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The dicyclohexylcarbodiimide-binding protein *c* of ATP synthase from *Escherichia coli* is not sufficient to express an efficient H^+ conduction

(bacteriophage Mu-induced *unc* mutants/H^+ pathway)

PETER FRIEDL*, GERHARD BIENHAUS†, JÜRGEN HOPPE*, AND HANS ULRICH SCHAIRER*

*Gesellschaft für Biotechnologische Forschung mbH., Abteilung Stoffwechselregulation, Mascheroder Weg 1, D-3300 Braunschweig-Stöckheim, Federal Republic of Germany; and †Max Planck Institut für Biologie, Abteilung Overath, Corrensstrasse 38, D-7400 Tübingen 1, Federal Republic of Germany

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ABSTRACT **Bacteriophage Mu was inserted into the *unc* genes of *Escherichia coli*. The resulting mutation AS12 had a polar effect on the *unc* operon: membranes of the mutant AS12 contained the dicyclohexylcarbodiimide-binding protein *c* and the protein *a* as sole subunits of the ATP synthase. It was shown by peptide mapping and amino acid analysis of the fragments that protein *c* from mutant AS12 was identical with the wild-type protein *c*. The absence of subunit *b* in mutant AS12 drastically lowered the H^+ conduction dependent on the membrane-integrated moiety (F_0) of the ATP synthase. This suggests that both subunits *b* and *c* are necessary for an efficient expression of H^+ conduction.**

The membrane-bound ATP synthases of different organisms have common structural and functional properties (1–3). The membrane-associated part, F_1, of the enzyme bears ATPase activity; the membrane-integrated part, F_0, catalyzes H^+ conduction across the membrane. Both parts, F_0 and F_1, are necessary for energy-transducing reactions (i.e., reactions coupled with H^+ translocation across the membrane). Binding of *N,N'*-dicyclohexylcarbodiimide (DCCD) to F_0 blocks the H^+ conduction (4–6) and, thereby, inhibits both ATP synthesis and ATP hydrolysis by the ATP synthase.

DCCD binds covalently to an extremely hydrophobic protein *c* of molecular weight 8500 (5, 7). The molecular weight of different ATP synthases can roughly be estimated as 500,000. The DCCD-binding protein *c* comprises 5–10% of the enzyme complex (3, 8), indicating that this subunit is organized in an oligomeric structure. There is biochemical and genetical evidence for a corresponding functional unit, ruling out the possibility that each protein *c* is independently involved in H^+ conduction (9–13). It is tempting to speculate that the DCCD-binding protein *c* is organized as a trimer or hexamer and constitutes the protonophore of the F_0 moiety. Several groups tried to reconstitute H^+-conducting phospholipid vesicles or films from the purified protein *c* and phospholipids. The results were controversial, and it is not possible to estimate the value of the successful experiments (see *Discussion*). We present evidence that in *Escherichia coli* protein *c* is not sufficient to express an efficient H^+ conduction. This is shown by membranes of mutant AS12, which have a greatly reduced F_0-dependent H^+ conduction, although an intact DCCD-binding subunit *c* is present.

METHODS AND MATERIALS

Bacterial Strains. Strains AS12, AC12, DG 7/1, and BH212 were derived from *E. coli* K12 $Y_{mel}(\lambda)$ F^-(*lac I fadR but12 rha ilv metE*). Phage Mu $c^{ts}62$ was used for Mu-induced mutagenesis (14); the mutation-inducing phage Mu can be inserted randomly into the *E. coli* genome (15) and confers a strong transcriptional polarity effect in distal genes of the operon into which it is inserted (16). Strain AS12 contains a Mu-insertion in its *unc* genes. The control strain AC12 was constructed by phage P1 transduction of the wild-type *unc* allele into strain AS12, thereby eliminating the phage Mu. The congenic background to the Mu-containing strain AS12 was regained by inserting the phage Mu into the *his*-operon. So the only genetic difference between strains AS12 and AC12 is the location of the phage Mu.

Preparative Procedures. The cells were grown over night at 32°C in Vogel–Bonner (37) minimal medium with 0.4% glucose as the sole carbon source. Preparation of membranes (17) and F_1-depleted membranes (18), purification of F_1 (19), reconstitution of F_1-containing membranes (13), production of antisera and purification of IgG (20), and P1 transduction (21) were performed as described.

K^+-Loaded Vesicles. For the preparation of K^+-loaded vesicles, the F_1-depleted membranes were resuspended in 250 mM K_2SO_4/0.1 mM EDTA, pH 7.5, at a protein concentration of 2–5 mg/ml. The suspension (1 ml) was sonicated for 45 sec (Labsonic 1510, Microtip; 30 W power output) and diluted to 1 mg of protein per ml; 80 μM DCCD was added to the control samples, and all samples were incubated at 37°C for 20 min. After addition of 5 mM $MgSO_4$, the incubation was continued for 5 min, and then the samples were centrifuged at 4°C with 200,000 × *g* for 30 min. The tubes and the pellets were twice rinsed with distilled water, and the tubes were dried carefully with paper and put on ice.

Measurement of H^+ Conduction. For the measurement of H^+ conduction, the K^+-loaded vesicles were gently suspended in 0.25 ml of 250 mM Na_2SO_4/5 mM $MgSO_4$, pH 7.5, and immediately assayed. Two techniques were used.

A. To 1 ml of 250 mM Na_2SO_4/5 mM $MgSO_4$/5 mM 4-morpholinepropanesulfonic acid, pH 7.0, was added 1–10 μl of vesicle suspension, and the fluorescence (excitation at 410 nm; emission at 490 nm) of the sample was set at zero. After the addition of 2.5 μM 9-amino-6-chloro-2-methoxyacridine, the final fluorescence level was arbitrarily chosen as 100%. Efflux of potassium was started by addition of 200 pmol of valinomycin in 2 μl of methanol. The initial rate of fluorescence quenching was extrapolated to 1 min and expressed as units of fluorescence quenching; 1 unit of fluorescence quenching activity is defined as the amount of fluorescence of the acridine dye that is

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Abbreviations: F_1, membrane-associated ATPase (E.C. 3.6.1.3) part of the ATP synthase; F_0, H^+-conducting membrane-integrated moiety of the ATP synthase; DCCD, *N,N'*-dicyclohexylcarbodiimide.

quenched per mg of membranes with an initial rate of 100% in 1 min (see refs. 13, 17, and 26).

B. To 3 ml of 250 mM Na_2SO_4/5 mM $MgSO_4$ was added 50 μl of vesicle suspension. After equilibration of the sample at a pH of about 6.5, the efflux of potassium was started by addition of 200 pmol of valinomycin in 2 μl of methanol. The pH change of the suspension—*ca.* 0.05–0.2 pH units—was monitored continuously with an Ingold complex electrode LOT 421 connected to a PHM research pH meter (Radio Copenhagen) and a Servogor S recorder. The suspension was gently stirred with a magnetic stirrer and maintained at 25°C. The assay was calibrated by addition of 5 or 10 μl of a HCl standard solution (1 mM).

Other Reagents and Analytical Procedures. ATP- and respiration-dependent quenching of acridine dye fluorescence (17); assay of ATPase activity (19); labeling and isolation of the DCCD-binding protein *c* (22, 23); amino acid analysis, chemical cleavage, and fractionation of the peptides (12); $NaDodSO_4$/polyacrylamide electrophoresis (24); blotting of proteins from polyacrylamide gels onto nitrocellulose sheets and immunological detections of antigens (35); and protein determination (25) were performed as described.

RESULTS

Energy Transduction of AS12 Membranes. Membranes of AS12 showed no ATPase activity or ATP-dependent energy transduction in the fluorescence test; the respiration-dependent energy transduction was comparable to that of the wild type. The ATP-dependent energy transduction of several F_1-defective *unc* mutants could be restored by substitution of mutant F_1 by wild-type F_1. This kind of restoration failed with membranes of mutant AS12 (Table 1); so, in mutant AS12 both F_1 and F_0 were defective. There may be several reasons why the reconstitution of ATP-dependent energy-transduction failed. The contact between F_1 and F_0 could be impaired, or the F_0 part could have lost its H^+-conducting activity.

H^+ Conduction in Wild-Type and Mutant Membranes. H^+ conduction by F_0 was measured as the charge-compensating H^+ influx. F_1-depleted vesicles were filled with K_2SO_4 and suspended in a Na_2SO_4 solution. Valinomycin catalyzed an electrogenic K^+ efflux, which caused an equivalent H^+ influx. The H^+ conduction was assayed by the quenching of acridine dye fluorescence and directly by a pH electrode (Table 2). Both methods are equivalent (see also figure 4 in ref. 26, p. 6128). In membranes of mutant AS12, the H^+ conduction was reduced to 5–7% of the wild-type activity. There was a problem to differentiate between H^+ conduction by F_0 and by background leakage. Only DCCD-sensitive rates were compared, because DCCD completely blocks F_0-dependent H^+ conduction (4–6, 26). The DCCD-insensitive background comprised *ca.* 5–10% of the total rate. A further control was the use of the well-defined *unc* mutant DG 7/1. In this mutant, the H^+ conduction by F_0 is blocked because of an altered subunit *c*: the carbodiimide-reactive aspartyl residue in position 61 of the amino acid sequence was substituted by a glycine residue (23). We still observed a small but significant DCCD-sensitive rate, revealing a DCCD-sensitive background not due to F_0. Thus, all rates were composed of (*i*) a DCCD-insensitive background, (*ii*) a DCCD-sensitive background not due to F_0, and (*iii*) the F_0-dependent rate. The latter was evaluated as follows: only DCCD-sensitive rates were compared, the DCCD-sensitive rate of DG 7/1 membranes (i.e., the F_0-independent background) being set as 0%; the DCCD-sensitive rate of AC12 membranes (wild type) minus the DCCD-sensitive rate of DG 7/1 membranes was set as 100%; and the residual activity of AS12 membranes was calculated by substracting the DCCD-sensitive rate of DG 7/1 membranes from the DCCD-sensitive rate of AS12 membranes.

Table 1. H^+ translocation by reconstituted F_1–F_0 complexes

Membranes of strain	ATP-dependent H^+ translocation, U_{F1}/mg*
AC12	66
AS12	<0.1
BH212	43
DG7/1	<0.1

F_1-depleted membranes were incubated with purified wild-type F_1, and the H^+ translocation was measured as the initial rate of ATP-dependent quenching of 9-amino-6-chloro-2-methoxyacridine fluorescence. BH212 is an *unc* mutant defective in F_1; DG 7/1 is defective in F_0.

* $Units_{F1}$/mg = amount of fluorescence of acridine dye quenched per mg of membranes, with an initial rate of 100% in 1 min.

Table 2. H^+ conduction by F_0

Membranes of strain	DCCD-sensitive H^+ influx: U_{F1}/mg*	DCCD-sensitive H^+ influx: nmol H^+/min/mg of membranes
AC12	215	77
AS12	19	9
BH212	226	96
DG7/1	9	2

F_1-depleted membranes were filled with K_2SO_4 and transferred to K^+-free medium; after addition of valinomycin the charge-compensating H^+-influx was measured as the initial rate of quenching of 9-amino-6-chloro-2-methoxyacridine fluorescence or directly by a pH electrode.

* U_{F1}/mg = amount of fluorescence of acridine dye quenched per mg of membranes, with an initial rate of 100% in 1 min.

It was important to rule out the possibility that the altered physiology of *unc* mutants had an influence on the H^+ permeability of the membranes. So we included the *unc* mutant BH212 in our experiments. The ATP synthase of BH212 contains a wild-type F_0 and a defective F_1. F_1 is lacking ATPase activity because subunit α is altered (unpublished results). This mutant has the same F_0 as does the wild-type but has the physiological background typical for *unc* mutants. Comparison of AC12 membranes with BH212 membranes showed that the altered physiology of *unc* mutants had no significant influence on the H^+ permeability of the membranes. Thus, the reduced H^+ permeability of mutant AS12 was solely due to a defect in F_0. The mutant was obtained by mutagenesis with phage Mu; the insertion of its DNA into the *unc* operon may drastically lower the expression of the promoter distal *unc*-genes causing the lack of several subunits.

ATP Synthase Subunits in Membranes of AC12 and AS12. The expression of ATP synthase genes in whole cells of wild type and *unc* mutant AS12 and of other polar *unc* mutants will be described elsewhere. Here the membranes of AC12 and AS12 were assayed for the ATP synthase subunits. Membrane proteins were separated by $NaDodSO_4$/polyacrylamide electrophoresis and blotted onto nitrocellulose sheets. The sheets were incubated with rabbit IgG raised against the purified ATP synthase subunits. The bound antibodies revealed the presence of the subunit and were localized by binding of ^{125}I-labeled anti-rabbit IgG and subsequent autoradiography. Fig. 1 shows that membranes of mutant AS12 do not contain any of the F_1 subunits. In F_1-depleted membranes of the wild type, all three subunits of F_0 could be demonstrated, whereas in identically

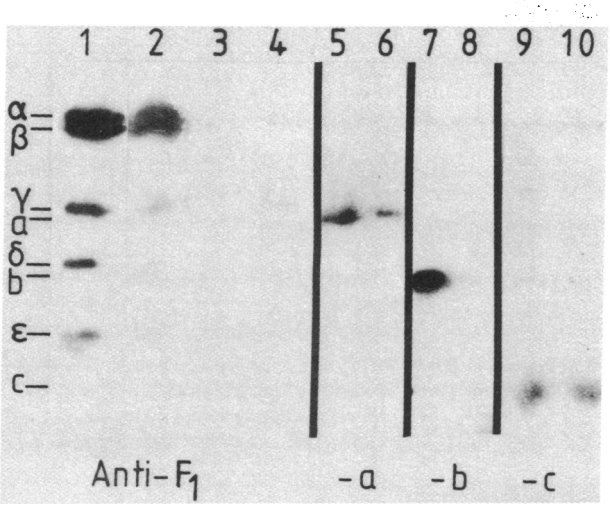

FIG. 1. Membrane proteins were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and blotted onto nitrocellulose sheets. ATP synthase subunits were localized by binding rabbit IgG raised against the isolated subunits. The bound antibodies were visualized by ^{125}I-labeled goat anti-rabbit IgG and subsequent autoradiography. F_1-ATPase (lane 1), membranes of AC12 (lane 2) and AS12 (lane 3), and F_1-depleted membranes of AC12 (lane 4) were assayed with antibodies against F_1; F_1-depleted membranes of AC12 (lanes 5, 7, and 9) and AS12 (lanes 6, 8, and 10) were assayed with antibodies against subunit *a* (lanes 5 and 6), subunit *b* (lanes 7 and 8), and subunit *c* (lanes 9 and 10).

treated membranes of mutant AS12, subunit *b* was missing. This was also true for cell-free extracts of mutant AS12 (data not shown) so that, in mutant AS12, the insertion of the phage Mu into the *unc* operon stopped or greatly reduced the synthesis of subunit *b*.

Von Meyenburg and Hansen (27) proposed the following sequence for the *unc* genes: *a*-(*c*,*b*)-(α,δ)-γ-(β,ε). We had to rule out the possibility that the phage had been inserted very close to the COOH terminus of protein *c*, so that protein *a* and a shortened, defective but still antigenic protein *c* was made.

Integrity of the DCCD-Binding Subunit *c*. Any alternation of the subunit *c* from mutant AS12 was excluded by peptide mapping of the isolated protein *c*. Comparable amounts of protein *c* could be isolated from mutant AS12 and wild type. After cyanogen bromide cleavage of the mutant protein *c*, the same set of fragments was obtained as with the wild-type protein. No new peptides were observed, and the recovery of the individual peptides was the same as in the wild type. The amino acid analysis of the fragments was the same for wild type and mutant (Table 3). Therefore it is concluded, that the amino acid sequence of the mutant protein is identical to the wild-type sequence.

Table 4. Specificity of DCCD binding

Incubation, nmol DCCD/mg of membrane	[^{14}C]DCCD bound at subunit *c*, mol/mol	
	AS12	AC12
0.26	0.19	0.20
5	0.25	0.28

Membranes of mutant AS12 and wild-type AC12 were incubated at the indicated concentrations of DCCD (nmol/mg of membrane protein); the DCCD-binding subunit *c* was isolated, and the amount of bound [^{14}C]DCCD was measured. Protein content of subunit *c* preparation was determined by quantitative amino acid analysis.

Specificity of DCCD-Binding. Under standardized labeling conditions, even small differences in protein conformation can be detected by an altered specificity of DCCD-binding (22, 28). The same amount of DCCD was incorporated into mutant and wild-type protein *c* under identical labeling conditions (Table 4), indicating a similar conformation for both proteins.

DISCUSSION

The F_0 part of the *E. coli* ATP-synthase is composed of three subunits (17, 36): proteins *a*, *b*, and *c* with apparent molecular weights of 28,000 (17) or 24,000 (33, 36), 19,000, and 8500. Subunit *b* is missing in membranes of the mutant strain AS12. Subunit *a* and the DCCD-binding subunit *c* are present.‡ The lack of subunit *b* reduces the F_0-dependent H^+-conduction to 5–7% of the wild-type activity. This result suggests that subunit *b* is

‡ Further evidence for the presence of a functional subunit *a* comes from a positive complementation of mutant AS12 by a plasmid harboring the *unc*-operon with a deletion in the gene coding for protein *a* (v. Meyenburg, personal communication).

Table 3. Amino acid composition of cyanogenbromide fragments

	Mole ratio of peptides, no.													
	2		3		4		6		7		8		9	
Amino acid	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Aspartic acid	1.94	(2.16)	0.90	(1.00)	—	—	1.03	(1.00)	1.09	(1.08)	—	—	—	—
Threonine	—	—	—	—	—	—	1.00	(0.88)	—	—	—	—	—	—
Serine	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Homoserine	0.64	(0.60)	0.40	(0.45)	0.79	(0.60)	0.65	(0.90)	0.42	(0.40)	0.71	(0.70)	—	—
Glutamic acid	1.00	(0.90)	—	—	—	—	3.19	(3.33)	—	—	—	—	—	—
Proline	—	—	—	—	—	—	1.80	(1.78)	1.10	(1.00)	—	—	—	—
Glycine	—	—	—	—	—	—	6.70	(6.98)	0.98	(1.00)	2.01	(2.00)	—	—
Alanine	—	—	—	—	3.00	(3.00)	6.02	(6.02)	1.09	(1.07)	1.01	(1.02)	—	—
Valine	—	—	—	—	1.00	(1.00)	1.10	(0.87)	0.94	(0.97)	1.77	(1.74)	1.00	(1.00)
Isoleucine	—	—	—	—	—	—	5.57	(5.74)	1.02	(0.97)	0.90	(0.91)	—	—
Leucine	0.98	(0.97)	2.10	(1.90)	—	—	6.08	(6.08)	1.08	(0.98)	2.03	(1.94)	—	—
Tyrosine	—	—	0.55	(0.50)	—	—	—	—	—	—	0.60	(0.40)	—	—
Phenylalanine	—	—	—	—	—	—	2.97	(2.88)	—	—	—	—	0.94	(0.95)
Arginine	—	—	—	—	—	—	1.92	(1.88)	—	—	—	—	—	—
Lysine	—	—	—	—	—	—	1.02	(1.21)	—	—	—	—	—	—

The isolated DCCD-binding subunit *c* was cleaved by CNBr, and the fragments were separated by gel chromatography. CNBr fragments were subjected to amino acid analysis. Further experimental details are described in ref. 23. The values of the wild-type AC12 are in parentheses (B) following the values of the mutant AS12 (A).

necessary to express F_0-dependent H^+-conduction and that the DCCD-binding protein *c* alone cannot express an efficient H^+ conduction.

The latter statement is only valid if the mutant protein *c* remained unaffected by the mutation AS12. The integrity of protein *c* from the mutant AS12 was proven by peptide mapping and amino acid analysis of the fragments. The specificity of DCCD-binding has been shown to be sensitive to conformational changes of protein *c*. The protein *c* of mutant AS12 and wild type showed an identical specificity of DCCD binding, indicating that in both strains the conformation of the protein is similar. Thus, the reduction of membrane H^+ permeability was caused solely by the lack of subunit *b*.

Mainly two results supported the idea that the DCCD-binding protein acts as a protonophore: reconstitution experiments of Nelson *et al.* (29) with the protein of chloroplast ATP synthase and experiments of Criddle *et al.* (30) with the protein of yeast mitochondrial ATP synthase. Similar experiments with the DCCD-binding protein of *E. coli* (30) and of the bacterium PS3 (11) failed, as did our own efforts with the protein of *E. coli* using different solvents for protein *c* and testing various reconstitution procedures for preparing proteoliposomes (unpublished results). Although some objections can be made against the experiments of Nelson *et al.*,[§] both the results of Criddle *et al.* and of Nelson *et al.* indeed show a specific effect of protein *c* on membrane conductance. But nothing can be said about the physiological relevance of the observed rates. Is it just a residual activity of 1–2% compared to the F_0-activity in the mitochondrial membrane or is it a significant 50% reconstitution? For example, Okamoto *et al.* (26) not only isolated and reconstituted the H^+-conducting moiety of the ATP synthase from bacterium PS3, they also were able to demonstrate that the reconstituted activity was about 25% compared with that of F_1-depleted submitochondrial particles (see also ref. 36). Possibly Criddle *et al.* measured only a similar residual activity as we did with membranes of mutant AS12.

The DCCD-sensitive residual activity of AS12 membranes is certainly a function of F_0 and causes some speculations about its nature. (*i*) The lack of subunit *b* alters the quarterny structure of the *c* oligomer. The effect is too small to be detected by a change in the specificity of DCCD-binding but is big enough to reduce the activity to the residual level. This would mean that the oligomer of protein *c* constitutes the H^+ pathway that is stabilized by subunit *b*. Subunit *b* itself is not directly involved in the H^+ movement. (*ii*) Both proteins *c* and *b* build up the H^+ pathway; without subunit *b*, protein *c* exerts an atypical reduced activity. (*iii*) Protein *c* builds up a frame for the subunit *b*, which constitutes the H^+ pathway; without subunit *b*, a new small activity of protein *c* appears. The binding of DCCD to protein *c* does not block a functional group for H^+ conduction; instead, the binding induces a conformational change that inactivates the protonophore.

[§] Objections are: (*i*) The inhibition of the postulated effect by DCCD was shown, not the effect itself—the conductance of liposomes plus bacteriorhodopsin versus the conductance of liposomes plus bacteriorhodopsin plus protein *c*; (*ii*) nothing is said about the amount of protein *c* used in the test; (*iii*) the stated argument that the protein *c* maintains its native structure during isolation and reconstitution is not valid (31); and (*iv*) it was not finally shown whether or not the protein *c* preparation was homogeneous (see the amount of histidine and cysteine in table 2 of ref. 32, p. 13).

At the moment it is not possible to favor one of these possibilities. The biochemical analysis of other *unc*-mutants may contribute further elucidation of the H^+ conduction catalyzed by F_0.

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