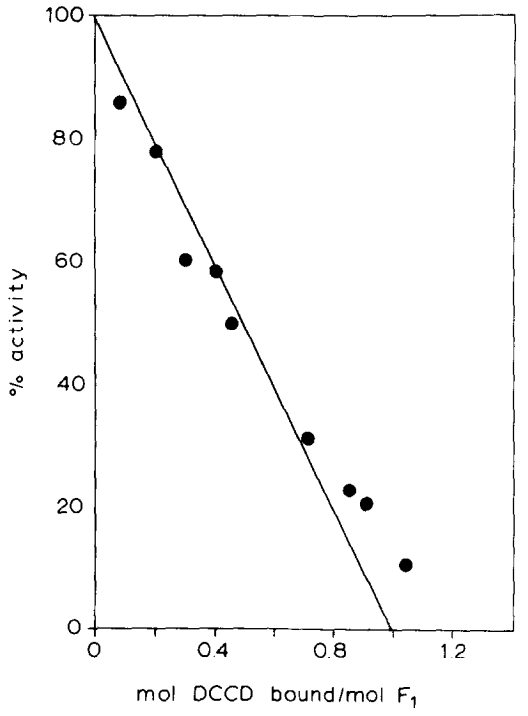

FIGURE 1: Inhibition of ECF_1 as a function of DCCD incorporated. ECF_1 (0.7 mg/ml) in 50 mM Mops, pH 7.0, 50 mM KCl was incubated in the presence of 10 µM [^{14}C]DCCD (7.4 x 10^4 cpm/nmol) at room temp. At appropriate time intervals, 50 µl aliquots were withdrawn and unbound [^{14}C]DCCD was removed by the column-centrifugation-elution technique (Penefsky, 1977). Total incubation time was 3 h.ATPase activity was measured as described under Experimental Procedure. The amount of [^{14}C]DCCD incorporated was calculated assuming a molecular weight of 380,000 for the ECF_1 complex. The straight line plots full inhibition by 1 mole DCCD/mole F_1.

and subsequent $NaDodSO_4$ polyacrylamide gel electrophoresis. For example, incubation of ECF_1 with 10 µM [^{14}C]DCCD for 60 min, resulted in 50% inhibition of ATPase activity, with 0.47 mole DCCD/mole F_1 bound to the enzyme after the gel filtration step. The amount of [^{14}C]DCCD incorporated into the β subunits, determined from $NaDodSO_4$ polyacrylamide gels after labeling under these conditions, was 0.35 mole/mole F_1, with less than 0.02 mole/mole F_1 in α and γ subunits together. Yoshida et al. (19) have shown that the major and probably only site of modification of DCCD in ECF_1 is Glu 193 of the β subunit. These authors also report a loss of bound reagent upon denaturation of the protein.

We have previously established that the water-soluble carbodimide-EDC reacts with ECF_1 to crosslink the ε subunit to one of the β subunits in relatively high yield (8) (referred to as β' in the following text). The β'-ε

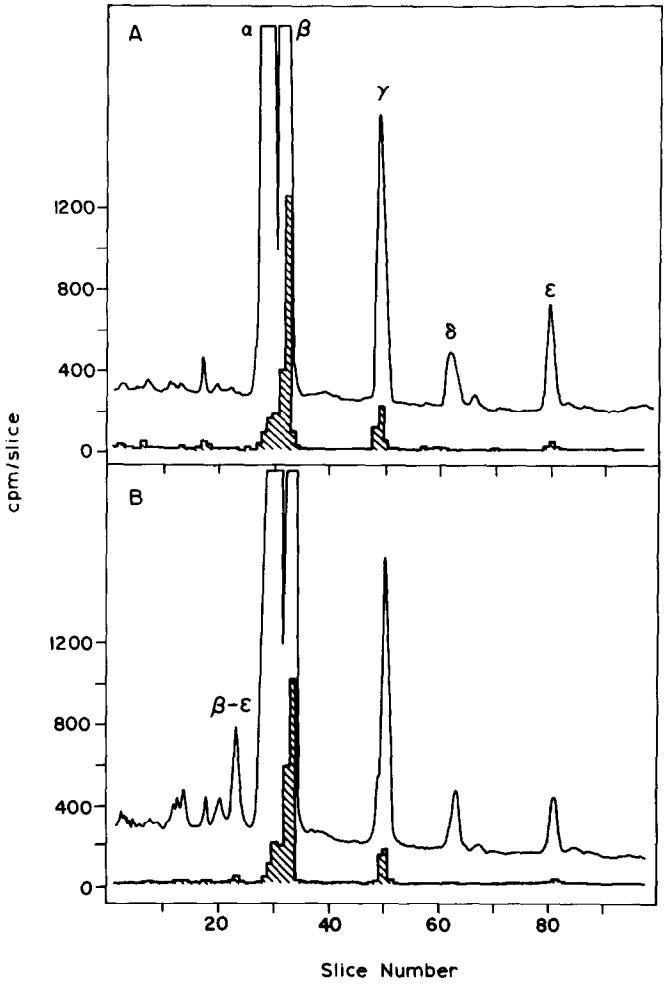

FIGURE 2: Generation of a β-ε crosslink in [^{14}C]DCCD-labeled ECF_1 by EDC. ECF_1 (1.5 mg/ml) was incubated with 135 μM [^{14}C]DCCD (22 x 10^3 cpm/nmol) at room temperature for 3 h. Unreacted reagent was removed by the column-centrifugation-method (see Experimental Procedure) and the [^{14}C]DCCD-labeled ECF_1 was further incubated in the presence of 6.5 mM EDC. After 60 min the samples were subjected to $NaDodSO_4$ gel electrophoresis. The distribution of radioactivity was determined after staining and destaining the gel with Coomasie blue. Solid line: scan at 560 nm; shaded area: radioactivity. A: Control sample, 19 μg applied. B: EDC-treated sample, 19 μg applied.

crosslinked product can be clearly resolved from (free) uncrosslinked β subunits by $NaDodSO_4$ polyacrylamide gel electrophoresis. Thus, the reaction of ECF_1 with EDC provides a convenient way of segregating one of the three β subunits (β') per monomer for examination, e.g. for determination of the distribution of modifying reagents such as DCCD. Figure 2 shows such an experiment.

ECF_1 was reacted with a relatively high concentration of DCCD (130 μM for 3 hr), one half of the sample was then reacted with EDC to generate the crosslinked product (β'-ε) and the other half was used as a control (not treated with EDC). Unbound DCCD was removed by gel filtration and both samples were examined by $NaDodSO_4$ polyacrylamide gel electrophoresis. The EDC treated sample shows a crosslinked product (β'-ε) (Fig. 2b) not present in the control (Fig. 2a), which contained 50% of the ε subunit (quantitated by estimating the amount of free ε and comparing this with the control, using the γ subunit as a reference). By inference, the crosslinked product must contain 50% of β' or 17% of the total β subunit in the complex. This crosslinked product stained only poorly with Coomassie brilliant blue. There was radioactivity associated with free β subunits (total of 1650 cpm) but no significant labeling of β' in the β'-ε crosslinked product. Given that 17% of the total β subunit of F_1 was in the crosslinked product, there should have been 280 cpm in this band if labeling was random with all three β subunits equally available to reagent. The difference in reactivity of the β' and free β subunits to DCCD is clearly demonstrated by the time course experiment in Figure 3. Aliquots of ECF_1 were reacted with [^{14}C]DCCD for different lengths of time before being reacted with EDC to generate the crosslinked product. Samples were then analyzed for distribution of radioactivity by $NaDodSO_4$ gel electrophoresis. Figure 3 compares the incorporation of [^{14}C]DCCD in free β subunits (circles) and the β'-ε crosslinked product (triangles) as a function of total DCCD incorporated. The amount of DCCD in the free β subunits reached 1.5 moles/mole F_1 with essentially no labeling of the β'-ε crosslinked product.

A trivial explanation of the above result would be that reaction of the β' subunit with DCCD affects its crosslinking to ε. Thus, only unlabeled β' could be in the crosslinked product with [^{14}C]DCCD reacted β' now running as free subunit. To test this possibility, the extent of crosslinking of β to ε was monitored as a function of the amount of DCCD bound to F_1. Figure 4 shows that the extent of crosslinking of β' to ε is virtually independent of the amount of DCCD in F_1.

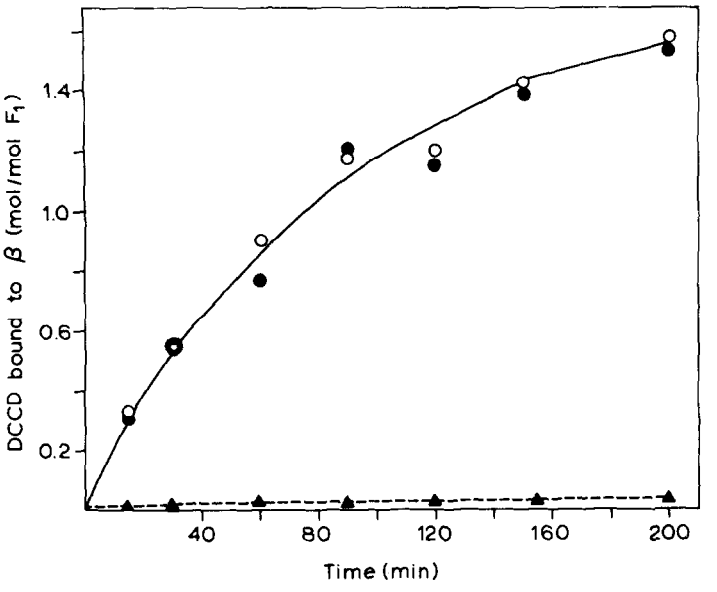

FIGURE 3: Incorporation of [^{14}C]DCCD into subunit β as a function of time. ECF_1 was treated with 135 μM [^{14}C]DCCD as described in Figure 2. At the indicated times 90 μl aliquots were withdrawn and unreacted reagent was removed. One portion at the [^{14}C]DCCD-labeled ECF_1 was further incubated with 6.5 mM EDC for 60 min. Control samples were not treated with EDC. The samples were then subjected to NaDodSO$_4$ gel electrophoresis and the amount of [^{14}C]DCCD incorporated into subunit β (circles) and β-ε crosslink (triangles) was determined after slicing the gel. Open circles: Control samples. Closed circles: EDC-treated samples.

DISCUSSION: Studies presented here show that maximal inhibition of the ATPase activity of ECF_1 requires around 1 mole of DCCD per mole of enzyme, with incorporation of the reagent mostly in the β subunit but with a small amount

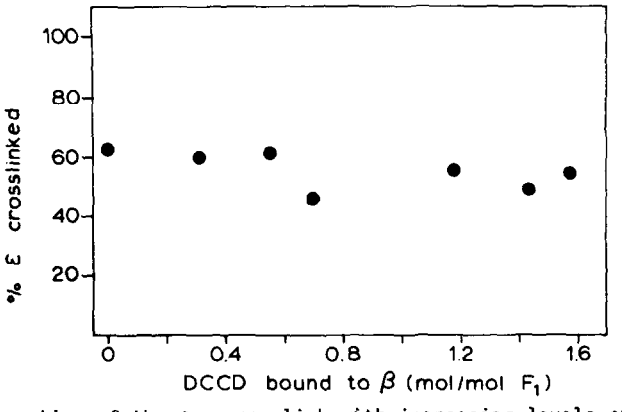

FIGURE 4: Formation of the β-ε crosslink with increasing levels of DCCD bound to subunit β. The amount of ε crosslinked to β was determined from the experiment presented in Figure 3 by integrating the densitometric traces of the gel scans. The area of ε from control-samples (not EDC-treated) was taken as 100%.

of labeling of both α and γ subunits. In this respect our results agree with the previous findings of Satre et al. (16). However, it is clear that the modification of ECF_1 with DCCD is more complicated than thought by these authors. The [^{14}C]DCCD, bound to ECF_1 after gel filtration to remove unbound reagent, appears to be associated with the protein in at least two ways. One fraction remains associated with the enzyme through denaturation of the protein for $NaDodSO_4$ polyacrylamide gel electrophoresis, and is probably incorporated as a stable N-acylisourea derivative. The remainder is released upon denaturation and gel electrophoresis and may be bound to the enzyme as an unstable O-acylisourea which becomes available to water and is hydrolyzed off during unfolding of the protein in $NaDodSO_4$. Similar conclusions have been reached by Yoshida et al. (19).

The stoichiometry of modification of ECF_1 in relation to the inhibition of ATPase activity is important. ECF_1, like F_1 from other sources, is thought to contain three catalytic sites per monomer, one in each of the three β subunits (1-3). DCCD has been shown to react selectively with the β subunit in ECF_1 at a single site, residue Glu 193 (19,20). This modification of DCCD is shielded to a small extent by ADP and ATP and to a major extent by the presence of Mg^{++} ions in the reaction mixture. These findings have led to the proposal that DCCD reacts in or very close to the active site for ATP hydrolysis.

The fact that 1 mole of DCCD per mole F_1 is sufficient to inhibit ATPase activity (to greater than 90%) is a clear indication of the cooperativity of active sites on the enzyme. Results presented here show that DCCD does not distribute equally among the three β subunits per F_1 complex. One of these three β subunits can be distinguished by its crosslinking to ε when the enzyme is reacted with EDC. This β subunit, differentiated as β', is not modified by DCCD as judged by incorporation of [^{14}C]DCCD under conditions of maximal inhibition of ATPase activity and where up to 1.6 moles of DCCD are covalently bound (in the two remaining β subunits) per mole F_1.

The altered reactivity of β' to DCCD could be a direct shielding, with binding of the ε subunit blocking access of DCCD to its site, or it could result from a conformational change in the β' subunit caused by interaction with ε. In either case, this differential reactivity to DCCD is the first experimental evidence for the expected asymmetry of this enzyme with the 3:3:1:1:1 stiochiometry of its five component subunits.

The differentiation of β subunits by DCCD may have important functional implications. It has been clearly established that DCCD and 4-chloro-7-nitrobenzofurazan (NBDCl) inhibit ATPase and ATP synthase reactions differently (21,22). Conditions of reaction of F_1 with DCCD have been reported where 95% of the ATPase activity was inhibited but with 40-50% of the ATP synthase reaction of the enzyme being retained (22). One explanation for this result is that DCCD modifies a group (Glu 193) important for ATP hydrolysis but not involved in ATP synthesis. The results presented here suggest an alternative explanation, i.e. that the site functional in ATP synthesis and sites acting in ATP hydrolysis are on different β subunits. DCCD or NBDCl could block ATPase activity by interacting with β subunits structured for the hydrolase reaction while leaving unmodified that β subunit primed for the synthase reaction by its interaction with ε and possibly γ.

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