F_0 OF *ESCHERICHIA COLI* ATP-SYNTHASE CONTAINING MUTANT AND WILD-TYPE CARBODIIMIDE-BINDING PROTEINS IS IMPAIRED IN H^+-CONDUCTION

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1. Introduction

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_1 and F_0, are necessary for energy-transducing reactions, i.e., reactions coupled with a H^+-translocation across the membrane. Binding of *N,N'*-dicyclohexylcarbodiimide to F_0 blocks the H^+-conduction [4–6] and thereby inhibits both ATP-synthesis and ATP-hydrolysis of the ATP-synthase.

The carbodiimide binds covalently to an extremely hydrophobic protein, c, of M_r 8500 [5,7]. The M_r of different ATP-synthases can be roughly estimated as 500 000. The carbodiimide-binding protein comprises 5–10% of the enzyme complex [3,8], indicating that this subunit is organized in an oligomeric structure. Is there a corresponding functional unit or is each protein independently involved in H^+-conduction? Our approach to answer this question uses a mixed population of c, consisting of intact and defective proteins.

From *Escherichia coli* numerous ATP-synthase mutants have been isolated ([9,10] and references therein). The mutant DG 7/1 has a defective protein c [11]. We constructed diploid strains carrying the wild-type allele of protein c on the chromosome and the mutant allele on a F′ plasmid and vice versa. The ATP-dependent H^+-translocation of F_1F_0 and the valinomycin/K^+-induced H^+-influx via F_0 were measured in the heterogenotes and compared with the activities of the diploid homogenotes. The DG 7/1 mutant allele was partially dominant over the wild-type allele. This indicates that the carbodiimide-binding proteins of the ATP-synthase are functionally dependent on each other.

2. Materials and methods

Strains DG 7/1 and BH 212 are derived from *Escherichia coli* K12 Y_{mel} (λ) F^- (*lacI fadR but12 rha ilv metE*). DG 7/1 is an *unc* mutant defective in H^+-conduction [11], BH212 an *unc* mutant with a defective F_1 ATPase [9]. The *unc*-alleles of these strains were integrated into an F′ plasmid and introduced into *recA* derivatives of DG 7/1 and BH212. The genetic construction of the diploid strains 7/1 × F7/1, 212 × F212, 212 × F7/1, 7/1 × F212 will be described in detail elsewhere.

The cells were grown over night in Vogel-Bonner minimal medium [16] with 0.4% glucose as carbon source. Preparation of F_1-depleted membranes [17], purification of F_1 [18] and protein determination [19] were performed as described. The ATP-dependent quenching of acridine-dye fluorescence was performed as in [14] except that in the final test volume (1 ml) F_1-depleted membranes (20–50 μg) were preincubated for 5 min with F_1 (100 μg).

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 2–5 mg protein/ml. A suspension of 1 ml was sonicated for 45 s (Labsonic 1510, micro-tip, 30 W output), diluted to 1 mg protein/ml, 80 μM dicyclohexylcarbodiimide 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 tube and the pellet were rinsed twice with distilled water, carefully dried with paper and put on ice.

For the measurement of H^+-conduction the K^+-loaded vesicles were gently suspended in 0.25 ml 250 mM Na_2SO_4, 5 mM $MgSO_4$ (pH 7.5) and immediately assayed:

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Assay A: To 1 ml 250 mM Na_2SO_4, 5 mM $MgSO_4$, 5 mM MOPS (pH 7.5) were added 1–10 μl vesicle suspension and the fluorescence (excitation 410 nm, emission 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% (= 1 full scale of the recorder). Efflux of K^+ was started by addition of 200 pmol valinomycin in 2 μl methanol. The initial rate of fluorescence quenching was extrapolated to 1 min and expressed as units of fluorescence quenching activity (U_{fl}). One unit of fluorescence quenching activity is defined as the amount of membranes which quenches the fluorescence of the acridine dye with an initial rate of 100% (1 scale) in 1 min.

Assay B: To 3 ml 250 mM Na_2SO_4, 5 mM $MgSO_4$ were added 50 μl vesicle suspension. The efflux of K^+ was started by addition of 200 pmol valinomycin in 2 μl methanol. The ΔpH of the suspension was monitored continuously with an Ingold complex electrode LOT 421 connected to a PHM 64 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 HCl standard solution (1 mM).

DNase, ATP were purchased from Boehringer (Mannheim), EDTA, MOPS, Tris, dicyclohexylcarbodiimide from Serva (Heidelberg), all other chemicals in highest purity available from Merck (Darmstadt), 9-amino-6-chloro-2-methoxyacridine was a gift from Dr P. Overath.

3. Results and discussion

The ATP-synthase of the mutant DG 7/1 contains a defective F_0 and a functional F_1, whose ATPase-activity, however, is not inhibited by dicyclohexylcarbodiimide. It was recently shown that the defect in H^+-conduction was caused by substituting a glycine for the carbodiimide-reactive aspartyl residue in position 61 of the amino acid sequence of protein c [11]. The ATP-synthase of the mutant BH212 contains a wild-type F_0 and a defective F_1. The enzyme is lacking ATPase-activity because the α subunit of F_1 is altered (Bienhaus and Schairer unpublished). We chose the mutant BH212 as 'wild-type'-reference because both strains, DG 7/1 and BH212, are *unc*-mutants and thus have the same physiological background. The alleles DG 7/1 and BH212 were integrated into F′ plasmids and introduced into *recA* derivatives of DG 7/1 and BH212. The construction of the diploids 7/1 X F7/1, 212 X F212, 7/1 X F212 and 212 X F7/1 will be described in detail elsewhere.

Table 1
H^+-translocation by reconstituted F_1F_0 complexes

Strain	ATP-dependent H^+-translocation (E_{F1}/mg)
212 X F212	40
212 X F7/1	4
7/1 X F212	2
7/1 X F7/1	<1

F_1-depleted membranes were reconstituted by incubation with purified wild-type F_1 and the H^+-translocation was measured as the initial rate of ATP-dependent quenching of acridine dye fluorescence as in section 2

The ATP-dependent H^+-translocation via the hybrid F_0 could only be measured in a reconstituted F_1F_0 complex. First the defective F_1 of the BH212 allele had to be removed and to be substituted by a functional wild-type F_1. H^+-Translocation was measured as quenching of acridine-dye fluorescence and the fluorescence test quantitatively evaluated. The validity of this method has been proved [12–14]. The results are shown in table 1: 212 X F 7/1 shows 10% of the control activity, the reciproke combination 7/1 X F212 5%.

The H^+-conduction via F_0 was measured as H^+-influx. F_1-depleted vesicles were filled with K_2SO_4 and suspended in a Na_2SO_4 solution. Valinomycin catalysed an electrogenic K^+-efflux, which caused an equivalent H^+-influx. The H^+-conduction was assayed as quenching of acridine-dye fluorescence and directly by a pH-electrode. Both methods lead to equivalent results. There are two ways of differentiating between H^+-conduction via F_0 and background leakage: inhibition by dicyclohexylcarbodiimide abolishes the F_0-dependent activity and comparison with the carbodiimide-sensitive rate of strain 7/1 X F7/1 shows the small carbodiimide-sensitive background not due to F_0. The results are shown in table 2. In both heterogenotes the H^+-conduction is greatly reduced: 212 X F 7/1 shows 5–15% of the wild-type activity, with 7/1 X F212 no activity could be measured.

The results clearly demonstrate that the mutant allele DG 7/1 is partially dominant over the wild-type allele. Two possible explanations should be discussed: a defective assembly of the F_1F_0 complex due to the

Table 2
H^+-conduction via F_0

Strain	H^+-influx	
	(E_{Fl}/mg)	(nmol $H^+ . min^{-1} . mg^{-1}$)
212 X F212	128	130
212 X F7/1	11	30
7/1 X F212	3	11
7/1 X F7/1	6	13

F_1-depleted membranes were filled with K_2SO_4 and transfered to K^+-free medium; after addition of 200 pmol valinomycin the electro-impelled H^+-influx was measured as the initial rate of quenching of acridine dye-fluorescence and directly by a pH electrode. Further details are in section 2

altered mutant protein c; or a functional cooperation of several proteins.

Preliminary evidence suggests, that the mutant ATP-synthase is still assembled, since the whole F_1F_0 complex of the mutant DG 7/1 can be precipitated by antibodies raised against F_1 (unpublished). Labelling experiments with dicyclohexyl [^{14}C] carbodiimide indicated the functional cooperation of several subunits in an oligomer. The enzymatic activities of the ATP-synthase (ATP-dependent quenching of acridine-dye fluorescence [Hoppe, unpublished], ATP–$^{32}P_i$ exchange and ATPase activity [15]) are already maximally inhibited if 16.6–50% of the protein c has bound the carbodiimide. The inactivation of the oligomer(s) by carbodiimide-binding of just one or two proteins can be most easily explained by a functional cooperation of several subunits.

Instead of carbodiimide-labelling we used the presence of the mutant DG 7/1 allele in a wild-type cell. In the heterogenotes there is only a small fraction of F_0 free of mutant protein c while the majority of F_0 contains 'mixed' oligomers consisting of mutant and wild-type protein. In the heterogenotes the rates of H^+-translocation and H^+-conduction are strongly reduced. A similar negative complementation has been shown for other oligomeric proteins, compare for example the effect of the i^{-d} allele on *lac*-repressor activity in heterogenotes [20]. We cannot say if the functional unit consists of 3 or 6 c subunits, because a quantitative, statistical interpretation of our results is not possible. Although the mutant DG 7/1 ATP-synthase is still assembled there is no information if the kinetics of the assembly remained unchanged. A slower assembly perhaps due to a slightly altered conformation of the mutant protein would favour the incorporation of wild-type protein into the 'mixed' oligomers of the heterogenotes. Furthermore, it seems that the gene dosis effect of one allele on the chromosome plus one allele on the F' plasmid is not a simple doubling. The heterogenote 212 X F7/1 has significantly more residual activity than the reciproke heterogenote 7/1 X F212.

The results support the assumption, that F_0 contains oligomers of c. The function of these oligomers is impaired if wild-type c is partially substituted by mutant c which has lost its catalytic function but has retained its ability to form oligomers. The number of proteins c forming one functional unit is not known.

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