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THE DCCD-REACTIVE ASPARTYL-RESIDUE OF SUBUNIT C FROM THE ESCHERICHIA COLI ATP-SYNTHASE IS IMPORTANT FOR THE CONFORMATION OF F_0

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The effect of various point mutations in subunits a and and c of the E. coli ATP-synthase was characterized. In each of the mutants there was no F_0-dependent H^+-conduction, but still an ATPase-activity comparable to wildtype activities. In addition, the subunit b could be extracted from the mutant's F_0 but not from the F_0 of wildtype. The effects are interpreted as a change in the conformation of F_0 caused by the different mutations.

The membrane-bound ATP-synthases of different organisms have common functional and structural properties (1). The enzymes are composed of two parts: F_1 is membrane-associated and bears ATPase-activity; F_0 is membrane-integrated and catalyzes the H^+-conduction across the membrane. Both parts are necessary for energy-transducing reactions, i.e. reactions coupled with an H^+ translocation across the membrane. Binding of N,N'-dicyclohexylcarbodiimide (DCCD) to F_0 blocks the H^+ conduction (2-4) and thereby inhibits both ATP-synthesis and ATP-hydrolysis of the ATP-synthase.

The intact ATP-synthase of E. coli has been purified (6,7). In agreement with genetic complementation studies (8) and DNA sequence data (9-11) it consists of 8 subunits, of which 5 (α, β, γ, δ, ϵ) can be asigned to F_1. The biochemical characterization of defined mutants (12,13) as well as highly purified preparations of an active F_0 (14,15) confirm that the residual 3 subunits (a, b, c) belong to

Abbreviations: F_1F_0, ATP-synthase of oxidative phosphorylation; F_1, ATPase moiety of F_1F_0; F_0, H^+ conducting moiety of F_1F_0; DCCD, N,N'-dicyclohexylcarbodiimide; SDS, sodium dodecylsulfate; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline; BSA, bovine serum albumin.

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F_0. Their stoichiometry was determined as 1:2:10-15 (16,30). The most thoroughly characterized component of F_0 is the DCCD-binding subunit c (for review see 17). Its primary structure is known for the protein from wildtype as well as from several unc-mutants (18-20). The importance of this subunit for H^+ conduction was early established (2-4). Recently it was shown that the two other subunits a and b are also indispensable for H^+ conduction via F_0 (21-23). Furthermore, these subunits are responsible for the binding of F_1 by F_0 (23).

But little is known about the interactions of F_0 subunits with each other. A high cooperativity of F_0 subunits was first demonstrated by substoichiometrical inhibition by DCCD (31) and in partial heterozygotes: in a point mutant of subunit c the H^+ conduction could not be restored by the wildtype allele (24). The modification of the asp^{61} residue in the subunit c of the E. coli ATP-synthase by either DCCD-binding or by mutations inhibits the F_0-dependent H^+-conduction. However, DCCD simultaneously inhibits the ATPase-activity of the enzyme whereas the mutations are without influence on ATPase-activity. This indicates that the F_1-ATPase of the mutants is not as tightly bound as the wildtype enzyme due to an altered conformation of F_0. In this report we show that the subunit interactions in F_0 are very sensitive to single amino acid exchanges. The alterations were caused by point mutations in subunits a and c. The change in the stability of F_0 was probed as the extractability of subunit b from F_0 by detergents. In the wildtype subunit b was very tightly bound to the membranes and only minute amounts could be extracted by a mixture of bile salts. Mutations in subunit a or c greatly increased the amount of extractable subunit b.

Material and Methods

Wildtype A 1 as well as the unc-mutant derivatives DG 7/1, 18/3, DG 3/2 and DG 25/3 were described previously (17,18,25). F_1-depleted membranes were prepared as described previously (5). Blotting and immunostaining (26) were slightly modified. The SDS-gelelectrophoresis was performed in 140x120x0.75 mm gels with 15 % acrylamide/0.4 % N,N'-methylene-bis- acrylamide in the sample gel and 0.15 % SDS in all

buffers (27). After electrophoresis the gel was directly placed on a pile consisting of a bottom polyvinyl chloride support (200x200x5 mm), a stainless steel electrode (160x140x1 mm) and 2 layers of cardboard (Schleicher & Schüll No. 2589) soaked with the electrophoresis buffer (including 20 % methanol). The blotting apparatus was completed by placing on top of the gel: a nitrocellulose sheet (Schleicher & Schüll No. BA 85) wetted with water, 5 layers of cardboard, another stainless steel electrode and an upper solid support plate. The whole pile was gently pressed by an appropriate weight. The distance between the electrodes was maximally 10 mm. The blotting was performed at 250 mA for 1 hour.

The nitrocellulose sheet was incubated for 1 hour in 20 ml PBS + 2 % BSA + 10 % horse serum (Boehringer) and for at least 3 hours in 10 ml PBS + 2 % BSA + 10 % horse serum + rabbit IgG (1:200) against the individual F_0-subunits. The sheet was washed for 5 min with each: PBS, 2 m NaCl,PBS, PBS + 0.05 % Triton-X-100 and PBS. The sheet was further incubated with FITC-labelled goat anti rabbit IgG antibodies (1/200) and washed again. The bound antibodies were visualized under UV light (366 nm) and photographed with a green filter. The same sheet could be reused for another immunostaining by applying a recently published procedure (28) for removing the antibodies without detachment of the antigen.

Extraction of F_0-subunits: 3 mg protein of F_1-depleted membranes were suspended in 1 ml buffer (20 mM Tris-HCl pH 7.5, 0.2 mm EDTA, 0.2 mm phenylmethansulfonylfluoride). An 0.1 x volume of detergent mixture (10 % cholate, 5 % deoxycholate) was added, the sample incubated for 20 min at 0°C and centrifuged with 200000 xg for 2 hours. The upper 800 µl of the supernatant were carefully withdrawn, the rest of the supernatant discarded and the membranes resuspended in the original volume buffer.

Materials: Acrylamide was from Serva (Heidelberg), bile salts from Sigma (München), FITC-labelled goat antibodies from Paesel (Frankfurt), DCCD from Boehringer (Mannheim), all other chemicals were from Merck (Darmstadt).

Results and Discussion

Strain A 1 is the wildtype containig a functional F_0. The *unc*-mutants DG 7/1, DG 18/3, DG 3/2 and DG 25/3 have a defect F_0. F_1-depleted membranes of mutants and wildtype were assayed for F_0-dependent H^+-conduction as described previously (24). The wildtype membranes had a normal H^+-conducting activity whereas the mutant membranes showed no F_0-dependent H^+-conduction (data not shown). In the first three mutants the defect could be defined on the molecular level: in the amino acid sequence of subunit *c* the DCCD-binding aspartyl residue 61 was substituted by a glycyl residue in mutant DG 7/1 (17) and by an asparaginyl residue in mutants DG 18/3 and DG 3/2 (18). The defect in mutant DG 25/3 is due to an altered subunit *a*. This was shown by complementation with plasmid pOM 11-1. The plasmid contains as the only *unc*-gene

the gene coding for subunit a (23). All these mutants are point mutants as indicated by the appearance of revertants.

The defect mutations of the DCCD-binding subunit c have so far always been interpreted as changes in an amino acid residue directly involved in H^+ conduction (29). This interpretation is particularly tempting for the mutants DG 18/3 and DG 3/2, where a "minimal" alteration occurred: the change from a free carboxyl group to an amidated carboxyl group should not increase the "bulkiness" of the amino acid residue. But the primary effect of these mutations could as well be a change in the conformation of F_0, thereby disrupting the H^+-pathway.

So far, there was no probe available to monitor the conformation of F_0. We found that in several unc-mutants with a defect F_0, the subunit b was no longer as tightly membrane-integrated as in the wildtype. A mixture of 0.5 % deoxylcholate and 1 % cholate in a low ionic strength buffer proved to be optimal for measuring a change in the extractability of subunit b from F_0. F_1-depleted membranes of wildtype and mutants were treated with the bile acid mixture - the extracts as well as the residual membranes were analyzed for the presence of subunits b and c. After SDS-gelelectrophoresis the separated polypeptides were transferred onto nitrocellulose sheets which were subsequently stained by rabbit antibodies against the subunits b and c and by FITC-labelled goat anti-rabbit IgG antibodies. Subunit c provides the greatest amount of protein in F_0 (30) and was thus chosen as indicator for either the extraction of whole F_0 or for the dissociation of F_0 followed by the extraction of at least b and c.

In wildtype as well as mutants 25 % of the total membrane protein was extracted by the bile acids. Identical amounts of protein from both extract and membranes were subjected to gelelectrophoresis, so the extracted protein was four times concentrated compared to the membrane protein. Fig. 1 shows that there was no extraction of subunit c from wildtype membranes (lanes 1,2) and only minute amounts of this subunit

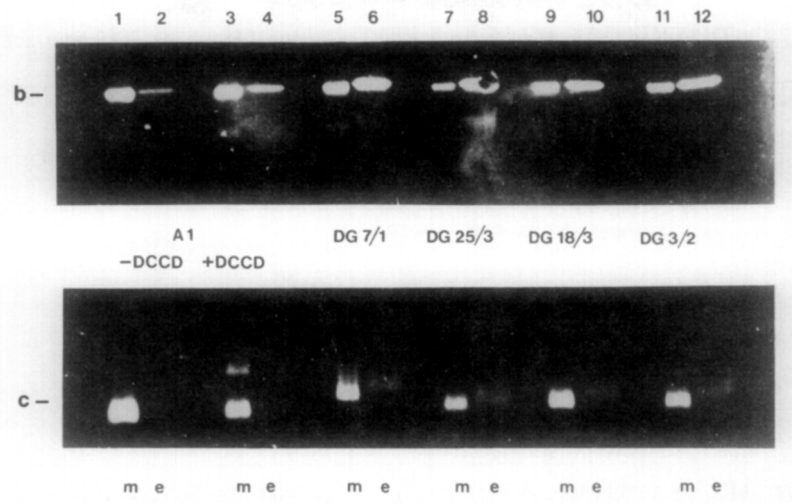

Figure 1: Distribution of subunits b and c between residual membranes and detergent extracts. Proteins of membranes and extracts were subjected to SDS-gelelectrophoresis, the separated proteins were blotted onto nitrocellulose sheets and the subunits b and c visualized by immunostaining. Strains: A 1 lanes 1,2; A 1 after DCCD-treatment lanes 3,4; DG 7/1 lanes 5,6; DG 25/3 lanes 7,8; DG 18/3 lanes 9,10; DG 3/2 lanes 11,12. Fractions: m = residual membranes, e = detergent extracts. Detailed procedures as described in Material and Methods.

were extractable from mutant membranes (lanes 5-12). Only minor amounts of subunit b could be extracted from wildtype membranes (lane 1,2), whereas substantial amounts of this subunit were extracted from the membranes of mutants DG 7/1 (lanes 5,6), DG 25/3 (lanes 7,8), DG 18/3 (lanes 9,10) and DG 3/2 (lanes 11,12). This increase of extractable subunit b was by far higher than the minute increase of extractable subunit c and can thus hardly be interpreted as extraction of total F_0. The binding of DCCD to the wildtype protein did not change the extractability of subunit b significantly. So the binding of DCCD does not seem to introduce a structural change great enough to be probed as an altered extractability of subunit b. Whether DCCD acts by blocking a catalytically important group or by causing more subtile conformational changes is still unclear.

It was shown that the structure of F_0 is very sensitive to alterations of the individual F_0 subunits. The resulting increased extractability of subunit b can be interpreted in two ways: (i) the mutation directly abolished a binding site for subunit b on the altered subunit

Table I: ATPase activity of membranes, its inhibition by DCCD and specificity of DCCD-binding

Strain	Altered Subunit	Membrane-Bound ATPase-activity [U/mg]	DCCD-Sensitivity	Specificity of DCCD-binding mol DCCD/mol of subunit c
A 1	-	0.6	80 %	0.24
7/1	c	0.6	1 %	-
18/3	c	0.8	1 %	-
3/2	c	0.6	1 %	-
25/3	a	0.6	1 %	0.23

Preparation of membranes, ATPase assay, inhibition by DCCD were described previously (5). The specificity of DCCD-binding was tested by incubating the membranes with[^{14}C] DCCD at a concentration of 3.75nmol DCCD per mg membrane protein and by measuring the amount of radioactivity bound to the isolated subunit c (21).

without affecting the conformation of F_0 or (ii) the mutation changed the conformation of F_0 and thereby weakened the binding of subunit b. The second possibility would help to explain some characteristic features of the mutant ATP synthases. The disappearance of the negatively charged aspartyl residue did not inhibit the ATPase activity of the mutant strains DG 7/1, DG 18/3 and DG 3/2. Neither did the binding of DCCD to subunit c of mutant DG 25/3 although its specificity was equivalent to the specificity of DCCD-binding in the wildtype (Table I). Furthermore, attempts to isolate the mutant ATP-synthases failed because F_1 dissociated from F_0 during the purification (Friedl, unpublished results). The dissociation of the enzyme complex as well as the lack of inhibition of the ATPase-activity could easily be explained by a weakened binding of F_1 due to a conformational change in F_0. In conclusion we favour the interpretation that the increased extractability of subunit b is a result of a conformational change in the mutant F_0. In any case, defined amino acid exchanges in any of the F_0 subunits can not be interpreted straightforwardly as the altered group being involved in the catalytic events. Such a conclusion could only be drawn, if a conformational change has been excluced. So the demonstrated

structural change in the F_0 of the mutants DG 7/1, DG 18/3 and DG 3/2 again turns up the question, whether the aspartyl-group 64 in the amino acid backbone of the DCCD-binding subunit c is merely a structurally important group and/or if it is directly involved in the catalysis of H^+ conduction.

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