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Amino Acid Sequence of the Proteolipid Subunit of the Proton-Translocating ATPase Complex from the Thermophilic Bacterium PS-3

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The proteolipid subunit of the ATPase complex was identified in whole membranes of the thermophilic bacterium PS-3 by means of a covalent modification with the ^{14}C-labelled inhibitor dicyclohexylcarbodiimide. The proteolipid could be purified from the membrane in free and carbodiimide-modified form by extraction with chloroform/methanol and subsequent carboxymethylcellulose chromatography in mixtures of chloroform/methanol/water.

The complete amino acid sequence of the 72-residue polypeptide could be determined by automated solid-phase Edman degradation of the whole protein, and of fragments obtained after cleavage with cyanogen bromide and *N*-bromosuccinimide. Chemical cleavages and separations of the resulting fragments by gel chromatography were performed in 80% formic acid.

The amino acid sequence shows a concentration of hydrophobic amino acids in two segments of about 25 residues at the amino-terminal and carboxy-terminal ends. The polar residues are clustered in the middle of the polypeptide chain. The bound [^{14}C]dicyclohexylcarbodiimide label is recovered exclusively at position 56, which is occupied by a glutamyl residue. The proteolipid from PS-3 exhibits homology to the corresponding ATPase subunit from mitochondria. The carbodiimide-reactive glutamyl residue occurs at the position as in the mitochondrial proteins.

A reversible ATP-driven proton pump has been identified in the energy-transducing membranes of mitochondria, chloroplasts and bacteria (for review see [1–3]). With respect to the proton-translocating properties of this ATPase complex, interest has focussed on a proteolipid constituting the major component of the ATPase membrane factor, F_0. This low-molecular weight subunit is the target of the inhibitor dicyclohexylcarbodiimide [4–7], which blocks the enzymatic activities of the whole complex [5,7–9] as well as the proton-translocation performed by the membrane factor, F_0 [10–12]. Furthermore, the isolated proteolipid subunit has been reported [6,13] to catalyze an inhibitor-sensitive proton-translocation when reinserted into artificial lipid membranes.

The ATPase proteolipid has been isolated from various mitochondria [7,14], chloroplasts [6] and bacteria [5,12]. At present their amino acid sequences are being studied in order to elucidate the typical features of this subunit as well as the evolutionary invariant amino acid residues [15,16]. The results of those protein-chemical studies are expected to give a first hint on the mechanism of proton-translocation.

The ATPase complex from the thermophilic bacterium PS-3 has been thoroughly studied by Kagawa's group [1,17]. A proton-conducting membrane factor, TF_0, has been highly purified consisting of only two subuints: a polypeptide of M_r 13000 probably involved in the binding of factor F_1 and the proteolipid [11]. The inhibition of proton-translocation of factor F_0 by chemical modification of specific amino acid residues has been studied [18]. The proteolipid has been purified from factor TF_0 [12].

The present communication describes the isolation of the ATPase proteolipid from whole membranes of bacterium PS-3. Bound [^{14}C]dicyclohexylcarbodiimide served as marker during the purification. The amino acid sequence was determined, and the carbodiimide-binding residue identified. Some of these results already have been published in preliminary form [16].

Enzyme. ATPase or ATP phosphohydrolyse (EC 3.6.1.3).

MATERIALS AND METHODS

Materials

A stock of the thermophilic bacterium PS-3 was kindly provided by Dr Y. Kagawa. [^{14}C]Dicyclohexylcarbodiimide was synthesized from [^{14}C]urea (Amersham Buchler) (55 Ci/mol) via dicyclohexylurea [19, 20]. 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 was prepared as described [21]. All the other chemicals were of the highest available purity. Pyridine, *N*-methylmorpholine and triethylamine were distilled over ninhydrin. Trifluoroactic acid was distilled over 96% sulfuric acid, and then mixed with 0.1% (v/v) water.

Preparation of the Dicyclohexylcarbodiimide-Binding Protein

The bacterium PS-3 was cultured with vigorous aeration at pH 7 and 70 °C in a medium containing 0.4% yeast extract, 0.3% NaCl and 0.8% peptone from soybean flour (Merck) [22]. 1.5 kg of cells (wet weight) were obtained from a 500-l culture grown into late log phase. A membrane fraction was prepared after lysozyme digestion of the cells as described [22], and stored at −20 °C. The ATPase proteolipid was extracted from whole membranes with chloroform/methanol (2/1; v/v) [5] and further purified by carboxymethylcellulose chromatography [14] according to described procedures. Minor modifications are detailed under Results.

Labelling with [^{14}C]Dicyclohexylcarbodiimide

Membranes were suspended in 50 mM Tris-acetate, pH 7.5, at a protein concentration of 5 mg/ml, and incubated with [^{14}C]dicyclohexylcarbodiimide for 18 h at 0 °C. For determination of bound [^{14}C]dicyclohexylcarbodiimide, an aliquot of 0.1 ml was diluted 10-fold with 50 mM Tris-acetate, pH 7.5. The membranes were sedimented at 30000 × *g* for 5 min, and then washed six times with chloroform/methanol/ether (2/1/12). The dried protein was dissolved in 2% sodium dodecylsulfate. Aliquots were used for determination of protein and radioactivity. Prior to electrophoresis, mercaptoethanol and Tris-base were added to a final concentration of 5% and 20 mM, respectively.

Analytical Procedures

ATPase activity was determined at 56 °C and pH 8.5 with 10 mM ATP as substrate [7]. Protein was determined by the Lowry method [23] using bovine serum albumin as standard. ^{14}C radioactivity was measured in a Packard PRIAS liquid scintillation spectrometer. All samples were first dissolved in 0.5 ml of a medium containing 1% sodium dodecylsulfate, 0.1 M Tris-acetate pH 8, and then 3 ml of Emulsifier (Packard) were added. Dodecylsulfate-gel electrophoresis was performed in vertical slab gels using a continuous buffer system (0.5% dodecylsulfate, 0.1 M Tris-acetate pH 8). The gel was polymerized from 15% acrylamide and 1.25% diallytartardiamide in the presence of buffer and 48% urea. Fluorography of the stained and dried gels was done according to Bonner and Laskey [24].

Chemical Cleavage and Fractionation of Peptides

4 mg of the ether-precipitated proteolipid were dissolved in 0.8 ml 98% formic acid containing 1 M CNBr. Then 0.2 ml H_2O was added, and the mixture incubated for 18 h at room temperature. The solvents were removed in a flash evaporator at 40 to 50 °C. For cleavage with *N*-bromosuccinimide, the peptide was dissolved in 0.25 ml of 80% formic acid, and 2 mg reagent was added per mg peptide. The reaction was carried out for 4 h at 37 °C [25]. The whole mixture was submitted to gel chromatography.

The peptide fragments were fractionated on Bio-Gel P-30, minus 400 mesh, (150 × 0.8 cm) in 80% formic acid. Flow rates of 1.3 − 1.5 ml/h were obtained at a hydrostatic pressure of 150 to 200 cm. Fractions were collected every 30 min. The effluent was continuously monitored for absorption at 280 nm (Isco UA5) and for refractory index (Siemens, differential refractometer). An aliquot (1 to 2%) of each fraction was analysed on silica plates developed with chloroform/methanol/25% NH_3 (2/2/1) and sprayed with 0.025% fluorescamine in acetone.

N-terminal amino acids were determined by the dansylation method [26]. The N-terminal formyl group was removed by a 4-h incubation in 0.5 M methanolic HCl at room temperature. Amino acid analyses were performed with an automated Biotronic (LC 2000) amino acid analyzer by a one-column procedure. Peptide or protein samples dissolved in 98% formic acid were dried in glass tubes, and hydrolyzed in 0.2 ml 6 M HCl for 24, 28 and 72 h at 105 °C. Methionine was determined as methioninesulfone after performic acid oxidation [27].

Automated Solid-Phase Edman Degradation Coupling

The whole proteolipid was immobilized by coupling its carboxyl group(s) to 3-aminopropyl glass. The protein (2 to 4 mg) dissolved in 3 ml chloroform/methanol (2/1) was incubated for 15 min with 5 mg dicyclohexylcarbodiimide and 4 mg *N*-hydroxysuccinimide at room temperature. Then 170 mg of the aminated glass (equilibrated with 1 M pyridine/HCl pH 5.5) were added, and the incubation continued

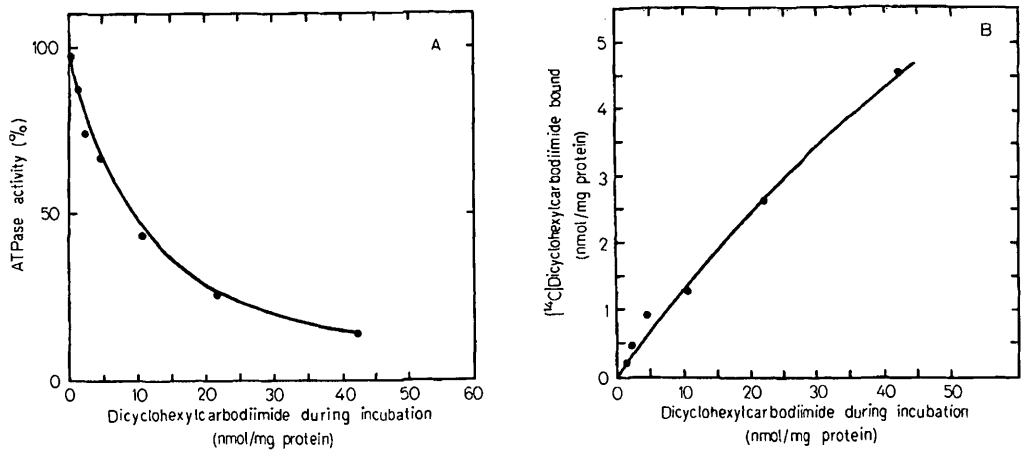

Fig. 1. *Inhibition of ATPase activity and recovery of bound label at various concentrations of [^{14}C]dicyclohexylcarbodiimide.* Inhibition of ATPase activity by dicyclohexylcarbodiimide. Membranes suspended in 50 mM Tris-acetate pH 7.5 at a protein concentration of 5 mg/ml were incubated with various concentrations of [^{14}C]dicyclohexylcarbodiimide for 18 h at 0 °C. Aliquots were withdrawn for determination (A) of the ATPase activity and (B) of covalently bound [^{14}C]dicyclohexylcarbodiimide (cf. Materials and Methods)

under gentle shaking over night. C-terminal coupling of peptides was performed according to Laursen [28] with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in dimethylformamide/water at pH 5.5. Peptides obtained after *N*-bromosuccinimide cleavage and containing a C-terminal-modified tyrosine were coupled in dimethylformamide to the aminated glass by a procedure resembling the homoserinelactone coupling method [28, 30]. The loaded glass was washed in all cases with first 5 ml methanol, then 3 ml 98% formic acid, and finally 5 ml methanol and 2 ml ether. The glass was dried *in vacuo*.

Sequencing

The amino acid sequences were determined with a solid-phase sequencer (model 12, Sequemat, Watertown, U.S.A.) using the small column (3 × 100 mm) and the standard single-column program as delivered by the factory [29] with the following minor modification. The cleaved-off thiazolinone amino acids are normally collected during a 30-min wash with trifluoroacetic acid and two 1-min washes with methanol. In the modified program collection was performed only between minute 10 and 20 of the trifluoroacetic acid wash. Thus, the amino acid derivatives were obtained in a volume of 0.8 ml in a Eppendorf microtest tube. The fractions were dried for 30 min at 65 °C *in vacuo* over KOH. The initial yields of the cleaved-off phenylthiohydantoin derivatives were usually 40–60% of the amount attached to the glass beads.

Analysis of Phenylthiohydantoins

The thiazolinone amino acids were converted into the phenylthiohydantoin derivatives by a 15-min incubation with acetylchloride/methanol (2/8) at 65 °C [29]. The samples were subsequently dried for 30 min at 65 °C *in vacuo* over KOH, and then taken up in 10 to 50 µl acetonitril. The phenylthiohydantoine amino acids were identified by a one-dimensional thin-layer chromatography on 10 × 10-cm silica plates (HPTLC-Fertigplatten, Kieselgel 60 F_{254}, mit Konzentrierungszone; Merck). The plates were developed first with chloroform/ethanol (98/2), then in the same direction with chloroform/methanol (9/1) for the identification of the polar amino acid derivatives, and finally with 1,2-dichloroethane/ethanol (5/2) for the identification of the arginine phenylthiohydantoin [31]. The presence of a arginine was confirmed by amino acid analysis after back hydrolysis of the sample in 50 µl 67% HI at 150 °C for 1 h [32]. All amino acid phenylthiohydantoins could be unambiguously identified down to a lower limit of 0.5 nmol per sequencer cycle. A fivefold amount was necessary in the case of the serine derivative, which is partially decomposed during sequencing.

RESULTS

Binding of [^{14}C]Dicyclohexylcarbodiimide and Inhibition of ATPase Activity in Whole Membranes

The ATPase activity of whole membranes from bacterium PS-3 is maximally inhibited by [^{14}C]dicyclohexylcarbodiimide at concentrations exceeding 40 nmol/mg protein (Fig. 1 A). Half-maximal inhibition is observed at 5–10 nmol/mg. Somewhat more than 10% of the applied radioactive carbodiimide is recovered as membrane-bound radioactivity (Fig. 1 B). At 10 nmol inhibitor/mg protein during incubation, the bound carbodiimide label corresponds to 0.8–

1.3 nmol inhibitor/mg membrane protein. Gel-electrophoretic analysis indicates that at this concentration 40% of the bound label is associated with a polypeptide of molecular weight 7000 to 8000 (data not shown). A similar size has been found for the carbodiimide-reactive subunit of the ATPase complex from mitochondria [7,14], chloroplasts [6,9] and bacteria [5,11].

Table 1. *Protein yield and enrichment of bound [^{14}C]dicyclohexylcarbodiimide label during the purification of the proteolipid*

Fraction	Protein	[^{14}C]Dicyclohexylcarbodiimide	Purification	Yield
	mg	nmol/mg	-fold	%
Total membrane	1000	0.83		
7000-M_r subunit		0.332[a]	0	100
Chloroform/methanol extract	1.5	38.5	116	17.5
After CM-cellulose chromatography	0.7	39.5	119	8.3

[a] This value represents [^{14}C]dicyclohexylcarbodiimide bound to the 7000-M_r subunit of the ATPase. 40% of the membrane-bound [^{14}C]dicyclohexylcarbodiimide is located in the band corresponding to the proteolipid subunit. For this quantification an unstained gel was cut into 1-mm slices. The protein was then eluted from the slices by an overnight incubation in 0.5 ml 1% sodium dodecylsulfate buffer pH 8.0 at 55 °C. Radioactivity was determined as described in Materials and Methods.

Purification of the Dicylohexylcarbodiimide-Binding Protein

As compiled in Table 1, the bound carbodiimide label is enriched more than 100-fold in the proteolipid extracted from the membranes with chloroform/methanol (2/1). Fractionation of the whole extract on carboxymethylcellulose (Fig. 2) removes most part of the contaminating lipid, but the protein-bound

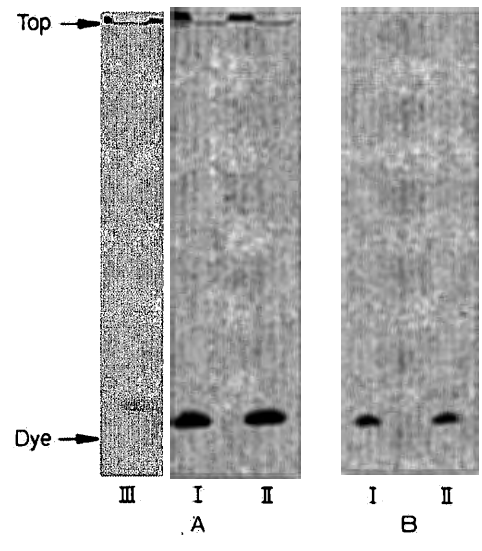

Fig. 3. *Dodecylsulfate/gel electrophoresis of the purified proteolipid.* 20 µg protein labelled with [^{14}C]dicyclohexylcarbodiimide were subjected to dodecylsulfate/gel-electrophoresis After visualizing the protein band by the Coomassie stain, the gel was prepared for fluorography essentially as described by Bonner and Laskey [20]. (A) Coomassie stain; (B) fluorography. (I) Chloroform/methanol extract; (II) CM-cellulose eluate; (III) purified proteolipid from *E. coli*

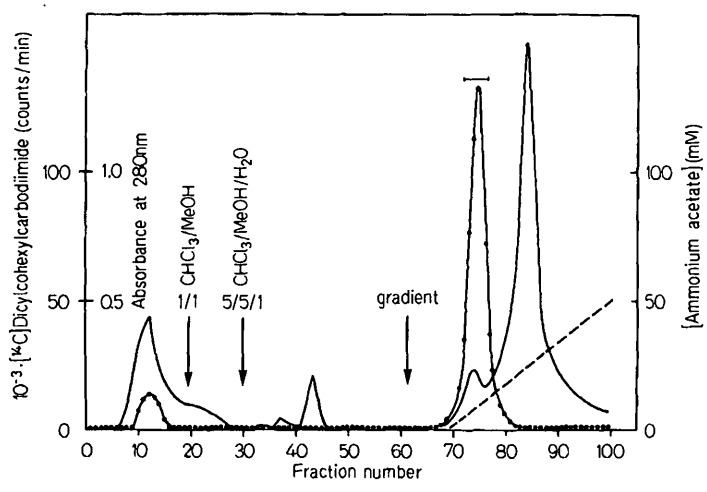

Fig. 2. *Chromatography of the chloroform/methanol extract on carboxymethylcellulose.* A column (2 × 13 cm), equilibrated in chloroform/methanol (2/1) at neutral pH [4] was loaded with 30 mg proteolipid extract at a concentration of 1 mg protein/ml. The column was developed at a flow rate of 40 ml/h. Fractions of 5 ml were collected. The dicyclohexylcarbodiimide-binding protein was eluted with a linear gradient of ammonium acetate in chloroform/methanol/water (5/5/1) with a total volume of 200 ml. The second peak eluted after the radioactivity does not contain protein as judged by the method of Lowry and amino acid analysis. (●——●) Radioactivity of [^{14}C]dicyclohexylcarbodiimide; (— —) absorbance at 280 nm; (----) gradient of ammonium acetate; the bar represents pooled fractions

Table 2. *Amino acid analysis of the whole protein and the peptide obtained after chemical cleavage.*
The values in parenthesis represent the number of amino acid residues obtained after sequence analysis. For fragments BI, BII and NI hydrolysis was for 72 h

Amino acid	Amount in							
	whole protein	CNBr fragments				*N*-bromosuccinimide fragments		
		BI		BII		NI	NII	
	mol/mol							
Aspartic acid	1.1 (1)	0.99 (1)						
Methionine sulfone	2.2 (2)							
Threonine	3.11 (3)	2.7 (3)						
Serine	2.84 (3)	1.8 (2)		0.96 (1)		0.78 (1)		
Homoserine		0.6 (1)						
Glutamic acid	5.06 (5)	4.3 (4)		1.16 (1)		0.88 (1)		
Proline	2.35 (3)	1.82 (2)		0.65 (1)		0.92 (1)		
Glycine	10.34 (11)	7.88 (8)		2.98 (3)		2.0 (2)	1.02 (1)	
Alanine	8.67 (9)	6.7 (7)		2.05 (2)		2.1 (2)		
Valine	7.52[a] (8)	3.64 (4)		3.02[a] (4)		2.9[a] (4)		
Isoleucine	7.97[a] (9)	4.57 (5)		3.2[a] (4)		2.85[a] (4)		
Leucine	9.65 (10)	7.14 (7)		3.05 (3)		2.4 (3)	1.04 (1)	
Tyrosine	0.79 (1)		(–)	0.7 (1)		–		
Phenylalanine	3.10 (3)		(–)	2.95 (3)		2.6 (3)		
Arginine	3.98 (4)	2.89 (3)		1.0 (1)		0.3 (–)	0.89 (1)	
N-terminal amino acid	–	Ser		Phe		Phe	Leu	

[a] Despite prolonged hydrolysis times (72 h) amounts of isoleucine and valine obtained were too small.

carbodiimide label is only marginally raised. Accordingly, after dodecylsulfate-gel electrophoresis of both the extract and the chromatographically purified protein only one polypeptide band of M_r 7000 was observed comigrating with the carbodiimide label (Fig. 3).

The purified protein migrates as single band during gel-electrophoresis in the presence of phenol/formic acid [14]. It is eluted as single symmetric peak after gel-chromatography on Bio-Gel P-30 in 80% formic acid (data not shown). It was noticed, however, that the carbodiimide label is slightly displaced compared to the protein.

SEQUENCE ANALYSIS

Analysis of the Complete Protein

The amino acid composition of the purified proteolipid (Table 2) shows an abundance of hydrophobic residues. Cysteine, lysine and histidine are absent. The ultraviolet spectrum is consistent with the absence of tryptophan (data not shown). Two methionines are determined as methioninesulfone.

The whole protein, either the crude chloroform/methanol extract of the membrane or the chromatographically purified product, was attached to aminopropyl glass via its carboxyl group(s) by the carbodiimide-coupling method. Between 40 and 50% of the employed protein were covalently fixed to the solid support. Dansylation of the whole protein did not reveal a N-terminal amino acid. However, when the protein bound to the loaded glass was preincubated with methanolic HCl, methionine was identified as the first residue. Thus, the proteolipid most likely starts with formylmethionine [33]. In two sequencer runs, employing 200 and 300 nmol bound protein, the first 55 residues of the amino acid sequence could be established (see Fig. 7). This N-terminal segment of the protein includes only two acidic residues (glutamic acid at positions 32 and 39). Whereas the yield of the released amino acid derivatives remained apparently constant after residue 32, a considerable decrease in yield was observed after residue 39. Thus, part of the protein probably was bound solely via residue 39 to the solid support. After residue 40 a background of derived leucine, valine, alanine, and glycine was obtained after thin-layer chromatography. The chromatograms could be evaluated semiquantitatively, however, since the samples of up to 12 consecutive cycles were analysed on the sample plate under strictly constant conditions.

Cyanogen-Bromide Fragments

The proteolipid contains two methionines at position 1 and 48, which were both included in the N-terminal sequence established with the whole protein. A nearly complete cleavage after these residues was obtained with cyanogen bromide. Two fragments,

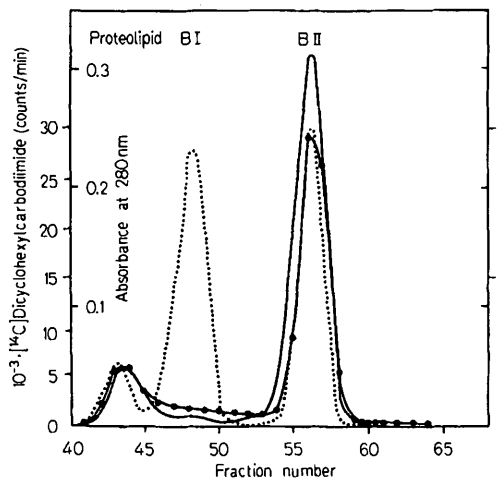

Fig. 4. *Gel chromatography of cyanogen-bromide fragments of the dicyclohexylcarbodiimide-binding protein.* 5 mg protein labelled with [^{14}C]dicyclohexylcarbodiimide were cleaved with CNBr (cf. Materials and Methods). Peptides were separted on a column (0.6 × 150 cm) with Bio-Gel P-30, (minus 400 mesh) in 80% formic acid at a flow rate of 1.5 ml/h. (——) Absorbance at 280 nm; (·····) refractory index; (●——●) radioactivity from [^{14}C]dicyclohexylcarbodiimide

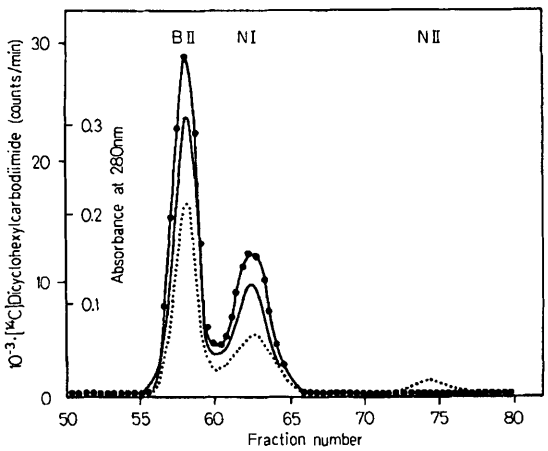

Fig. 5. N-*Bromosuccinimide cleavage of peptide B II.* 300 nmol of peptide BII were treated with 4 mg *N*-bromosuccinimide in 250 µl 80% formic acid for 4 h at 37 °C. Peptides were separated as described in Fig. 4

BI and BII, were isolated by gel-chromatography (Fig. 4). The large fragment contains homoserine and a N-terminal serine. Thus, it contains residues 2 to 48 of the polypeptide. This is confirmed by its amino acid composition (see Table 2). The smaller fragment BII, absorbs at 280 nm, due to the presence of the tyrosine. It is devoid of homoserine and starts with phenylalanine. Thus, it covers the C-terminal sequence of the protein, beginning with residue 49.

When cyanogenbromide cleavage is performed with the [^{14}C]dicyclohexylcarbodiimide-modified proteolipid, the ^{14}C-radioactivity is recovered exclusively in the small C-terminal fragment BII (Fig. 4).

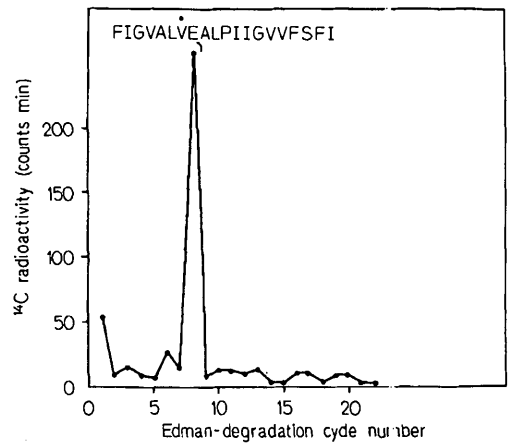

Fig. 6. *Determination of the [^{14}C]dicyclohexylcarbodiimide-binding residue.* [^{14}C]Dicyclohexylcarbodiimide-modified peptide NI (see Fig. 5) was subjected to automatic Edman degradation. Aliquots of the released phenylthiohydantoin derivatives of the respective amino acid were analysed for ^{14}C radioactivity. The one-letter notation for amino acids is used [45]

Attemps to sequence the whole fragments BI and BII were unsuccessful due to their insolubility in a variety of solvents including dimethylformamide, chloroform/methanol mixtures, trifluoroethanol, dimethoxyethane and water. The digestion of the large fragment BI with trypsin led to the cleavage in very low yields at the arginine residues as deduced from the occurrence of new N-terminal residues threonine, glutamic acid and proline in the whole digest. Unfortunately, the tryptic peptide with N-terminal proline could not be purified in reasonable amounts.

N-*Bromosuccinimide Fragments*

The C-terminal cyanogen-bromide fragment BII was further treated with *N*-bromosuccinimide. Gel-chromatography resolved three fractions (Fig. 5), corresponding to uncleaved material and two new fragment NI and NII, which originated in about 25% yield.

The small fragment NII is a tripeptide consisting of leucine, glycine and arginine (see Table 2). It does not absorb at 280 nm (see Fig. 5). The N-terminal residue is leucine. Arginine forms the C-terminus of the peptide, as shown by digestion with carboxypeptidase B [34]. The tripeptide was coupled with carbodiimide to the aminated glass, and the sequence leucine-glycine-arginine was obtained. It represents the C-terminal sequence of the proteolipid.

The larger fragment NI starts with phenylalanine as peptide BII, and it exhibits a strong absorbance at 280 nm due to the *N*-bromosuccinimide-modified tyrosine (Fig. 5). No tyrosine is found after amino acid analysis (see Table 2). Fragment NI could be attached

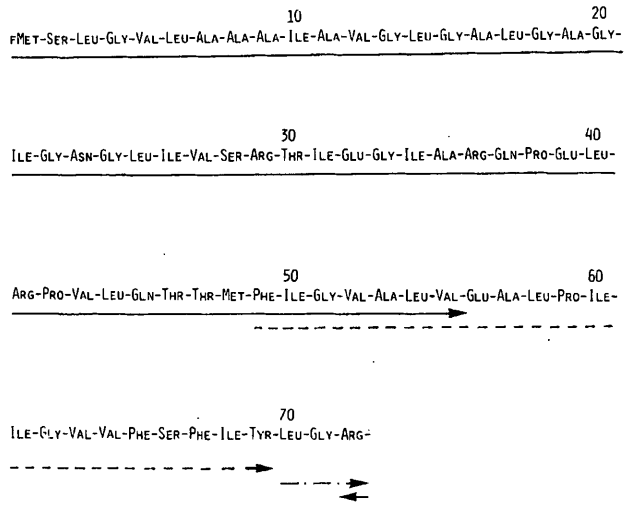

Fig. 7. *Amino acid sequence of the proteolipid subunit of the proton-translocating ATPase from the thermophilic bacterium PS-3.* The arrows indicate residues identical by automated solid phase Edman degradation of: (———→) the whole protein; (·····→) the large *N*-bromosuccinimide peptide NII of the cyanogenbromide peptide BII; and (·–·–·→) the small *N*-bromosuccinimide peptide NII. (←———) Identified by digestion of peptide NII with carboxypeptidase B. The presence of the tyrosine at the C-terminus of *N*-bromosuccinimide peptide NI (position 69) was deduced from the observations that this peptide absorbs at 280 nm and that is could be attached covalently to amino propyl glass [30]

to aminated glass by a procedure resembling the homoserinelactone-coupling method [28, 30]. The sequence was established up to step 20, where a isoleucine was identified (see Fig. 7). No phenylthiohydantoin amino acid was released in cycle 21. It has been found that the *N*-bromosuccinimide-modified tyrosine, which is covalently bound to the glass, is not recovered during Edman degradation [30]. It is therefore deduced that a tyrosine is located at position 21 of the peptide NI.

As shown in Fig. 5, the bound [^{14}C]dicylohexylcarbodiimide label is recovered in fragment NI. During sequence analysis, the ^{14}C radioactivity is released exclusively during step 8 (Fig. 6). At this position of the amino acid sequence a glutamyl residue has been identified (see Fig. 7).

DISCUSSION

The proteolipid isolated from whole membrane of the thermophilic bacterium PS-3 represents a subunit of the proton-translocating membrane factor of the ATPase complex, as indicated by the following evidence. (a) The protein reacts with reasonable specificity with dicyclohexylcarbodiimide, an inhibitor of the ATPase complex from all organisms analysed up to now [4–7, 9, 10]. (b) Its amino acid sequence shows homology to the ATPase proteolipid from other sources [15, 16]. (c) The amino acid composition corresponds reasonably to that of the proteolipid isolated from the ATPase membrane factor of bacterium PS-3 [12, 18].

The general properties of the proteolipid from the thermophilic bacterium PS-3 are similar to those of the corresponding ATPase protein from other organisms [5, 7, 14, 16]. The abundance of hydrophobic amino acid residues as well as the small size may explain its solubility in organic solvents. It thus could be purified by methods originally devised for the fractionation of lipids [35]. Like in other ATPase proteolipids tryptophan, histidine and cysteine are missing [5, 14, 16]. The absence of lysine is peculiar for bacterium PS-3. The relative abundance of arginine and glutamic acid (glutamine) has been observed also with other thermophilic proteins [36].

During amino acid sequence analysis, only chemical cleavage methods were applied. The fragments produced with cyanogen bromide were obtained in high yield. Lower yields were obtained with *N*-bromosuccinimide. Due to the low number of methionines and tyrosines present, the resulting fragments could be resolved by gel chromatography. 80% formic acid served as highly efficient solvent and conveniently volatile medium. Large parts of the amino acid sequence including the methionine at position 48 were obtained by sequencing the whole protein. The C-terminal sequence was obtained by analysing the two *N*-bromosuccinimide fragments produced by cleavage of the C-terminal CNBr fragment. The tyrosine at position 69 and the alignment of the fragments at this position were deduced indirectly, since no overlapping fragment could be analysed.

The amino acid sequence shows a clustering of the hydrophobic and hydrophilic residues in certain segments of the polypeptide chain. The N-terminus up to position 27 is hydrophobic with the exception of two uncharged polar residues (serine 2, asparagine 23). A second hydrophobic sequence of 24 residues is found at the C-terminus from position 48 to 71, which is interrupted only by one acidic group (glutamic acid 56) and a serine (serine 66). The C-terminal arginine introduces one positive and one negative charge. The hydrophilic residues are found to be concentrated in a 20-residue segment (position 28 to 47) in the middle of the polypeptide chain. The 11 hydrophilic residues in this polar loop are three arginines, two glutamic acids, two glutamines, three threonines and one serine. It is tempting to speculate that the two hydrophobic segments traverse the membrane, whereas the polar central segment is either exposed to the water phase or involved in the contact with the other subunits of the complex. Similar hydrophobic 25-residue segments have been found in bacteriorhodopsin [37, 38], which probably spans the membrane with seven helically organized segments [39].

The ATPase proteolipid from the thermophilic bacterium PS-3 shows clear homology to the corresponding protein from mitochondria and *Escherichia coli* [15,16]. Its amino acid sequence can be aligned with the other proteins without apparaent deletions or insertions. The thermophilic proteolipid containing only 72 residues is shorter than the proteins from the other cells analysed up to now. The polar N-terminal segment which is usually present is missing. Invariant positions exist in the case of glycine-18, glycine-22, glycine-33, arginine-36, proline-32 and alanine-57. Interestingly enough, the glutamic-acid-56 which can be modified by the inhibitor dicyclohexylcarbodiimide occurs at the only acidic position which is conserved in all analysed proteolipids. In mitochondria from *Neurospora crassa* and *Saccharomyces cerevisiae* [40] an invariant glutamyl residue, and in *E. coli* an isofunctional aspartyl residue, at this position [15,16] is selectively modified by the carbodiimide. Recently, the ATPase proteolipid from an *uncB*-type mutant of *E. coli* has been analysed [41]. The membrane factor of this mutant ATPase is defective in proton conductance. In the mutant proteolipid the carbodiimide-reactive aspartyl residue is substituted by a glycine. All these observations indicate that an acidic residue at this position of the proteolipid is essential for H^+ conductance, either since it participates itself in H^+ translocation or since it stabilizes the functional conformation of the H^+ conductor.

In the present studies maximum inhibition of membrane-bound ATPase activity was obtained, when about 40 nmol of the carbodiimide were bound per mg ATPase proteolipid. A similar value has been reported for expcriments with the purified ATPase complex and with the isolated membrane factor, F_0, from bacterium PS-3 [12]. Also in *E. coli* indirect evidence has been provided that the modification of one third of the ATPase proteolipid is sufficient for a complete inhibition of the proton-translocating activity of the membrane factor [6]. The proteolipid occurs in the ATPase complex as oligomer. In the mitochondrial ATPase complex the presence of a hexameric subunit has been suggested by several independent lines of evidence [7]. In the mitochondrial [7,42,43] and chloroplast [44] enzymes the ATPase activity appears to be inhibited when about one sixth of the proteolipid is modified by the carbodiimide. Despite this difference it appears clear, however, that the proteolipid monomers cooperate in the ATPase complex in such a way, that the modification of one or two of the monomers interrupts the function of the whole oligomer.

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