AMINO ACID REPLACEMENT IN DICYCLOHEXYLCARBODIIMIDE-REACTIVE PROTEINS FROM MUTANT STRAINS OF *ESCHERICHIA COLI* DEFECTIVE IN THE ENERGY-TRANSDUCING ATPase COMPLEX

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1. Introduction

The membrane-bound ATPase complex from mitochondria, chloroplasts and bacteria plays a crucial role in energy-transducing reactions [1–3]. One of the subunits from the F_0-component of this enzyme complex from *Escherichia coli,* the dicyclohexylcarbodiimide (DCCD)-reactive protein, has been purified to homogeneity [4,5] and the amino acid sequence of the proteolipid has been established [6]. Increasing evidence exists that this subunit in oligomeric, most likely hexameric, form [4,5,7] constitutes a H^+-channel within the F_0 part of the enzyme complex [8–11].

Several mutant strains have been isolated from *E. coli* which have a defect within the ATPase complex [12,13]. Of particular interest are *uncB* phenotypes in which H^+-translocation through the F_0 part is blocked even after removal of the F_1 portion [12], and in which the proteolipid does no longer react with DCCD. In a second type of mutant (DCCD-resistant strains) the inhibition of the ATPase activity can only be achieved with elevated levels of DCCD [14,15]. Therefore, it seemed promising to us that the characterization of those mutant proteolipids might give us some hints, which residues are necessary for the translocation of H^+ through F_0, leading to a better understanding of the molecular mechanism of H^+ translocation.

We have recently isolated and characterized the DCCD-reactive proteins from an *uncB* mutant (DG 7/10) and a DCCD-resistant strain (DC 1) [16]. We now describe the amino acid sequence of these mutant proteolipids. The data are in full agreement with our earlier suggestion that the DCCD-reactive aspartyl residue in position 61 of the polypeptide chain is replaced by a glycine residue in the mutant proteolipid from the *uncB* phenotype. In the DC1 protein an isoleucine/valine replacement occurred at position 28.

2. Materials and methods

2.1. *Bacterial strains and growth conditions*

E. coli K12 Y_{mel} (λ), F^- (*lacI, fadR, but12, rha, ilv, metE*), and its derivatives, the *uncB* type mutant DG 7/10 and the DCCD- resistant strain DC1, were kindly supplied by Dr H. U. Schairer [12,14]. These strains were grown in a minimal medium [5] supplemented with isoleucine, valine and methionine (0.1 mg/ml each) with 1% glucose as the carbon source. Cells were obtained from 200 l and 3000 l cultures grown to late log phase.

2.2. *Isolation and purification of the proteolipids*

The ATPase proteolipid was extracted from intact cells on a large scale with chloroform/methanol (2:1) as described previously [16]. Purification to homogeneity was achieved by DEAE-cellulose or CM-cellulose chromatography [5,16]. Minor contaminants, occasionally present in the proteolipid fraction from the ion exchange column, could be removed successfully by chromatography on Sephadex LH-60 [5,16].

2.3. *Separation and characterization of fragment peptides*

Amino acid analysis of the protein fragment

peptides was performed after hydrolysis of suitable aliquots of protein with 5.7 N HCl at 160°C for 30, 60, and 180 min (Durrum D-500, Palo Alto). Cyanogen bromide (CNBr)-cleavage was carried out with 200–500 nmol protein in 0.4–1.0 ml concentrated formic acid (Merck, Darmstadt), 1 M in CNBr (Serva, Heidelberg). The concentration of the formic acid was lowered to 80% by addition of 0.1–0.25 ml water. The reaction was allowed to proceed for 24 h at room temperature in the dark. After evaporation of the reaction mixture the fragment peptides were redissolved in 80% formic acid and size-fractionated on a column of Bio-Gel P-30 minus 400 mesh (Bio-Rad, Gauting/München) with 80% formic acid as eluant. Column effluents were continuously monitored at 280 nm and by differential refractometry (Waters R 401, Königstein). The eluted peptides were further characterized by end group determination with dansyl chloride [17].

2.4. *Immobilization of peptides to activated porous glass*

The homoserine peptides generated by CNBr-cleavage were lactonized by a 10 min treatment at 56°C with anhydrous trifluoro-acetic acid. After evaporation of the trifluoroacetic acid in a desiccator over KOH pellets, the corresponding homoserine lactone peptides were dissolved in 0.6 ml dimethylformamide, mixed with 200 mg of 3-aminopropyl glass and supplemented with 0.01 ml triethylamine. The mixture was kept overnight at 56°C [18]. The automated Edman degradation was performed with a home-built solid-phase sequencer. The degradation program, the processing of the released thiazolinones, is described in detail elsewhere [19]. The amino acid phenylthiohydantoins were identified by gas-chromatography (Shimadzu GC 6A FPD) and/or by multiple ion programmed mass spectrometry in electron impact mode (Finnigan 3200, München).

3. Results

Mutants of the *uncB* type (DG 7/10) are classified according to the following criteria: the mutation maps between asn and ilv in the uncA,B region of the *E. coli* chromosome [13]. The mutants contain an enzymatically active F_1 component, but no functional

Table 1
Amino acid composition of the DCCD-reactive proteins from wild type and mutant strains of *E. coli* and of the CNBr fragments B6

Amino acid	Molar ratios K12	DG 7/10	DC 1	B6 K12	B6 DC 1
Asp	5.0 (5)	4.1 (4)	5.1 (5)	(1)	1.3 (1)
Thr	1.1 (1)	1.1 (1)	1.1 (1)	(1)	1.0 (1)
Hse	– (0)	– (0)	– (0)	(1)	1.0 (1)
Glu	4.2 (4)	4.3 (4)	4.3 (4)	(3)	2.9 (3)
Pro	3.0 (3)	3.0 (3)	3.2 (3)	(2)	2.3 (2)
Gly	10.0 (10)	10.8 (11)	10.1 (10)	(7)	6.6 (7)
Ala	12.8 (13)	13.0 (13)	12.8 (13)	(6)	6.1 (6)
Val	6.1 (6)	6.1 (6)	6.9 (7)	(1)	1.8 (2)
Met	7.7 (8)	7.7 (8)	7.7 (8)	(0)	– (0)
Ile	7.9 (8)	7.9 (8)	6.9 (7)	(6)	4.5 (5)
Leu	12.1 (12)	12.2 (12)	12.1 (12)	(6)	6.0 (6)
Tyr	2.0 (2)	2.0 (2)	1.8 (2)	(0)	– (0)
Phe	3.9 (4)	3.9 (4)	4.0 (4)	(3)	2.8 (3)
Lys	1.0 (1)	1.0 (1)	1.0 (1)	(1)	0.9 (1)
Arg	2.1 (2)	2.1 (2)	2.0 (2)	(2)	2.1 (2)
Total residues	79	79	79	40	40

Serine, cysteine, histidine and tryptophan are missing. The integers in parentheses are deduced from sequence analysis. Changes in amino acid composition of the mutant peptides are underlined

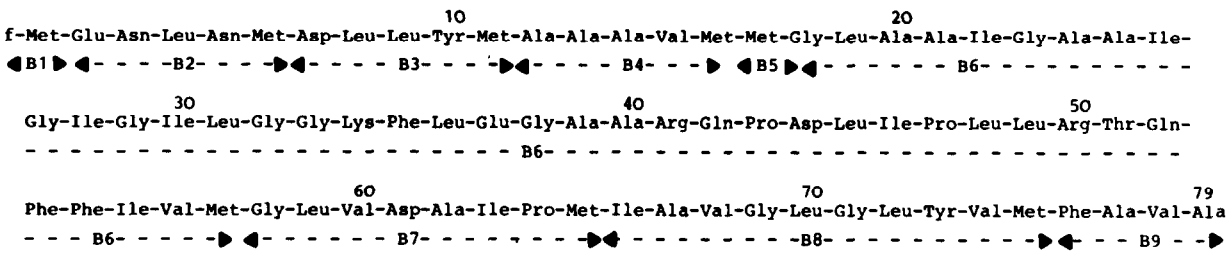

Fig.1. Amino acid sequence of the proteolipid from *E. coli* wild type K12. The arrows indicate the location of CNBr-peptides.

F_0. We have reported that in the case of the *uncB* mutant DG 7/1, the inability to bind DCCD is not due to the absence of the proteolipid in the F_0 part, but had to be explained by a modification of this protein [20]. The latter fact also holds for the mutant DG 7/10 [16]. The DCCD-resistant strain DC1 has a functional F_0 as well as F_1 part. However, the inhibition of the ATPase activity is impaired [14], indicating minor changes within the proteolipid.

The amino acid composition of wild type and mutant strains are given in table 1. Compared with the wild type, the proteolipid of mutant DG 7/10 contained one more glycine and one aspartic acid residue is missing. In the case of the mutant DC1 a valine residue seems to have replaced an isoleucine.

By CNBr-treatment the proteins were cleaved into various fragments. This is shown for the wild type in fig.1. The fragment B6, comprising the residues from

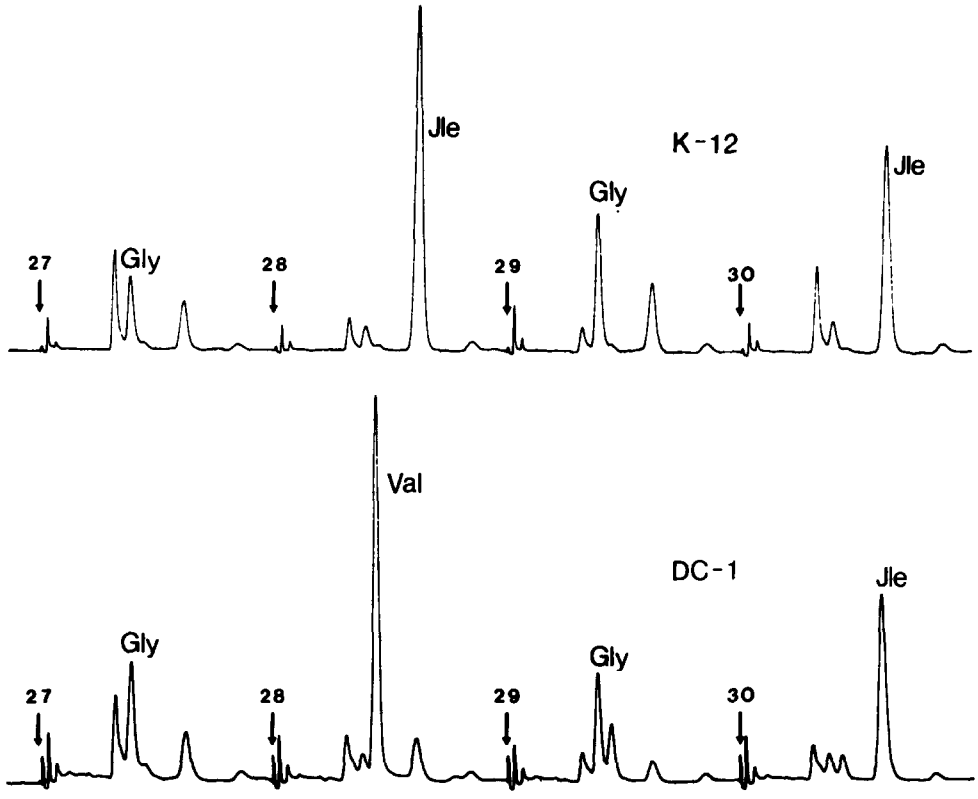

Fig.2. Gas-chromatograms of the phenylthiohydantoins derived from the Edman degradation of the CNBr-peptide B6 from wild type proteolipid (upper part) and the DC1 mutant proteolipid (lower part) (Shimadzu GC 6A FPD; flame photometric sulfur sensitive detector; isothermal run at 210°C; liquid phase: PTH-Chrom-S (Serva, Heidelberg)). Arrows indicate injection points.

Gly^{18}... Met^{57}, is by far the largest. Consequently, it could be separated easily from the other fragments by gel chromatography (data not shown). The N-terminal group and the amino acid composition of the fragments were determined systematically by the dansylation technique and amino acid analysis, respectively. Comparison of the amino acid composition of fragment B6 from the wild type with that of mutant DC1 revealed the isoleucine/valine exchange (table 1, last two columns). The isolated fragment B6 of mutant DC1 was immobilized and subsequently degraded. Fig.2 shows the significant part of the gas-chromatography (GC) traces of mutant and wild type degradation products. Although the quantities of phenylthiohydantoins obtained are slightly different for the two peptides, the marked difference in position 28 is obvious. All the other GC-traces of the various mutant peptides revealed no remarkable differences as compared with the wild type peptides.

The investigation of the CNBr-peptides of the proteolipid from DG 7/10 was focussed from the very beginning on fragment B7 (fig.1). The inability to bind DCCD, and the replacement of one aspartic acid residue by glycine strongly suggested an exchange at position 61. Similar as in the case of the wild type [21] the chromatography of the mutant fragments on Bio-Gel P-30 resulted only in a partial separation of the octapeptide B7 (Gly^{58}... Met^{65}) and the decapeptide B8 (Ile^{66}... Met^{75}) (data not shown). Nevertheless, our experience with the wild type protein [21] encouraged us to perform the Edman degradation with the peptide mixture, thus avoiding material-consuming and laborious purification steps. Fig.3 shows the significant parts of the GC-traces monitoring the degradation products starting with residue Gly^{58} until Ala^{62} for the wild type as well as the mutant peptide mixture. From the upper part of fig.3 (wild type) it can be deduced that the sequence Leu^{59}-Val-ASP-Ala^{62} of fragment B7 is accompanied by Ala^{67}-Val-GLY-Leu^{70} of fragment B8. By contrast,

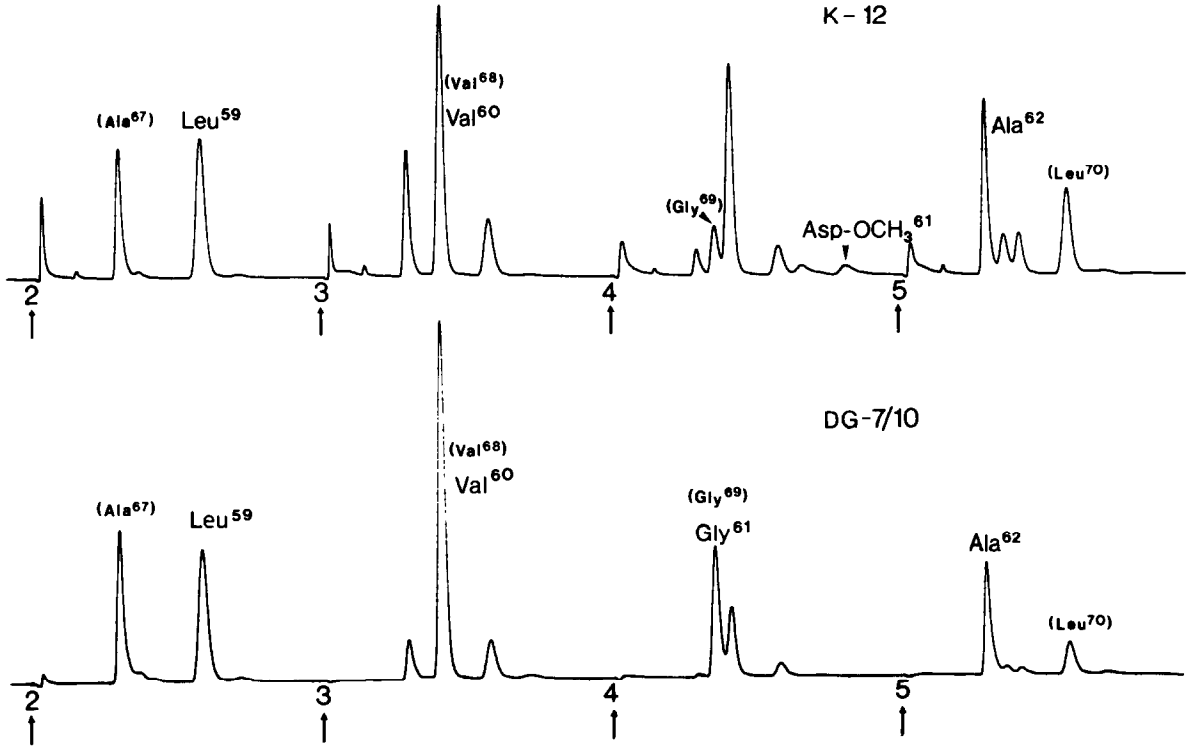

Fig.3. Gas-chromatograms of the phenylthiohydantoins derived from the Edman degradation of the CNBr-peptide B7 contaminated with B8 from wild type proteolipid (upper part) and of the DG 7/10 mutant proteolipid (lower part). For experimental conditions see fig.2 with the following exception: The support was coated with SP-400 for differentiation of Val- and Gly-phenylthiohydantoin. The residues in parenthesis are according to the contaminating peptide B8 (see fig.1). Arrows indicate injection points. The figures beneath the GC-traces correlate to Edman degradation steps.

the mutant sequence Leu[59]-Val-GLY-Ala[62] is accompanied by the identical sequence Ala[67]-Val-GLY-Leu[70] (fig.3, lower part). It may seem difficult to differentiate the Gly[61] of the mutant peptide from the contaminating sequence in which Gly[69] occurs at the same degradation step. However, the absence of an aspartic acid methylester phenylthiohydantoin as well as a quantitative determination of the glycine phenylthiohydantoin corresponding to position 61 firmly proves the altered sequence. All the other fragments had amino acid compositions and end groups, identical to that of the wild type.

A comparison of the CNBr-fragment patterns from the Bio-Gel P-30 of the mutant protein with that of the wild type revealed that, especially in the case of DG 7/10, small but reproducible differences in the cleavage yields were observed (data not shown). At the moment, it is rather speculative, to interpret such an observation in terms of changes in the protein confomation.

4. Discussion

The Asp/Gly-exchange in the proteolipid of the *uncB* mutant DG 7/10 was established to occur at the site which is exclusively responsible for the binding of the inhibitor DCCD in the wild type protein. This finding strongly supports the notion that the carboxyl group of the aspartyl residue at position 61 of the peptide chain is directly involved in the H^+-translocation mechanism. Further support for this view stems from sequence analyses of proteolipids derived from different sources [22]. With the exception of the *E. coli* proteolipid (Asp-Ala), this site is conserved by the sequence Glu-Ala in all organisms investigated so far. Recently, the proteolipid from a similar (or perhaps identical) *uncB* mutant (DG 7/1) of *E. coli* has been characterized. An identical amino acid replacement was found [23].

The Ile/Val replacement of the DC1 proteolipid was identified at position 28 (fig.1). Unlike in oligomycin-resistant mutants from *Neurospora crassa* and *Saccharomyces cerevisiae* [6] the replacement occurs at a location rather distant from the DCCD-reactive site, at least in terms of primary structure. Nevertheless, it is conceivable that in the native conformation this residue might be in close vicinity to the DCCD-reactive site. This view would point to a sterical influence between these sites giving rise to the impaired reactivity towards DCCD at the aspartyl residue. Finally, it is interesting to note that the replacement of isoleucine by a less bulky hydrophobic amino acid residue causes such a pronounced effect.

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