AMINO ACID SEQUENCE OF THE PROTEOLIPID SUBUNIT OF THE ATP SYNTHASE FROM SPINACH CHLOROPLASTS

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Received 30 October 1980

1. Introduction

In [1] dicyclohexylcarbodiimide (DCCD) was discovered to inhibit energy-transducing reactions in beef heart mitochondria. Subsequently, the hydrophobic carbodiimide was found to be covalently bound to a low M_r polypeptide soluble in neutral chloroform/methanol [2]. In the meantime this DCCD-binding proteolipid has been identified as main constituent of the ATP synthase membrane factor F_0, in mitochondria (e.g., [3,4]), chloroplasts [5] and bacteria [6–8]. Increasing evidence is being accumulated, that this subunit (M_r 8000) in oligomeric form [4,8] is intimately involved in the H^+-translocating properties of F_0 [9–11].

The ATP synthase complex from eukaryotic and prokaryotic cells shows remarkable similarities with respect to functional properties, overall structure and subunit composition (reviews in [9–11]). The amino acid sequences of the ATP synthase proteolipid subunit from various mitochondria [12] and bacteria [13,14] have been presented and their homology documented [15,16]. Here, the amino acid sequence studies have been extended to the proteolipid subunit of the ATP synthase from spinach chloroplasts. A comparison of phylogenetically distantly related proteolipid species hopefully will allow the identification of invariant features and of highly conserved amino acid residues, which are indispensible for the function and structure of this putative protonophore. Some properties [15] and the amino acid sequence [16] of the chloroplast proteolipid have been published in preliminary form.

2. Methods

Chloroplasts were isolated from commercially available spinach [17]. Chloroplasts from young leaves of greenhouse-grown spinach plants (*Spinacea oleracea*) were kindly donated by R. G. Herrmann, Botanisches Institut der Universität Düsseldorf. No differences were detected in the amino acid sequence of the proteolipid isolated from both sources.

Amino acid sequences were determined by automated solid-phase Edman degradation as detailed elsewhere [13,14]. The cleaved-off amino acid residues were identified as phenylthiohydantoine derivatives by thin layer chromatography [13]. Procedures for the cleavage of peptide bonds with cyanogenbromide and *N*-bromosuccinimide [12,14], for the separation of peptides by Bio-Gel P-30 chromatography in 80% formic acid [12,14], and for the coupling of peptides to 3-aminopropyl glass and to thioisocyanatoglass [14,18] have been described.

The cyanogenbromide fragment B-2 of the proteolipid was digested with trypsin as follows: A solution of the peptide in formic acid (200–400 nmol/50 μl) was diluted 20-fold with water, and adjusted to pH 8 with 10 N NaOH. The copious precipitate was washed with H_2O and then suspended in 0.5 ml H_2O. The pH was adjusted to 8.5 with pyridine, and 5% trypsin (w/w) was added. After incubating at 37°C for 2 h another 5% trypsin was added, and 2 h later the turbid mixture was dried in vacuo over KOH and P_2O_5. The residue was submitted to Bio-Gel P-30 chromatography in 80% formic acid.

Proteins were analysed by SDS–15% polyacrylamide gel electrophoresis [14]. Radioactivity was determined in the stained gels by fluorography [19].

3. Results

The ATP synthase proteolipid was purified by extraction of chloroplasts with neutral chloroform/methanol, 2/1 (v/v), and subsequent chromatography on carboxymethylcellulose. This method [6,20] already could be applied for the isolation of the proteolipid from mitochondria [20] and bacteria [13, 14]. Final impurities were removed by Bio-Gel P-30 chromatography in 80% formic acid [12]. Bound [^{14}C]DCCD served as marker during purification. As shown in table 1, the bound [^{14}C]DCCD label is enriched 22-fold in the crude proteolipid extract, 47-fold in the protein eluted from carboxymethylcellulose with chloroform/methanol/water, 5/5/1 (by vol.), and 64-fold after final purification by Bio-Gel P-30 chromatography in 80% formic acid. The final specific radioactivity corresponds to 5 nmol bound DCCD/mg protein and indicates that only ~4% of the proteolipid had been modified.

The proteolipid obtained during the last two purification steps shows a single stained protein band (M_r 8000) after SDS gel electrophoresis (fig.1B). Despite this fact, proteins were found to be removed by the gel chromatography in 80% formic acid which clearly were recognized as contaminants due to differences in light absorbancy at 280 nm and in amino acid composition.

Table 1
Enrichment of bound [^{14}C]DCCD label during purification of the ATP synthase proteolipid

Step	Total protein (mg)	Total ^{14}C radioactivity (cpm)	Specific [^{14}C]DCCD content (nmol DCCD/mg protein)
Chloroplasts	1190	9.4×10^6	0.08 (=1)
Chloroform/methanol extract	39	6.4×10^6	1.64 (22)
CM-cellulose	10.5	4×10^6	3.8 (47)
Bio-Gel P-30	n.d.	n.d.	5.1 (64)

Chloroplasts suspended at 10 mg protein/ml Tris-acetate (pH 7.4) were incubated with 3 nmol [^{14}C]DCCD (55 mCi/mmol)/mg protein for 4 h at 0°C. Bound [^{14}C]DCCD radioactivity was determined as in [4]. Protein was measured by the Lowry method using bovine serum albumin as standard

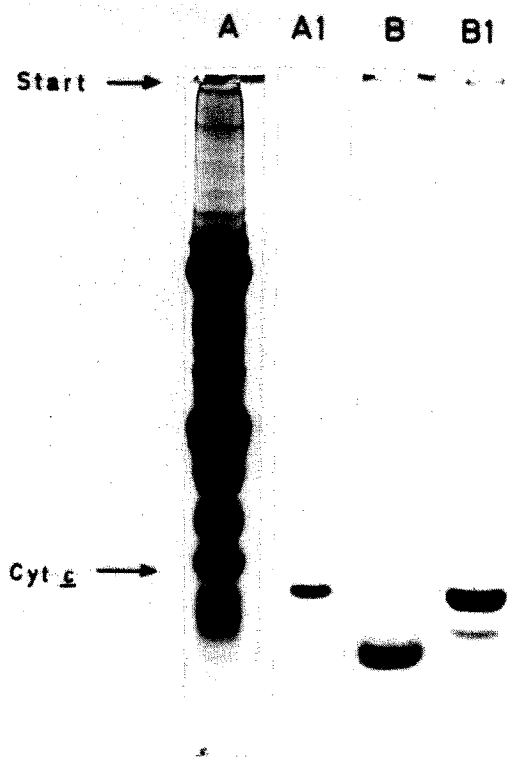

Fig.1. Analysis of [^{14}C]DCCD-labelled chloroplast protein (A, A1) and of the proteolipid purified by CM-cellulose chromatography (B, B1). The protein fractions described in table 1 were separated by SDS–polyacrylamide gel electrophoresis [14], and stained with Coomassie blue (A,B). Bound [^{14}C]DCCD was determined by fluorography [17] (A1, B1). The lower arrow indicates the migration of cytochrome *c*.

Remarkably, during SDS gel electrophoresis the bound [^{14}C]DCCD label does not comigrate with the stained protein band. Instead, one labelled component of higher M_r is observed in whole chloroplasts (fig.1A1). This component is most prominent also in all proteolipid fractions analysed during purification (fig.1B1). In addition a second labelled component is observed migrating slightly slower than the stained protein band. Similar results have been obtained during purification of the carbodiimide labelled proteolipid from pea chloroplasts [21]. Protein and carbodiimide label coincide, however, during Bio-Gel P-30 chromatography in 80% formic acid and during gel electrophoresis in the presence of phenol/formic acid [20] (not shown). Thus, it appears likely that the carbodiimide-modified proteolipid species exhibits a reduced electrophoretic mobility on SDS gels [4]. Additionally, the DCCD-modified species, but

not the free species, may form partially a defined aggregate in SDS buffer.

The amino acid sequence studies were performed with the proteolipid fraction obtained after carboxymethyl-cellulose chromatography. The contaminating proteins, still present at this stage of purification, were removed during peptide purification, and they did not interfere with the sequence determination performed with the whole protein.

The whole proteolipid was coupled in 50% yield via its carboxyl groups to 3-aminopropyl glass in chloroform/methanol, 2/1 (v/v), by the carbodiimide/hydroxybenzotriazol method [22]. In two experiments, using 250 nmol and 300 nmol bound protein, the amino acid sequence was determined unequivocally up to Arg_{50} (fig.2). The sequence up to Tyr_{66} was tentatively established with the exception of Thr_{52}, Ser_{56}, Leu_{63} and Thr_{64}. The Edman degradation started with methionine only when the glass-bound proteolipid had been pretreated with methanolic HCl [23]. This suggests strongly that formyl-methionine is present as amino-terminal residue.

The sequence of the carboxyterminal 21 residues was determined in the fragment B-3 obtained in 40% yield after cyanogenbromide cleavage. Peptide B-3 was poorly soluble in a variety of solvents (dimethylformamide, chloroform/methanol, trifluoroethanol, chlorethanol), and therefore, coupling to 3-aminopropyl glass by the carbodiimide method was inefficient (≤20%). Furthermore, Edman degradation of the coupled fragment proceeded with low yield (~5%), probably, because the amino-terminal glutamic acid was converted during the coupling to the pyroglutamyl residue. The fragment B-3 was further cleaved at its single tyrosyl residue with N-bromosuccinimide. The two fragments B-3/N-1 and B-3/N-2 were obtained in 40% yield. Both peptides were coupled to the aminated glass, B-3/N-1 via the spirolactone and B-3/N-2 by the carbodiimide method.

Tryptic digestion was performed with the large cyanogenbromide fragment B-2. The enzymatic cleavage was found to be inefficient. Besides the complete digestion products B-2/T-1, B-2/T-2, B-2/T-3 and B-2/T-4 (fig.2), uncleaved material and di- as well as tri-tryptic fragments could be identified by end-group determination and amino acid analysis. The fragments, with the exception of B-2/T-3, were not completely resolved during gel chromatography. The homoserine-containing fragments, however, were separated from each other, and they could be selec-

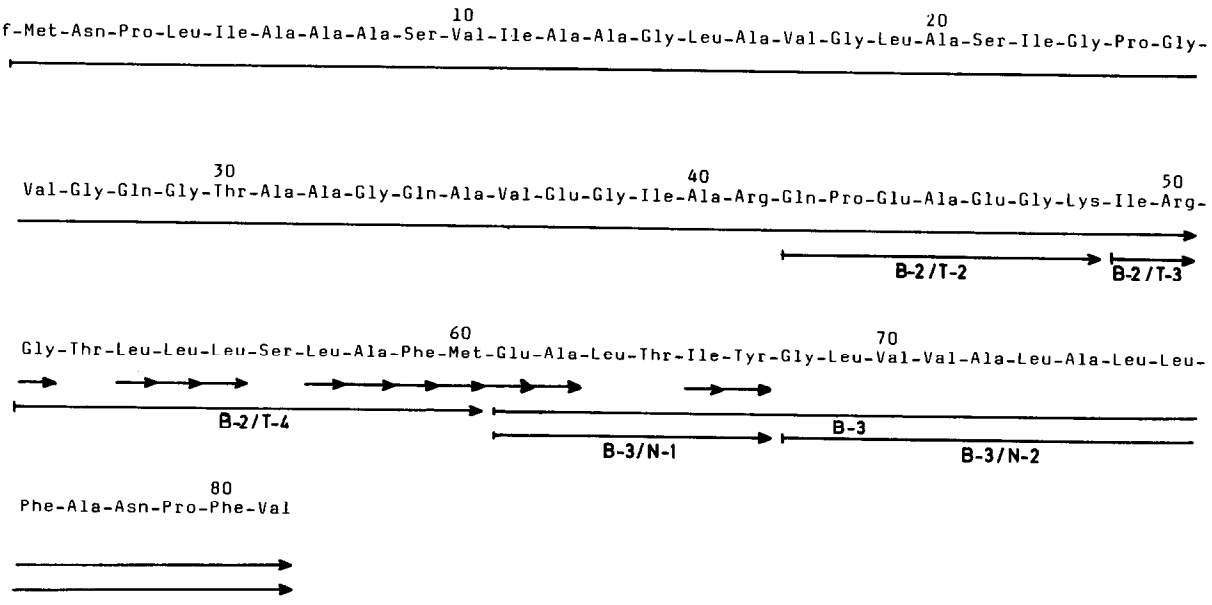

Fig.2. Amino acid sequence of the ATP synthase proteolipid from spinach chloroplasts. The arrows indicate residues identified by automated solid-phase Edman degradation of the whole protein as well as of fragments produced by cyanogenbromide (B-3), the application of first cyanogenbromide then trypsin (B-2/T-2, B-2/T-3, B-2/T-4), and first cyanogenbromide then *N*-bromosuccinimide (B-3/N-1, B-3/N-2).

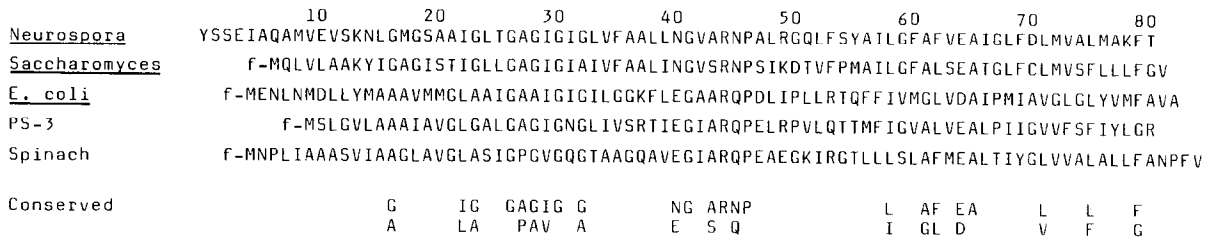

Fig.3. Comparison of the amino acid sequence of the ATP synthase proteolipid from mitochondria of *Neurospora crassa* and yeast [12], from the plasma membranes of *E. coli* [13] and the thermophilic bacterium PS-3 [14] and from spinach chloroplasts. The amino acids of the sequences are represented by the one-letter code [26]. Conserved positions contain the same or only 1 of 2 residues in all 5 proteins.

tively coupled to the aminated glass after lactonization. The sequences of the peptides B-2/T-4 and B-2/T-3+T-4 were determined. The amino-terminal glutamine residue of the triptryptic fragment B-2/T-2+T-3+T-4 disappeared during the coupling. Probably, a pyroglutamyl residue originated during the lactonization step. The first 6 residues of peptide B-2/T-2 could be determined after coupling the lysyl residue to isothiocyanatoglass [18].

When [^{14}C]DCCD-modified proteolipid was analysed, the ^{14}C radioactivity was recovered exclusively in cyanogenbromide fragment B-3, and then in the *N*-bromosuccinimide fragment B-3/N-1. The radioactivity was released during Edman degradation at step one together with the glutamyl residue corresponding to position 61 of the whole proteolipid (see fig.2).

4. Discussion

The amino acid sequence of the proteolipid from spinach chloroplasts presented here shows clear homology to the ATP synthase proteolipid subunit from both mitochondria [12] and bacteria [13,14] (fig.3). In comparison of the proteolipid from chloroplasts, mitochondria and bacteria, 6 positions of the amino acid sequences are found to be occupied by an identical residue. At 17 further positions only two different residues occur. Remarkably, only one basic, one acid and one polar uncharged side chain is conserved in the proteolipid from all sources.

The chloroplast proteolipid contains a polar sequence extending from residues 37–50 in the middle of the polypeptide chain. In this polar segment all 3 basic residues and 3 acidic residues occur. The only further acidic side chain is provided by the DCCD-reactive glutamyl residue at position 61 which is present in a hydrophobic sequence extending 30 residues long towards the carboxylterminus. A second hydrophobic sequence completely devoid of charged residues starts from the formylated aminoterminus and extends until Val_{36}. Six polar uncharged residues exist in the first hydrophobic segment, and 4 such residues are found in the second one. The clustering of hydrophilic and hydrophobic side chains in certain segments of the polypeptide chain is a typical feature of the proteolipid from all sources analysed up to now.

The proteolipid analysed here was isolated from whole chloroplasts. There can be little doubt, however, that this protein represents the proteolipid subunit of the membrane factor CF_0 of the chloroplast ATP synthase complex [5]. The extensive homology to the ATP synthase proteolipid from mitochondria and chloroplasts has been discussed above. Furthermore, DCCD has been found to inhibit chloroplast ATP synthase activity [24], and to bind to an 8000 M_r subunit [5,25]. Here, DCCD is shown to bind selectively to the Glu_{61} of the proteolipid. An acidic sidechain at the corresponding position invariantly is modified by DCCD in the proteolipid from all other sources analysed so far [12–14].

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