Volume 260, number 1, 122-126 FEB 08026 January 1990

Isolation and amino acid sequence of a novel 6.8-kDa mitochondrial proteolipid from beef heart

Use of FAB-MS for molecular mass determination

E. Terzi, P. Boyot, A. Van Dorsselaer, B. Luu and E. Trifilieff

Laboratoire de Chimie Organique des Substances Naturelles, URA CNRS 31, 5 rue Blaise Pascal, 67084 Strasbourg Cédex, France

Received 10 November 1989

We have isolated a 6.8 kDa proteolipid from an acidic chloroform/methanol extract of bovine cardiac muscle. The molecular mass of the polypeptide was measured by fast atom bombardment-mass spectrometry (FAB-MS) (m/z 6834.1). Its amino acid sequence was partly determined by direct sequencing and completed by characterization of cyanogen bromide and tryptic fragments (sequencing, FAB-MS and amino acid analysis). The polypeptide consists of 60 amino acid residues. Polyclonal antibodies raised in rabbit allowed its localization by electroimmunoblotting in mitochondria.

Proteolipid; Mitochondria; Fast atom bombardment-mass spectrometry; Amino acid sequence; (Bovine heart)

1. INTRODUCTION

Hydrophobic membrane proteins are usually difficult to isolate and to purify. Recently, we have developed a method for the purification of low-molecular-weight beef heart proteolipids which is characterized by the intensive use of organic solvents and exclusion of any detergent. The method was successful for the purification, from an acidic chloroform/methanol extract of bovine cardiac muscle, of phospholamban (which is an integral membrane protein of cardiac sarcoplasmic reticulum) [1], of several hydrophobic subunits of cytochrome *c* oxidase (subunits VIIIa and b) and of ATP synthase (A6L protein) [2].

In this paper, we describe the purification of a novel 6.8 kDa proteolipid from an acidic chloroform/methanol extract of beef heart. Its primary sequence was determined by classical protein chemistry methods and by use of FAB-MS (fast atom bombardment-mass spectrometry). The mitochondrial origin of this 60-residue polypeptide was shown by electroimmunoblotting.

2. MATERIALS AND METHODS

2.1. *Materials*

Bovine heart was obtained from the local slaughterhouse; Sephadex LH-20 and LH-60 were purchased from Pharmacia (Uppsala, Sweden); CM-Trisacryl was from IBF (Villeneuve-la-Garenne, France); sheep (anti-rabbit IgG) antibodies conjugated to horseradish peroxidase were from Biosys (Compiègne, France); nitrocellulose paper was from Millipore (Bedford, USA). All other reagents were obtained from the usual commercial sources at the highest available degree of purity.

Correspondence address: E. Trifilieff, Laboratoire de Chimie Organique des Substances Naturelles, URA 31, 5 rue Blaise Pascal, 67084 Strasbourg Cédex, France

The nucleotide sequence(s) presented here has (have) been submitted to the EMBL/GenBank database under the accession number no. Y07527

2.2. *Beef heart proteolipid extraction and purification*

Bovine cardiac muscle was homogenized with chloroform/methanol/4 N HCl (200/100/0.75, v/v/v) and the extract was fractionated by gel permeation chromatography on LH-60, followed by ion-exchange chromatography on CM-Trisacryl as described in [2] and summarized in fig.1.

2.3. *Purification of the 6.8-kDa polypeptide and of fragment peptides by HPLC*

Analyses were performed on a Waters Associates System of two 6000A pumps, an automated gradient controller and a U6K injector. Detection was made with a model 450 variable wavelength detector (Waters Associates).

2.3.1. Purification of the 6.8-kDa polypeptide

Proteins of the lyophilized fraction βT_2^A (fig.1) were separated on a C8 reverse-phase Brownlee Labs RP-300 aquapore column (250 × 7 mm) with a linear gradient from 30 to 60% of acetonitrile containing 0.1% TFA (solvent B), in 30 min, at a flow rate of 4 ml/min at 210 nm. Solvent A was water containing 0.1% TFA.

2.3.2. Purification of cyanogen bromide fragments

After cleavage of the native polypeptide with CNBr in 90% formic acid for 24 h, lyophilized CNBr cleavage peptides were dissolved in 20% acetic acid and fractionated by HPLC on a C8 reverse-phase Brownlee Labs RP-300 aquapore column (250 × 4.6 mm), at a flow rate of 1.7 ml/min and detected at 210 nm. A linear gradient from 5 to 50% acetonitrile (solvent B), in 30 min, was used. Solvent A was TEPA (1 l H_2O containing 1 ml triethylamine and 1 ml orthophosphoric acid).

2.3.3. Purification of tryptic peptides

The oxidized 6.8-kDa polypeptide was digested with trypsin for 6 h

Published by Elsevier Science Publishers B.V. (Biomedical Division)

at 37°C at pH 8.5 with the presence of 0.6% Triton according to Lees et al. [3]. Lyophilized tryptic peptides, dissolved in 20% acetic acid were isolated by HPLC on a Beckmann Ultrasphere-ODS column (4.6 mm × 15 cm), with a linear gradient from 0 to 30% of solvent B, in 90 min, detected at 206 nm. Solvent B was acetonitrile containing 0.1% TFA. Solvent A was water containing 0.1% TFA.

2.4. *Automated Edman sequence analyses*

Sequences were performed on an Applied Biosystems 470A protein sequencer. Phenylthiohydantoin derivatives of amino acids were identified by HPLC.

2.5. *Manual sequencing*

Manual sequencing was done according to the Chang method [4].

2.6. *Amino acid analysis*

Amino acid composition analysis was accomplished following total acid hydrolysis (6 N HCl, 110°C, 24 h) and precolumn phenylisothiocyanate derivatization using the PICO-TAG system of Waters Associates.

2.7. *Mass spectrometry*

2.7.1. FAB-MS on cleavage peptides

All spectra were obtained on a VG Analytical ZAB-HF double-focusing mass spectrometer. The accelerating voltage was 8 kV. The fast xenon atom beam was operated with an emission current of 0.1 mA at 8 eV. The mass spectrometer was operated at a resolution of 1000; in these conditions, average (or chemical) masses were measured in the state of the monoisotopical masses. Peptides were dissolved in 20% acetic acid plus methanol and then added to the matrix, which was usually 1-thioglycerol.

2.7.2. FAB-MS on the 6.8-kDa polypeptide and on CN_3

Positive ion mass spectra were obtained on a VG ZAB-SE double-focusing instrument and recorded on a VG 11-250 data system. Ionization of the sample was performed with about 1 μA of 30 keV energy cesium ions. The protein and peptides were dissolved in DMSO. The matrix used was 1-thioglycerol.

2.8. *Preparation of antisera to the 6.8-kDa polypeptide*

Two rabbits were injected subcutaneously on days 0, 14, 50, 63 and 71 with a mixture of 500 μl of a solution containing 100 μg of the protein and 500 μl of either Freund's complete adjuvant (day 0) or incomplete adjuvant (days 14 to 71). The rabbits were bled on day 78. The presence of antibodies was checked by ELISA.

2.9. *Immunoelectroblotting*

Immune blotting procedures were carried out according to Towbin et al. [5]. Protein samples were separated by Laemmli slab gel electrophoresis (15–25% linear gradient of acrylamide) and then transferred to nitrocellulose sheets at a constant voltage of 40 V for 3 h using a Mini Protean II Bio Rad apparatus. The immunostaining of the blot was done as previously described [6] using the polyclonal antisera at a dilution of 1/1000 and anti-rabbit IgG conjugated to horseradish peroxidase.

2.10. *Preparation of heart mitochondria*

Mitochondria were prepared as described by Mela and Seitz [7].

3. RESULTS AND DISCUSSION

Bovine cardiac muscle was extracted by an acidic chloroform/methanol mixture. The extract was purified as shown in fig.1 and as already described for the purification of phospholamban [1] and of low-molecular-weight beef heart proteolipids [2].

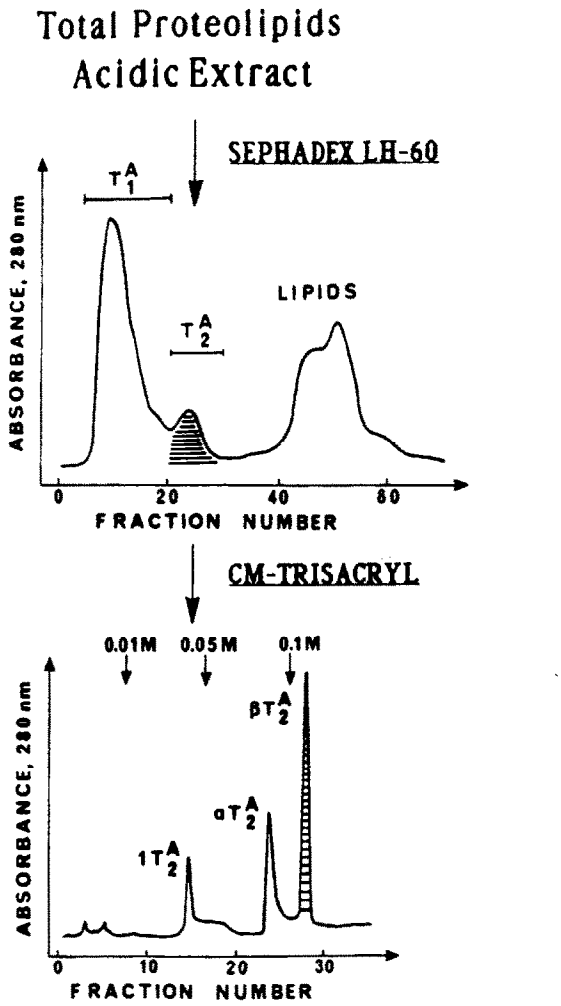

Fig.1. Purification of low-molecular-weight beef heart proteolipids.

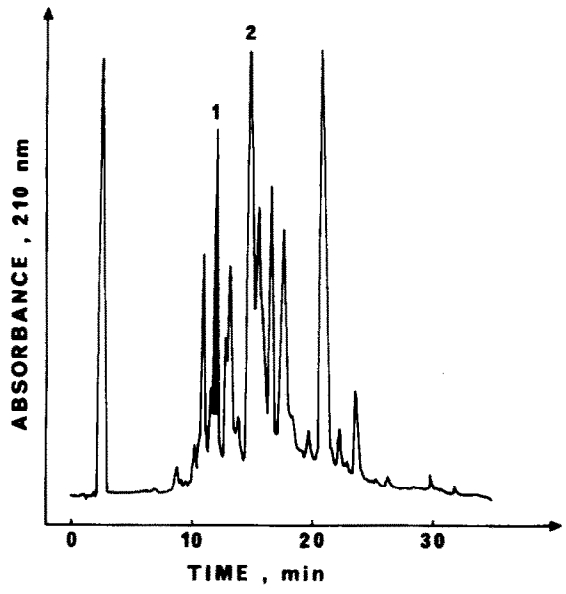

Fig.2. HPLC chromatogram of the βT_2^A protein peak. Peak 1 is the 6.8 kDa proteolipid.

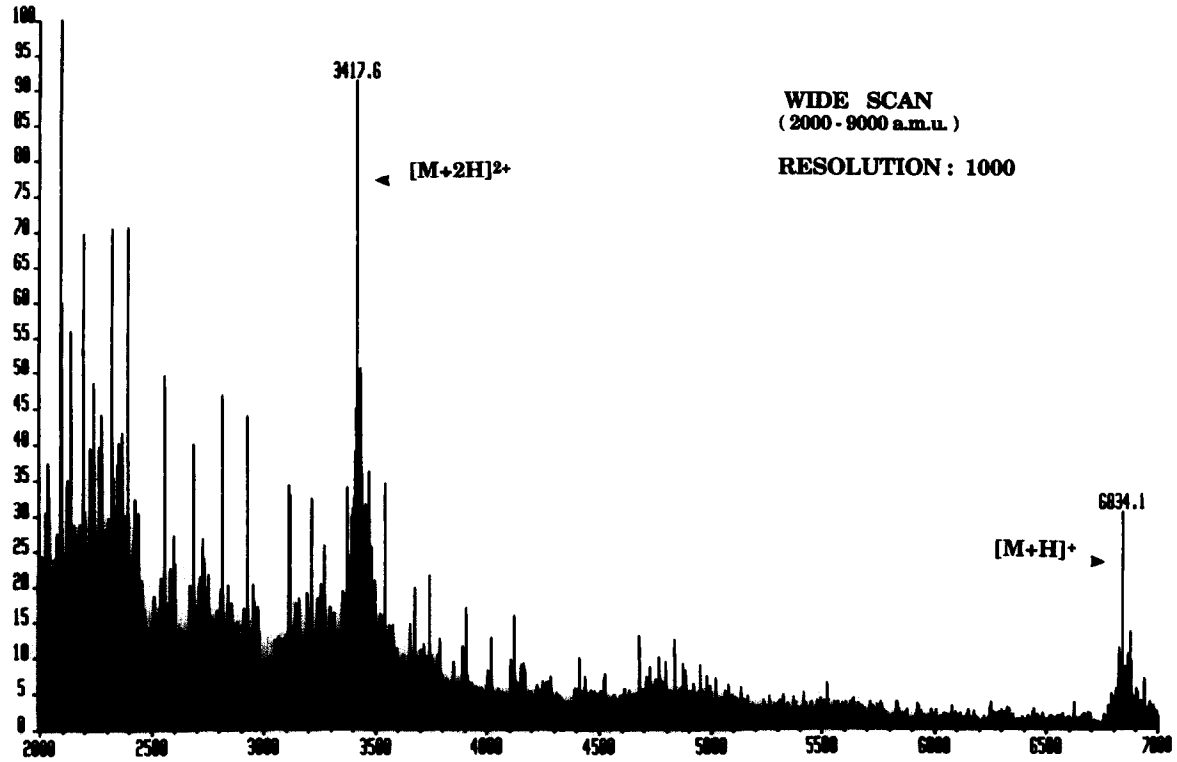

Fig.3. FAB-mass spectrum of the 6.8-kDa polypeptide. The protonated molecular ion appears at m/z 6834.1. A double-charged ion appears at m/z 3417.6.

The proteins of peak βT_2^A were further purified by HPLC on an aquapore RP-300 column (fig.2). In previous work, peak 2 was identified as subunit VIIIa of cytochrome *c* oxidase [2] and in this paper we report the sequence elucidation of peak 1.

To characterize the peak 1 protein, we measured its molecular weight by FAB-MS. Peak 1 yielded a protonated molecular ion $[M+H]^+$ measured at m/z 6834.1 (fig.3) indicating that the isolaed polypeptide contains about 60 residues. Its amino acid composition

Table 1

Amino acid compositions of the 6.8-kDa proteolipid and cleavage peptides

Amino acid	Intact protein	CN1 residues 1–13	CN2 residues 14–31	CN3 residues 32–60	T10 residues 50–60
Asx	2.78	0 (0)	0 (0)	0 (1)	0 (0)
Glx	6.33	0.57 (1)	3.30 (3)	0 (0)	0 (0)
Ser	7.02	1.38 (1)	0 (0)	3.57 (4)	1.98 (2)
Gly	5.43	1.02 (0)	1.90 (2)	1.15 (1)	1.38 (1)
His	5.05	0 (0)	0 (0)	2.72 (3)	2.74 (3)
Arg	5.50	0 (0)	0 (0)	2.57 (2)	0 (0)
Thr	3.63	0 (0)	1.65 (2)	0 (0)	0 (0)
Ala	13.78	0 (0)	1.09 (1)	7.01 (7)	3.82 (4)
Pro	3.60	1.32 (1)	1.11 (1)	1.10 (1)	1.08 (1)
Tyr	6.74	0 (0)	2.67 (3)	1.81 (2)	0 (0)
Val	4.93	1.20 (1)	1.09 (1)	1.10 (1)	0 (0)
Met	4.92	0.38 (1)*	0 (0)*	0 (0)*	0 (0)
Cys	0	0 (0)	0 (0)	0 (0)	0 (0)
Ile	5.88	2.22 (2)	1.08 (1)	1.98 (2)	0 (0)
Leu	8.80	2.47 (2)	1.23 (1)	1.24 (1)	0 (0)
Phe	3.17	0 (0)	0 (0)	0 (0)	0 (0)
Lys	12.44	1.44 (2)	0.88 (1)	4.75 (4)	0 (0)
Total residues*	100	11	16	29	11

* Trp and homoserine lactone (reaction of CNBr with Met) are not included (no tryptophan had been found by sequencing)

Numbers in parentheses are expected values from sequencing data

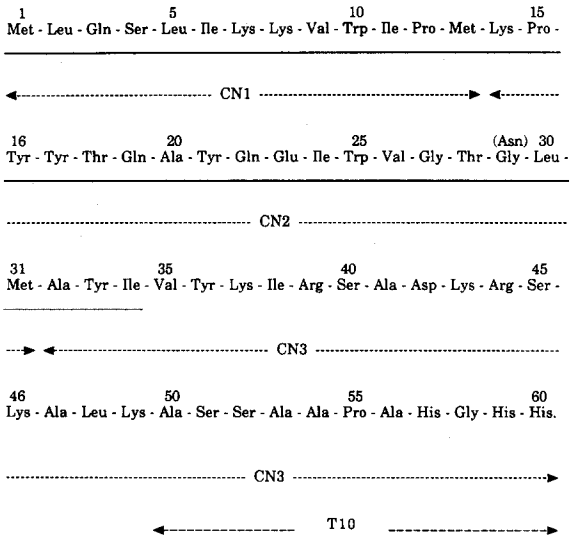

Fig.4. Amino acid sequence of the 6.8-kDa proteolipid. —, automated sequencing; CN_x, cyanogen bromide peptide; T*x*, tryptic peptide.

is given in table 1. The protein contains no Cys and has a fairly high content of hydrophobic amino acids.

Direct sequencing of the peak 1 polypeptide allowed the identification of 34 residues from Met 1 to Ile 34 (fig.4). Cycle 29 showed two possibilities: Asn or Gly.

The intact polypeptide was then cleaved by cyanogen bromide and three peptide fragments CN1, CN2 and CN3 were purified by HPLC. These peptides were characterized by their amino acid composition (table 1), by FAB-MS (table 2) and by a few cycles of manual Edman degradation (table 3).

Peptide CN1 was identified as fragment 1–13; its N-terminal sequence (Met-Ilx-Glx-Ser; table 3) showed that no cleavage occurred between Met 1 and Leu 2.

Table 2

Average and monoisotopic mass measurements of cleavage peptides by FAB-MS

Cleavage peptides	Measured mass		Calculated mass
	$[M+H]^+$	$[M+Na]^+$	$[M+H]^+$
CN1*	1539.0	1560.9	1538.9
CN2	2100.5	2121.8	2101.4
CN3	3134.6	–	3135.6
T10*	1042.6	1064.5	1042.5

* The masses of these peptides were measured at unit resolution which yields a monoisotopic value

Table 3

Results of manual Edman degradation on cleavage peptides

Cleavage peptides	Amino acid sequences
CN1	Met-Ilx-Glx-Ser-
CN2	X-Pro-Tyr-Tyr-
CN3	Ala-Tyr-Ilx-Val-
T10	Ala-X-X-

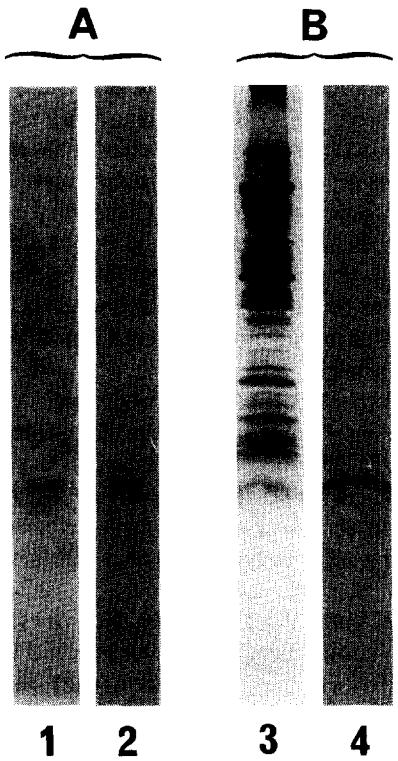

Fig.5. Electroimmunoblot analysis of pure 6.8-kDa polypeptide (A) and total beef heart mitochondrial protein (B) with the antiserum against the purified 6.8-kDa proteolipid. Lanes 1 and 3, gel strips stained after electrophoretic transfer. Lanes 2 and 4, nitrocellulose strips after immunostaining.

The analysis of CN1 (FAB-MS and amino acid composition) was in accordance with the result of automated sequencing run on the intact protein.

From the analysis of CN2 we could conclude that this peptide covers sequence 14–31. As no Asn could be detected in its amino acid composition (table 1), and as its measured chemical mass in FAB-MS ($[M + H]^+ = 2100.5$) agreed with the calculated mass of sequence 14-(Gly 29)-31 (table 2), we concluded that residue 29 was a Gly.

Automated Edman degradation run on peptide CN3 resulted in an sequence of 28 amino acids. As the sequence of the first three residues was identical with sequence 32–34, we deduced that CN3 represents the C-terminal part of the protein starting at residue Ala 32 and finishing at residue His 59.

The chemical mass of CN3 measured by FAB-MS was found to be 3134.6. The calculated mass $[M+H]^+$ of fragment Ala 32–His 59 is 2997.5. The difference between the two values if 137.1 and corresponds to the presence of an additional His (M_r = 137.14) at position 60. The presence of His 60 was confirmed by the amino acid analysis of CN3 (3 His; table 1) and by the measured chemical mass of the intact polypeptide (6834.1; fig.3) which is in complete accordance with the calculated mass for polypeptide Met 1–His 60 ($[M+H]^+ = 6835.1$).

The C-terminal sequence of the peak 1 protein was confirmed by analysis of tryptic peptides isolated by HPLC after the digestion of the protein. Peptide T10 (residues 50–60; fig.4) was sequenced up to His 59. Its amino acid analysis and its molecular mass measured by FAB-MS (table 1 and 2) were in complete accordance with the presence of His 60 as C-terminal residue.

A search in a protein data bank (MBRF PIR) indicated that the polypeptide of 60 residues we have isolated from bovine heart and sequenced, is unknown up to now and reveals no significant homology with any identified protein.

In order to find the subcellular origin of this new chloroform/methanol-soluble polypeptide in heart muscle, we raised polyclonal antibodies in rabbit by injecting the purified 60-residue protein. The reactivity of the obtained sera was checked by electroimmunoblotting with the pure polypeptide at a dilution of 1/1000 (fig.5).

Fearnley and Walker have shown that several hydrophobic mitochondrial proteins could be extracted with a chloroform/methanol mixture [8]. So, we checked, by immunoelectrophoresis, if the organic solvent-soluble polypeptide that we have isolated, was localized in mitochondria. The electroimmunoblot run on total beef heart mitochondrial proteins (fig.5) showed indeed that a protein band migrating at the same level as the pure 60-residue polypeptide was specifically recognized. With the same technique, we also showed that the same polypeptide was present in brain and liver mitochondria (data not shown).

In a previous paper, we have shown that the method of extraction and purification we have set up was efficient for the purification of several small subunits of cytochrome *c* oxidase and ATP synthase [2]. The identification of the 6.8-kDa proteolipid as a subunit of a mitochondrial enzyme complex is under investigation.

Acknowledgements: We are grateful to Dr D. Filliol for advice on mitochondria preparation and for providing brain and liver mitochondria, to Dr D. Fraisse from the SCA-CNRS for MS facilities and to Dr L. Denoroy from the SCA-CNRS for automated sequencing. E.T. received fellowship from Rhône-Poulenc.

REFERENCES

[1] Boyot, P., Luu, B., Jones, L.R. and Trifilieff, E. (1989) Arch. Biochem. Biophys. 269, 639–645.
[2] Boyot, P., Trifilieff, E., Van Dorsselaer, A. and Luu, B. (1988) Anal. Biochem. 173, 75–85.
[3] Lees, M.B., Chao, B.H., Lin, L.F.H., Samiullah, M. and Laursen, R.A. (1983) Arch. Biochem. Biophys. 226, 643–656.
[4] Chang, J.Y. (1983) Method Enzymol. 91, 455–466.
[5] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[6] Trifilieff, E., Luu, B., Nussbaum, J.L., Roussel, G., Espinosa de los Monteros, A., Sabatier, J.M. and Van Rietschoten, J. (1986) FEBS Lett. 198, 235–239.
[7] Mela, L. and Seitz, S. (1979) Methods Enzymol. 55, 39–46.
[8] Fearnley, I.M. and Walker, J.E. (1986) EMBO J. 5, 2003–2008.