THE PROTEOLIPID OF A MUTANT ATPase FROM *ESCHERICHIA COLI* DEFECTIVE IN H^+-CONDUCTION CONTAINS A GLYCINE INSTEAD OF THE CARBODIIMIDE-REACTIVE ASPARTYL RESIDUE

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1. Introduction

A carbodiimide-binding proteolipid subunit has been identified in the energy-transducing ATPase complex from eukaryotic and prokaryotic cells [1–4]. Increasing evidence is being accumulated that the proteolipid (M_r 8000) in oligomeric, most likely hexameric, form [2] constitutes the proton-conductor of the ATPase membrane factor, F_0 [5–7]. The amino acid sequence has been determined for the ATPase proteolipid from various mitochondria (*Neurospora crassa, Saccharomyces cerevisiae,* bovine heart), chloroplasts (spinach) and bacteria (*Escherichia coli,* PS-3) [8]. Dicyclohexylcarbodiimide (DCCD), an effective inhibitor of the proton-translocating activity of the ATPase complex [4,9,10], binds selectively to one acidic amino acid side chain (glutamic or aspartic acid) [8,11].

From *E. coli* numerous ATPase mutants of the *uncB* phenotype have been isolated [12]. These mutants contain a functional F_1 ATPase, but the membrane factor, F_0, is defective [12,13]. In contrast to the *E. coli* wild-type, removal of the F_1 ATPase from the mutant membrane does not result in a proton leak. We are presently engaged in screening several of these *uncB*-type mutants for alterations in the amino acid sequence of the ATPase proteolipid. An analysis of structural gene mutants hopefully allows an identification of those amino acid residues of the proteolipid, which are essential for the function and/or assembly of the proton-translocator. Furthermore, a structural gene mutant will be useful for the cloning and isolation of the proteolipid gene. As a first result of these protein-chemical studies, this communication describes an *uncB*-type mutant with a single amino acid exchange in the ATPase proteolipid. The purified CNBr fragments of the mutant and wild-type proteolipid were studied by thin-layer chromatography and amino acid analysis. The exchange could be localized in one fragment, whose amino acid sequence was determined. Interestingly enough, the carbodiimide-reactive aspartyl residue in position 61 of the polypeptide chain [11] was found to be substituted by a glycine in the mutant proteolipid.

2. Materials and methods

E. coli K12 Y_{mel} (λ), F^- (*lacI, fadR, but12, rha, ilv, metE),* and its derivative the *uncB*-type mutant *DG 7/1* were grown in CR medium with 1% glucose as carbon source, supplemented with isoleucine, valine and methionine, each at 0.1 mg/ml [13]. Cells (0.8–1.0 kg wet wt) were obtained from 200 l cultures grown to late log phase. The cells were incubated with lysozyme (1 mg/g wet wt) and DNase (20 μg/g wet wt) for 3 h at room temperature. Then a crude membrane fraction was sedimented at 8000 × *g* for 15 min. The ATPase proteolipid was isolated by extraction of whole membranes with chloroform/methanol 2:1 [3] and subsequent carboxymethyl-cellulose chromatography [14]. The pure *E. coli* proteolipid was eluted from the column during the wash with chloroform/methanol/water (5:5:1) [14].

The proteolipid (4 mg Lowry protein) was cleaved at its 8 methionyl residues [8,11] by incubation in 1 ml 1 M CNBr in 80% formic acid at room temperature for 18 h. The resulting fragments were submitted to chromatography on Bio-Gel P-30 (–400 mesh) in the presence of 80% formic acid.

Chromatographic fractions containing cyanogenbromide fragments (I) B-6, (II) B-7 and B-8, (III) B-4 and (IV) B-2, B-3 and B-9 were combined and dried over KOH in vacuo. The pooled fractions were dissolved in trifluoroacetic acid and dried over KOH in vacuo, 2 times, in order to convert C-terminal homoserine into the lactone. Then the individual peptides were further separated and purified by preparative thin-layer chromatography (DC Fertigplatten, 0.25 mm Kieselgel 60, mit Konzentrierungszone; Merck, Darmstadt) using *n*-butanol/acetic acid/pyridine/water (68:14:40:25) as solvent. The developed plates were superficially sprayed with 0.025% fluorescamine in acetone. Peptides were eluted from the silica gel with 98% formic acid. Analytical plates (HPTLC Fertigplatten, Kieselgel 60, mit Konzentrierungszone; Merck, Darmstadt) were developed with the same solvent as the preparative plates.

For determination of the amino acid composition, the whole proteolipid and CNBr fragments were hydrolysed in 6 N HCl for 24 h and 72 h at 105°C. Methionine was determined as its sulfone after performic acid oxidation [15]. The samples were analysed on a LC 2000 (Biotronik, München) amino acid analyzer. N-terminal amino acids were identified by the dansylation method [16].

Amino acid sequences were determined by automated solid-phase Edman degradation with the Sequemat, model 12, sequencer (Sequemat, Watertown, USA) using the standard program with minor modifications [17]. The whole protein (1.5 mg Lowry protein) dissolved in chloroform/methanol 2:1 was coupled to 3-aminopropyl glass [18] via carboxyl group(s) by the carbodiimide coupling procedure [19]. CNBr fragments B-7 were coupled to the aminated glass using the homoserine lactone coupling procedure [19]. The cleaved-off thioazolinone amino acids were converted into the phenylthiohydantoine derivatives by a 15 min incubation at 65°C with acetylchloride/methanol (2:8) [20], and then identified by thin-layer chromatography [21].

3. Results

Mutant *DG 7/1* was selected after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the procedures in [22]. The mutation maps between *asn* and *ilv* in the *uncA,B* region of the *E. coli* chromosome [12]. The mutant contains a functional F_1 ATPase, which, however, is not inhibited by DCCD. A functional membrane integrated part of the ATPase complex is lacking [13]. According to this phenotype, strain *DG 7/1* is classified as an *uncB*-type mutant.

The amino acid sequence of the ATPase proteolipid from *E. coli* wild-type K12 has been published in preliminary form [8,11]. During the present studies this sequence could be confirmed with the exception that at position 37 repeatedly a glutamic acid was identified instead of a glutamine. This proteolipid consists of 79 amino acid residues. It contains 8 methionines, including a N-terminal formylmethionine and a methionine–methionine sequence at position 16/17. Thus, besides formylhomoserine and homoserine, 7 fragments are produced by CNBr cleavage. Analysis by thin-layer chromatography reveals that the corresponding fragments from the proteolipid of the wild-type and mutant *DG 7/1* exhibit the same R_F value with the exception of fragment B-7 (fig.1). The mutant fragment migrates considerably faster. Amino acid analysis of the CNBr fragment B-7 from mutant *DG 7/1* shows an aspartic acid residue less and an extra glycine residue in comparison with the corresponding wild-type fragment (table 1). No significant differences could be detected when the amino acid compositions as well as the N-terminal residues of the other fragments were compared (data not

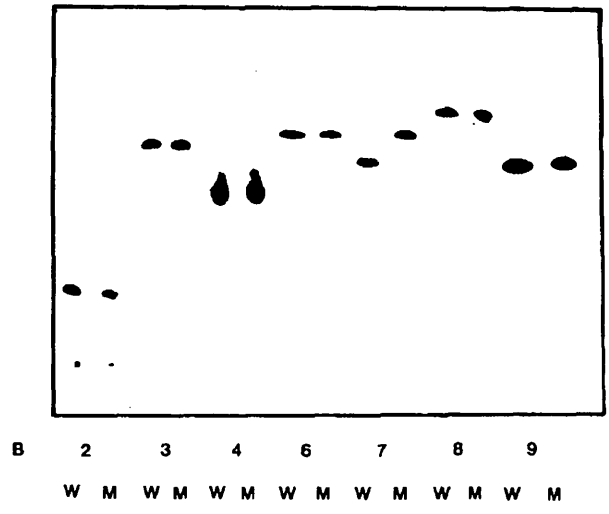

Fig.1. Thin-layer chromatography of CNBr fragments of the ATPase proteolipid of the wild type and *uncB*-type mutant *DG 7/1* from *E. coli*. The fragments from wild type (W) and mutant (M) are numbered according to their occurrence in the amino acid sequence [11]. Fragments B-1 (formylhomoserine) and B-5 (homoserine) are not included.

Table 1
Amino acid composition of the proteolipid and CNBr fragment B-7 from the wild-type and *uncB* mutant *DG 7/1* of *Escherichia coli*

Residue	Proteolipid (mol/mol)		CNBr fragment B-7 (mol/mol)	
	Wild-type	Mutant *DG 7/1*	Wild-type	Mutant *DG 7/1*
Aspartic acid	5.15 (5)	4.15	1.08 (1)	–
Methionine sulfone	7.7 (8)	7.4	–	–
Threonine	1.18 (1)	0.95	–	–
Homoserine			0.58 (1)	0.48 (1)
Glutamic acid	4.13 (4)	3.96	–	–
Proline	2.78 (3)	2.86	0.9 (1)	0.9 (1)
Glycine	9.6 (10)	10.6	1.0 (1)	2.01 (2)
Alanine	12.8 (13)	12.8	1.07 (1)	1.17 (1)
Valine	5.4 (6)	5.5	0.91 (1)	0.88 (1)
Isoleucine	6.8 (8)	6.95	0.93 (1)	0.99 (1)
Leucine	11.8 (12)	11.6	0.98 (1)	1.08 (1)
Tyrosine	1.69 (2)	1.88	–	–
Phenylalanine	3.76 (4)	3.8	–	–
Lysine	1.14 (1)	1.27	–	–
Arginine	2.16 (2)	1.91	–	–

The proteolipid contains no serine, histidine and tryptophan [4,11]. The numbers in parenthesis were determined by sequence analysis [11].

shown). The N-terminal formylmethionine and the methionine–methionine sequence (positions 16/17), which remained undetermined during the analysis of the cyanogenbromide fragments, were identified by sequencing the whole mutant protein up to position 43. When the amino acid composition of the whole mutant protein was analysed (table 1), the only significant differences occurred in the number of aspartic and glycine residues. Accordingly, the aspartic acid/glycine exchange most likely is the only alteration of the proteolipid produced by the mutation in strain *DG 7/1*.

Amino acid sequence analysis of CNBr fragment B-7 from wild-type and mutant *DG 7/1* revealed (fig.2) that at one position (cycle 4) an aspartic acid is substituted by a glycine. The amino acid exchange occurs at position 61 of the *E. coli* proteolipid.

4. Discussion

The present studies established an amino acid exchange in the amino acid sequence of ATPase proteolipid from the *uncB*-type mutant *DG 7/1* of *E. coli*. Remarkably, the aspartyl residues substituted by a glycine in the mutant proteolipid occurs at the same position which previously has been shown to correspond to the carbodiimide-reactive residue [11]. This finding is in accordance with the observation that the mutant proteolipid can not be modified by [^{14}C] dicyclohexylcarbodiimide (unpublished results). Recently, the amino acid composition of another *uncB*-type mutant (*GD 7/10*) has been reported, which no longer binds the carbodiimide [23]. Since an extra glycine and a missing aspartic acid was determined as the only alteration, it is a distinct possibility that this strain contains the same mutation as strain *DG 7/1*. It has to be mentioned, however, that we have analysed several *uncB*-type mutants, which do not bind carbodiimide, and which exhibit no alteration in the ATPase proteolipid, when the amino acid compositions of CNBr fragments are analysed.

At the moment, the primary lesion produced by the aspartic acid/glycine exchange in mutant *DG 7/1* remains undetermined. Preliminary evidence suggests, that the mutant proteolipid is still assembled with the F_1 ATPase, since the whole ATPase complex, F_1F_o, can be precipitated with antibodies raised against the F_1 protein (P. Friedl, unpublished results). This suggests that the amino acid exchange in the mutant results in a non-functional proton-translocator, rather than in a non-assembled membrane factor. This would indicate that an acidic group at position 61 of the

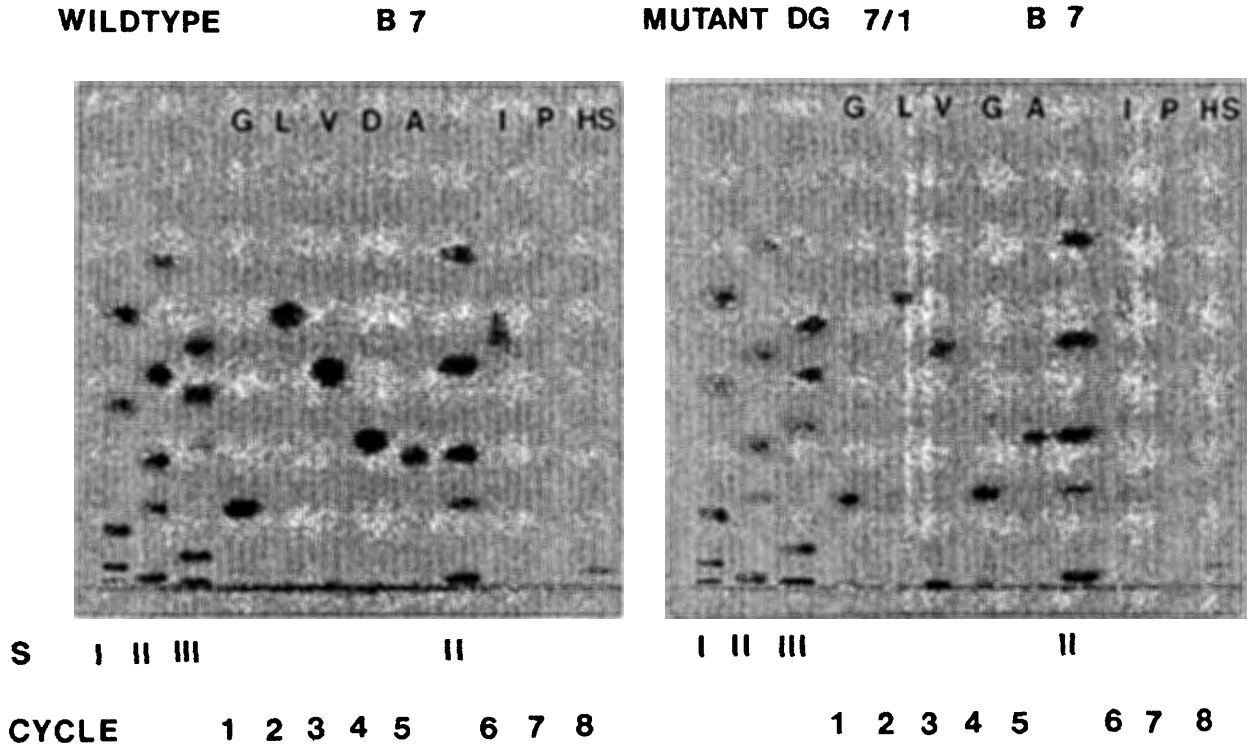

Fig.2. Amino acid sequence of CNBr fragment B-7 of the proteolipid from wild-type and *uncB*-type mutant *DG 7/1*. The amino acid derivatives released during cycle 1–8 of automated solid-phase Edman degradation were identified by thin-layer chromatography using chloroform/ethanol 98/2 as the developing solvent. The calibration standards (S) of the phenylthiohydantoine amino acids contained (I) leucine (L), methionine, lysine, threonine, glutamine, arginine, (II) proline (P), valine (V), alanine (A), glycine (G), serine, asparagine and (III) isoleucine (I), phenylalanine, aspartic acid methylester (D), glutamic acid methylester, tyrosine, glutamic acid, aspartic acid. HS designates the derivative of homoserine.

proteolipid is essential for the proton-conducting activity of the ATPase membrane factor. The importance of this acidic residue has been inferred already from the observation, that this is the only acidic residue which has been conserved during evolution [8].

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