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Identification of Amino-Acid Substitutions in the Proteolipid Subunit of the ATP Synthase from Dicyclohexylcarbodiimide-Resistant Mutants of *Escherichia coli*

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The amino acid sequence of the proteolipid subunit of the ATP synthase was analyzed in six mutant strains from *Escherichia coli* K12, selected for their increased resistance towards the inhibitor *N,N'*-dicyclohexylcarbodiimide. All six inhibitor-resistant mutants were found to be altered at the same position of the proteolipid, namely at the isoleucine at residue 28. Two substitutions could be identified. In type I this residue was substituted by a valine resulting in a moderate decrease in sensitivity to dicyclohexylcarbodiimide. Type II contained a threonine residue at this position. Here a strong resistance was observed.

These two amino acid substitutions did not influence functional properties of the ATPase complex. ATPase as well as ATP-dependent proton-translocating activities of mutant membranes were indistinguishable from the wild type. At elevated concentrations, dicyclohexylcarbodiimide still bound specifically to the aspartic acid at residue 61 of the mutant proteolipid as in the wild type, and thereby inhibited the activity of the ATPase complex.

It is suggested that the residue 28 substituted in the resistant mutants interacts with dicyclohexylcarbodiimide during the reactions leading to the covalent attachment of the inhibitor to the aspartic acid at residue 61. This could indicate that these two residues are in close vicinity and would thus provide a first hint on the functional conformation of the proteolipid. Its polypeptide chain would have to fold back to bring together these two residues separated by a segment of 32 residues.

ATP synthases isolated from prokaryotic and eukaryotic organisms generally contain a proteolipid subunit constituting the major component of the ATP synthase membrane factor, F_0 [1–5]. This low-molecular-weight subunit is the target of the inhibitor dicyclohexylcarbodiimide which blocks the enzymatic activity of the whole complex by inhibition of the proton-translocating activity of the membrane factor, F_0 [6–9]. Proton-translocating properties have been attributed to this proteolipid subunit [10,11].

The amino acid sequence of the proteolipid from a variety of sources has been determined including mitochondria (*Neurospora crassa*, *Saccharomyces cerevisiae*, beef heart), chloroplasts (spinach), and bacteria (*Escherichia coli*, PS-3) [12–14]. A comparison of these evolutionary distant but homologous sequences allows the identification of typical features as well as of invariant, i.e. functionally important, residues of this ATPase subunit.

An acidic position which occurs in the middle of a hydrophobic segment appears to be of special importance. It is the only acidic residue which is conserved in all organisms. Furthermore, this group is specifically and covalently modified by the inhibitor dicyclohexylcarbodiimide [15]. Recently, an ATPase mutant from *E. coli* has been described whose membrane factor is unable to translocate protons. Here this acidic residue is exchanged for a glycine [16].

Mutants containing a dicyclohexylcarbodiimide-resistant ATP synthase have been isolated in *E. coli* [17,18]. By genetic analysis the resistance mutation was shown to be located in the *unc*-gene region. At concentrations effective in the wild type, the carbodiimide does not inhibit the mutant enzyme, and does not bind covalently to the proteolipid subunit. The present analysis of six inhibitor-resistant strains showed

Abbreviations. $(cHxN)_2C$, *N,N*-dicyclohexylcarbodiimide; Mops, 4-morpholinepropanesulfonic acid.

Enzyme. ATPase or ATP phosphohydrolase or ATP synthase (EC 3.6.1.3).

single amino acid substitutions in the proteolipid. The carbodiimide-reactive aspartyl residue of this protein, however, was found to be unaffected. It is proposed that the residue of the proteolipid altered in the resistant strains is involved in the noncovalent binding of dicyclohexylcarbodiimide which has to proceed the covalent reaction of the carbodiimide with the aspartyl residue. Other explanations appear to be unlikely since the amino acid substitutions are inconspicuous (isoleucine/valine; isoleucine/threonine) and since functional properties of the mutant ATP synthase are undisturbed according to several criteria.

MATERIALS AND METHODS

Carboxymethylcellulose, CM-52, was purchased from Whatman. Bio-Gel P-30, minus-400 mesh (control number 106044), was obtained from BioRad Laboratories. 3-Aminopropyl-glass and *β-N*-aminoethyl(3-aminopropyl)-glass were prepared as described [19]. [^{14}C]Dicyclohexylcarbodiimide was synthesized from [^{14}C]urea (Amersham Buchler) (55 Ci/mol) via dicyclohexylurea [20,21]. Pyridine, *N*-methylmorpholine, *N,N'*-dimethylformamide, and triethylamine were distilled over ninhydrin. Trifluoroacetic acid was distilled over 96% sulfuric acid, and then mixed with 0.1% (v/v) water. 8-Amino-6-chloro-2-methoxyacridine was a gift from Dr P. Overath (MPI for Biology, Tübingen). All other chemicals were of highest available purity.

Bacterial Strains

Strain A (*lacI*, *fadR*, *but12*, *rha*) is derived from *Escherichia coli* K12 Y_{mel} (λ) F^-. Mutants with an increased resistance towards dicyclohexylcarbodiimide were isolated as described [18]. The inhibitor properties of the dicyclohexylcarbodiimide on the ATPase activity of the resulting strains were tested. Mutants with an increased resistance were subjected to P_1 transduction into strain DG 20/1 (*uncA*$^-$, *ilv*$^-$, *met*$^-$). Transduction was performed according to Lennox [22]. Five strains with an altered resistance were obtained: DC13, DC19, DC24, DC25, DC54.

Growth of Cells

Cells were grown in CR medium with 1% glucose as the carbon source. For the mutants 0.1 mg/ml methionine was included. Cells were cultivated in 500-l fermenters until late log phase. Typically 5 kg cells (wet weight) were obtained.

Preparation of the Proteolipid Subunit

The cells were incubated with lysozyme (1 mg/g wet weight) and DNase (20 μg/g wet weight) for 3 h at 30 °C. Then a crude membrane fraction was sedimented at 8000 × *g* for 15 min. The ATPase proteolipid was isolated by extraction of the crude membrane fraction with chloroform/methanol (2:1, v/v) [8] and subsequent chromatography on carboxymethylcellulose in mixtures of chloroform/methanol/water. The pure *E. coli* proteolipid was eluted from the column during the wash with chloroform/methanol/water (5:5:1, v/v/v) [16,23]. The purity of the isolated proteins was checked by sodium dodecylsulfate electrophoresis [14] and Bio-Gel P-30 chromatography in the presence of 80% formic acid. Lipid content as determined by the measurement of organic phosphorous content [24] is less than 1 μg/mg protein. Typical yields are 2–3 mg of pure protein from 100 g of cells (wet weight).

Labelling with Dicyclohexylcarbodiimide

2 g (wet weight) fresh cells (log phase) were suspended in 10 ml buffer (50 mM Mops pH 7.0, 10 mM $MgCl_2$, 175 mM KCl, 0.2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 20 mM benzamidine). The cells were broken by a passage through a Ribipress at 18000 lb × in^{-2} (124 MPa) at 10–15 °C. After reducing the viscosity by incubation with DNase and removal of the cell debris by centrifugation at 20000 × *g* for 20 min, membranes were sedimented for 1 h at 235000 × *g*. The membrane pellet was suspended with buffer (50 mM Tris pH 8.0, 10 mM $MgCl_2$, 175 mM KCl, 0.2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 20 mM benzamidine) at a protein concentration of about 10 mg/ml. For reaction with dicyclohexylcarbodiimide the protein concentration was adjusted to 1 mg/ml with the same buffer. Dicyclohexylcarbodiimide was added in 2 μl ethanol. Incubation was carried out overnight at 0 °C. Labelling with [^{14}C]dicyclohexylcarbodiimide was performed at 5 mg protein per ml under the same conditions. Aliquots of 30 μl were used for measurement of the ATPase activity [18] and the energy-dependent quenching of 9-amino-6-chloro-2-methoxyacridine fluorescence [1, 18]. One unit of ATPase activity is defined as the amount of enzyme which catalyzed the hydrolysis of 1 μmol of ATP per min at 30 °C and pH 8.

The initial rate of fluorescence quenching was extrapolated to 1 min and expressed as units of fluorescence-quenching activity. 1 unit of fluorescence-quenching activity is defined as the amount of membrane which quenches the fluorescence of the acridine dye with an initial rate of 100% in 1 min [1].

Analytical Procedures

Protein concentration was determined by the method of Lowry [25] using bovine serum albumin as the standard. Since the method of Lowry leads to an

underestimation of the protein content [8], in several cases amino acid analysis was used instead. ^{14}C-radioactivities were measured in a Packard PRIAS liquid scintillation spectrometer. All samples were first dissolved in 0.5 ml buffer (1% sodium dodecylsulfate, 0.1 M Tris acetate pH 8.0) and then 3 ml of Emulsifier (Packard) were added.

Chemical Cleavage and Fractionation of Peptides

Cleavage with CNBr and subsequent chromatography on Bio-Gel P-30 was performed as described [14].

N-terminal amino acid residues were determined by the dansylation method [26]. The N-terminal formyl group [27] was removed by a 4-h incubation in 0.5 M HCl dissolved in dry methanol at room temperature.

Amino acid analysis was performed with an automated amino acid analyzer (Biotronic LC 2000). Methionine was determined as methionine sulfone after performic acid oxidation [28].

Thin-layer chromatography of the peptides was performed as described [16].

Automated Solid-Phase Edman Degradation

The whole proteolipid was immobilized by coupling its carboxyl group(s) to 3-aminopropyl-glass. The proteins (2–4 mg) dissolved in 2–5 ml chloroform/methanol/water (5:5:1) were shaken with 175 mg aminated glass (equilibrated with 1 M pyridine/HCl pH 5.5) in the presence of 8 mg dicyclohexylcarbodiimide and 4 mg *N*-hydroxysuccinimide. Coupling yields were nearly quantitative. The large cyanogenbromide fragments B6 were attached to *β*-*N*-aminoethyl(3-aminopropyl)-glas using the homoserine lactone procedure [29]. As these fragments are insoluble in pure *N*,*N*′-dimethylformamide they were first dissolved in 10 µl trifluoroacetic acid. 500 µl *N*,*N*′-dimethylformamide were then added yielding a clear solution. 170 mg aminated glass and 100 µl triethylamine were then added. The mixture was incubated for 6 h at 65 °C. The loaded glass beads were washed twice with 1 ml trifluoroacetic acid, then with methanol and diethylether and dried. Coupling yield ranged from 20 to 40%. Sequencing and analysis of the phenylthiohydantoin were performed as described [14,16]. The initial yield of the phenylthiohydantoin cleaved off was about 40% of the amount attached to the glass beads.

RESULTS

Sensitivity of the Mutant ATP Synthase towards Dicyclohexylcarbodiimide

Fig. 1 A shows the inhibition of the membrane-bound ATPase activity at various inhibitor concentrations. For the wild type half-maximal inhibition was found at 3–5 nmol inhibitor/mg protein, and complete inactivation occurred at amounts higher than 20 nmol/mg protein. With the mutant, two classes can be distinguished. Class one, consisting of mutants DC13, DC19, DC24, shows 50% inhibition at 30 nmol inhibitor/mg protein. The ATPase activity is maximally inhibited at concentrations higher than 160 nmol/mg protein. Class-two mutants (DC25, DC54) exhibit higher resistance. Half-maximal inhibition is achieved at 200 nmol inhibitor/mg protein.

A more specific test for the functional ATP synthase complex is provided by the measurement of the ATP-dependent proton translocation. Fig. 1 B shows the inhibition of the initial rate of ATP-dependent acridine-dye fluorescence quenching in inside-out membrane vesicles. It has been shown that the initial

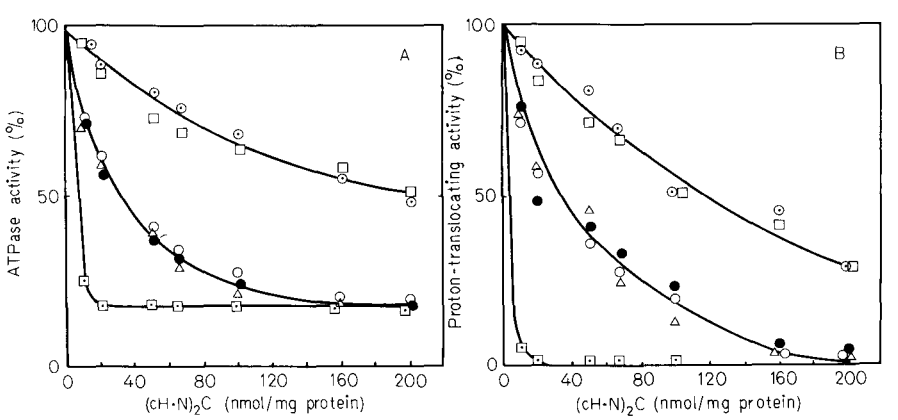

Fig. 1. *Inhibition of the ATPase activity and the energy-dependent quenching of the acridine-dye fluorescence.* Wild-type and mutant membrane preparations were incubated with the indicated amount of dicyclohexylcarbodiimide (see Materials and Methods). 30 µl of the incubation mixture were used for the determination of the following. (A) Inhibition of the ATPase activity; (⊡) wild type; (△) DC13; (○) DC19; (●) DC24; (⊡) DC25; (⊙) DC54. (B) Inhibition of the energy-dependent quenching of the fluorescence of 9-amino-6-chloro-2-methoxyacridine. The reaction mixture (1 ml total volume) contained 2 µM 9-amino-6-chloro-2-methoxyacridine, 1 µM valinomycin, 25 mM Mops, 0.3 M KCl, 10 mM $MgCl_2$ pH 7.3. The reaction was started by the addition of 10 µl 0.2 M ATP solution

rate is proportional to rate of proton translocation [30]. Again the high and the low resistance class of mutants can be distinguished by their response to increasing inhibitor concentrations. The values required for 50% inhibition are comparable to that obtained by the ATPase activity measurements. The ATP-dependent proton translocation, however, could be inhibited completely in the case of the wild type and the class-one mutant membrane.

Unaltered Functional Properties of the ATP Synthase in the Mutant Strains

ATPase activities for the wild-type ATPase and mutant ATPase ranged from 1.7 to 2.0 U/mg. As a second criterion for the functional integrity of ATP synthase of the mutants, ATP-dependent H^+ translocation was determined by the initial rates of energy-dependent acridine-dye fluorescence quenching. Values ranged from 14 to 18 fluorescence-quenching units/mg [1]. Again no detectable differences between wild type and mutants could be found.

According to these two sets of experiments the function of the mutant ATP synthases is not apparently influenced by the mutation leading to an increased resistance towards dicyclohexylcarbodiimide.

Binding of [^{14}C]Dicyclohexylcarbodiimide to Mutant Proteolipid

As demonstrated in the first paragraph, dicyclohexylcarbodiimide at higher concentrations still inhibits ATP synthase activity of the mutant strains. Considering the unspecific binding of the inhibitor to many polypeptides in the *E. coli* membrane [8], it was important to examine whether or not in the mutant membranes the specificity of binding is retained at the level of the ATP synthase and the proteolipid subunit at increased inhibitor concentrations. Conditions under which the mutants' ATP synthase is maximally inhibited would require very high concentrations of radioactive dicyclohexylcarbodiimide. Therefore, the analysis was restricted to lower concentrations. Table 1 shows the reactivity of mutant proteolipid representative for class one and two at 5 nmol ^{14}C-labelled inhibitor/mg membrane protein, which concentration is highly effective for the wild type. In addition, the effect of a fourfold higher amount of [^{14}C](cHxN)$_2$C was investigated for the mutant membranes. With the wild type, at 85% inhibition of proton translocating activity, 0.2 mol of [^{14}C]-(cHxN)$_2$C were bound/mol of the isolated proteolipid. This value correlates with earlier reports that at maximal inhibition of the ATPase activity one third of the proteolipid oligomer is modified by the inhibitor [8]. At an inhibitor concentration of 5 nmol/mg of protein, the mutant proteins only bound 0.07 and 0.05 mol/mol, respectively, resulting in a 34% and 10% inhibition. The amount of proteolipid-bound inhibitor is roughly proportional to the inhibition of the proton-translocating activity. At the 4.4-fold higher concentration of inhibitor during incubation a slight increase in proteolipid-bound [^{14}C](cHxN)$_2$C was obtained as expected from the dose-inhibition curves shown in Fig. 1 B. These results are in agreement with the idea that also in the inhibitor-resistant mutants the (cHxN)$_2$C is still bound to the proteolipid albeit to a much lower extent. This low residual binding correlates with the low inhibition.

Table 1. *Reactivity of the mutant protein towards dicyclohexylcarbodiimide*
Membranes (30 mg protein) were labelled with [^{14}C]dicyclohexylcarbodiimide. The dicyclohexylcarbodiimide-binding proteins were purified in microscale as described under Materials and Methods. The protein content is based on amino acid analysis. The values in parenthesis represent the percentage of the inhibition of H^+ translocation activities. n.d., not determined

Strains	Proteolipid-bound [^{14}C](cHxN)$_2$C at	
	5 nmol/kg	22 nmol/mg
	mol/mol	
A1 (wild type)	0.2 (85)	n.d.
DC13 (class one)	0.07 (34)	0.11
DC25 (class two)	0.05 (10)	0.078

The specificity of the [^{14}C](cHxN)$_2$C binding in mutant proteolipid was examined by sequence analysis. Earlier studies have shown that in the wild type the bound carbodiimide radioactivity is recovered exclusively with the aspartic acid at position 61 of the polypeptide chain [13]. After CNBr cleavage at the eight methionine residues of the proteolipid, this carbodiimide-reactive aspartyl residue occurs at position 4 of cyanogen bromide fragment B7 (residues 58–65) (see Fig. 4). [^{14}C](cHxN)$_2$C-labelled proteolipid from mutant DC13 (class one) and DC25 (class two) were cleaved with CNBr, and the resulting fragments separated on a Bio-Gel P-30 column as shown in Fig. 2 B for mutant DC13. As a control, labelled wild-type proteolipid was analysed in parallel (Fig. 2A). Radioactivity was eluted in three peaks, occurring in the same fractions and the same proportions with both proteolipids. The first peak corresponds to uncleaved proteolipid and the second to the partial fragment of B7 plus B8 (E. Wachter, unpublished results). The third peak contains about 80% of the total ^{14}C-radioactivity, and is eluted together with a mixture of peptides B7 and B8 (see also Fig. 3). During sequence analysis of this fraction (insert of Fig. 2 A, B) the [^{14}C](cHxN)$_2$C radioactivity is recovered at position 4 in both the mutant DC13 and

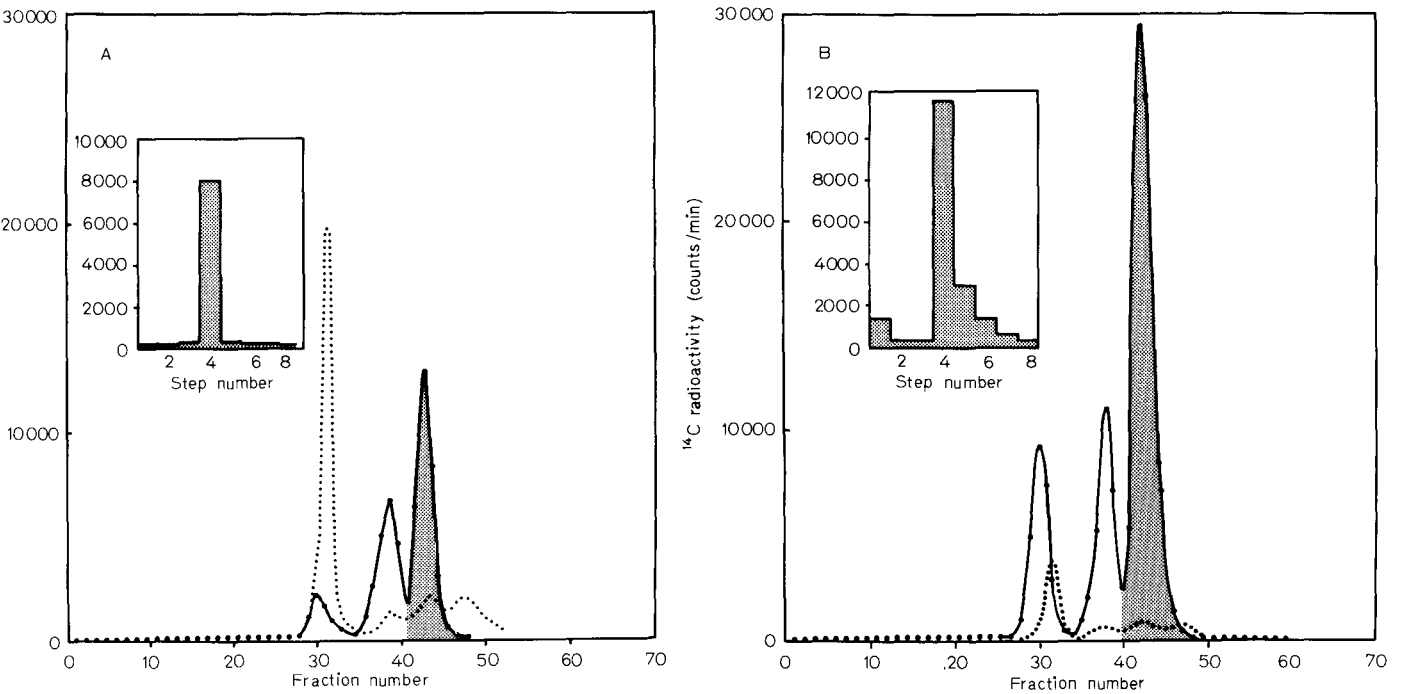

Fig. 2. *P30 gel chromatography of cyanogen-bromide fragments of the dicyclohexylcarbodiimide-binding proteins and determination of the residue.* Membranes were labelled with [^{14}C]dicyclohexylcarbodiimide at a concentration of 22 nmol/mg of membrane protein. The isolated proteolipid was cleaved with CNBr. Fractions obtained after P30 gel chromatography (0.4 × 150 cm) were analyzed for ^{14}C-radioactivity. Fractions of 150 µl were collected. (A) Wild type (0.2 mg); (·····) refractive index; (●——●) ^{14}C-radioactivity. (B) Mutant DC13 (0.05 mg). Insert: for determination of the [^{14}C]dicyclohexylcarbodiimide-binding residue fractions containing the peptide B7 (shaded peak) were collected. The peptides were coupled by the homoserine-lactone method to *β-N*-aminoethyl(3-aminopropyl)-glass and subjected to automatic Edman degradation. Aliquots of the released phenylthiohydantoin derivatives were analyzed for ^{14}C-radioactivity

the wild type. Comparable results were obtained with class-two mutant DC25. Thus, the specificity of dicyclohexylcarbodiimide-binding is retained in the proteolipid from inhibitor-resistant mutants.

Identification of Amino Acid Substitutions in the Mutant Proteolipids

The ATP synthase proteolipid was purified from the $(cHxN)_2C$-resistant strains described in the previous paragraphs as well as from mutant DC1 partially characterized in an earlier publication [18].

Amino acid analysis of the whole proteins is shown in Table 2. Clearly one extra threonine is recognized in mutants DC25 and DC54 and the number of isoleucines is reduced by about 0.7 units. The class one mutants show an increased amount of valine (DC1, DC13, DC19, DC24) and a reduced amount of isoleucine. For further analysis the proteolipids (4 mg Lowry protein) were cleaved with cyanogen bromide. The resulting peptides could be partially separated by chromatography on Bio-Gel P-30 in 80% formic acid. Fig. 3 shows a typical elution pattern obtained for all the proteins used in these experiments (wild type and mutants). The fragments are numbered according to their occurrence in the sequence (see Fig. 4). The large fragment B6 could be obtained in pure form, while peptides B7 and B8 as well as peptides B2, B3, B4 and B9 are eluted together. These peptides could be finally purified by preparative thin-layer chromatography [16]. The individual mutant peptides and the corresponding wild type fragments exhibited the same R_F-values. This is in contrast to the recently described protein of the *uncB*-type mutant DG7/1, where an alteration in peptide B7 could be detected by thin-layer chromatography [16]. Amino acid analysis of all the peptides was performed. No changes in amino acid content could be detected in the peptides B2, B3, B4, B7, B8 and B9 (data not shown). As these peptides are small, an amino acid exchange in these peptides should be detected by amino acid analysis. The amino acid analysis of the large fragment B6 is shown in Table 3. The data clearly show that peptides B6 from the mutants DC1, DC13, DC19, DC24 contain an extra valine residue while one isoleucine is missing. The analysis of the B6 fragment from the mutant B25 and DC54 suggest an isoleucine-threonine exchange. It should be noted that both exchanges can originate from a point mutation. The exchanged amino acid was localized by sequenching the whole protein and/or the peptide B6. In all six mutants analysed, the amino acid exchange was found to be located at posi-

Table 2. *Amino acid analysis of whole mutant and wild type proteolipid*
The values in parenthesis represent the number of amino acid residues obtained by sequence analysis. Hydrolysis was carried out for 24, 48, 72 h. Methioninesulfone was determined after 24 h hydrolysis

Amino acid	Amount in strain						
	A1	DC1	DC13	DC19	DC24	DC25	DC54
	mol/mol						
Aspartic acid	5.15 (5)	4.83	5.02	5.1	5.06	5.21	5.19
Methionine sulfone	7.7 (8)	7.01	7.4	7.84	7.5	7.02	7.46
Threonine	1.18 (1)	0.97	1.30	1.0	1.04	**2.05**	**2.28**
Glutamic acid	4.13 (4)	4.26	4.23	4.4	4.3	4.1	4.1
Proline	2.78 (3)	2.8	2.6	2.9	2.7	2.7	2.8
Glycine	9.0 (10)	9.6	9.33	9.6	9.7	9.57	9.44
Alanine	12.8 (13)	12.8	12.8	12.8	12.8	12.8	12.8
Valine	5.4 (6)	**5.6**	**6.2**	**6.0**	**6.0**	5.4	5.65
Isoleucine	6.8 (8)	**6.34**	**5.79**	**6.2**	**6.2**	**6.11**	**6.38**
Leucine	11.8 (12)	11.2	11.7	11.9	11.9	11.95	11.8
Tyrosine	1.69 (2)	1.76	1.83	1.9	1.9	1.94	1.71
Phenylalanine	3.76 (4)	3.8	3.84	3.80	3.9	3.85	3.78
Lysine	1.14 (1)	1.08	1.04	1.10	1.1	1.06	1.13
Arginine	2.16 (2)	1.9	1.88	1.96	1.99	1.88	1.96

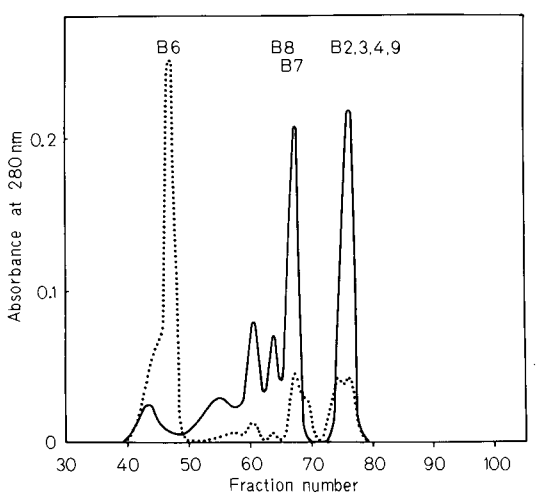

Fig. 3. *P30 gel chromatography of the cyanogen-bromide fragments of the dicyclohexylcarbodiimide-binding protein from the mutant DC13.* 5 mg of protein were cleaved with CNBr. Peptides were separated on a column 0.8 × 150 cm with P30 (minus-400 mesh) in 80% formic acid at a flow rate of 1.5 ml/h. Fractions of 750 µl were collected. (——) absorbance at 280 nm. (·····) Refractive index

tion 28 of the whole protein. The isoleucine residue located at this position in the wild type is exchanged by a valine residue in all class-one mutants. A threonine residue at this position is found in proteolipids from class two. Substitution by valine leads to a moderate resistance towards modification by dicyclohexylcarbodiimide, while a threonine results in a strong resistance. Interestingly, among the six mutants investigated in these studies only these two types of mutants could be detected. It can be stated that amino acid 28 is the only exchanged residue, since no other alterations were detected during sequence analysis of the whole protein which covered about two thirds of the entire sequence. No alteration in the remaining small C-terminal peptides was found by thin-layer chromatography and amino acid analysis.

DISCUSSION

In the present studies amino acid exchanges have been identified in the proteolipid subunit of the ATP synthase from *Escherichia coli* showing increased resistance towards the inhibitor dicyclohexylcarbodiimide. Out of about 20 strains selected for inhibitor-resistant growth [18], six mutant strains showed inhibitor-resistant ATPase activity *in vitro*. All six mutants were found to be altered in the ATP synthase proteolipid subunit. Only two different mutant proteolipids were detected, one containing an isoleucine-valine exchange and the other an isoleucine-threonine exchange. Interestingly, in both cases the same isoleucine at position 28 of the amino acid sequence is substituted.

Although only six mutants have been analysed the finding of just two different amino acid substitutions is unexpected, especially since only one position is affected, and since a point mutation in the isoleucine codons could lead to a variety of amino acid substitutions in addition to the observed two exchanges. This suggest that besides the covalently modified aspartic acid 61 only the observed alterations influence the binding of the inhibitor. It is more reasonable to assume, however, that alterations of other residues

Table 3. *Amino acid analysis of the large cyanogen-bromide fragment B-6*
Hydrolysis was carried out for 24 and 72 h

Amino acid	Amount in strain						
	A1	DC1	DC13	DC19	DC24	DC25	DC54
	mol/mol						
Aspartic acid	1.0 (1)	1.05	1.0	1.05	0.98	1.01	1.05
Threonine	0.88 (1)	0.93	0.88	0.98	0.88	**1.83**	**1.75**
Homoserine	0.90 (1)	0.99	0.91	0.98	0.96	1.0	0.88
Glutamic acid	3.33 (3)	3.4	3.42	3.45	3.3	3.4	3.44
Proline	1.78 (2)	1.76	1.8	1.8	1.70	1.9	2.01
Glycine	6.98 (7)	6.75	6.8	7.02	6.8	7.0	6.85
Alanine	6.02 (6)	6.02	6.02	6.02	6.02	6.02	6.02
Valine	0.87 (1)	**1.6**	**1.66**	**1.8**	**1.76**	0.84	0.73
Isoleucine	5.74 (6)	**4.4**	**4.65**	**4.6**	**4.6**	**4.65**	**4.1**
Leucine	6.08 (6)	5.86	6.04	6.14	5.9	5.9	6.03
Phenylalanine	2.88 (3)	2.83	2.88	2.90	2.96	2.86	2.83
Lysine	1.21 (1)	1.06	1.2	1.09	0.87	1.3	1.22
Arginine	1.88 (2)	1.93	1.9	1.93	1.84	1.88	1.9

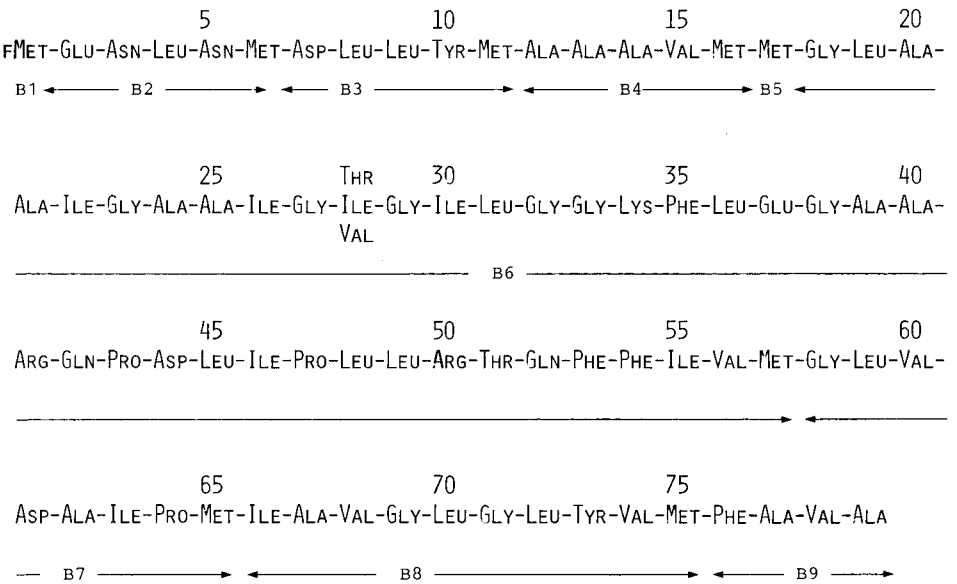

Fig. 4. *Amino acid sequence of the dicyclohexylcarbodiimide-binding protein from wild type and mutants.* B1 to B9 indicate the fragments produced by cyanogen bromide

possibly involved in $(cHxN)_2C$-binding lead to defects in the assembly or function of the proteolipid. It may be relevant in this respect that the isoleucine 28 occurs in a glycine-rich segment (see Fig. 4) of the proteolipid sequence which appears to be highly conserved when the proteolipid species from phylogenetically distant organisms are compared [12, 13]. In the mitochondrial proteolipid species also an isoleucine is present at the corresponding position. While the $(cHxN)_2C$-sensitivity of the *Neurospora* ATPase is comparable to that of *E. coli*, the beef heart enzyme is much more sensitive [4]. On the other hand the ATPase from thermophilic bacterium PS-3 [14] exhibiting a sensitivity comparable to *E. coli* contains an asparagine at the corresponding position of the proteolipid subunit. Hypersensitivity to $(cHxN)_2C$ was observed in three types of oligomycin-resistant mutants from *Neurospora* (Sebald, W., unpublised observations), containing amino acid substitutions in the ATPase proteolipid in close vicinity to the $(cHxN)_2C$-binding glutamyl residue. Thus, the isoleucine 28 is apparently only one of several residues determining the sensitivity towards the inhibitor.

The two amino acid exchanges observed in the $(cHxN)_2C$-resistant *E. coli* mutants are inconspicuous. The amino acids isoleucine and valine have a similarly low polarity, whereas threonine has a polar uncharged side chain. This may be correlated with the observation

that the valine-containing mutants exhibit moderate resistance and the threonine-containing mutants high resistance towards $(cHxN)_2C$. The side chains of both the valine and the threonine are slightly smaller than that of the isoleucine. A larger hydrophobic surface possibly supports the binding of the cyclohexyl group of the inhibitor.

Remarkable, the residue 28 altered in the mutants is located in the primary structure 33 residues away from aspartic acid at residue 61, to which dicyclohexylcarbodiimide binds covalently, most likely by forming the stable *N*-acylurea [15]. Nevertheless, the changed residues found at position 28 specifically reduce the binding affinity of the proteolipid for the inhibitor. At increased concentration the inhibitor still binds to aspartic acid 61, and thereby inhibits ATP synthase activities. Functional properties of the ATP synthase appear to be unchanged in the mutants as deduced from the unaltered levels of ATPase activity and ATP-dependent H^+-translocation. Considering further the conservative amino acid substitutions in the class-one mutants, an allosteric effect on the binding of $(cHxN)_2C$ appears unlikely. Thus, it is proposed that the isoleucine residue 28 forms part of the binding site for the inhibitor. This provides a first, but admittedly indirect, indication, that the polypeptide chain folds back to juxtapose isoleucine 28 and aspartic acid 61 in the functional conformation of the proteolipid. The situation may be more complicated, however, since the proteolipid occurs in the ATP synthase as oligomer [1,4,8], and the binding site for $(cHxN)_2C$ may be provided by different monomers. It will be interesting to see in future experiments whether isolated monomeric mutant proteolipids retain their decreased affinity for the inhibitor.

The amino acid sequences of the dicyclohexylcarbodiimide-binding proteolipid from all organisms analysed up to now show a clustering of hydrophobic and hydrophilic amino acid residues [12–14]. Generally, hydrophobic segments of about 25 residues are interrupted by a polar segment of 12 to 20 residues. This is reminiscent to bacteriorhodopsin from *Halobacterium halobium* [31–33]. The hydrophobic segments may traverse the membrane in an α-helical conformation, whereas the polar loop may be either exposed to the water phase or may be in contact with other subunits of the ATP synthase complex. The above proposal that isoleucine 28 and aspartic acid 61 are in close proximity would fit excellently to such a 'hair pin' model.

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