PARTIAL PROTEOLYSIS OF THE NATURAL ATPase INHIBITOR FROM BEEF HEART MITOCHONDRIA

Isolation and characterization of an active cleavage product

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1. Introduction

The activity of the mitochondrial ATPase (F_1) is controlled by a small peptide ($M_r \sim 10\,000$) referred as the natural ATPase inhibitor (IF_1) [1–3]. IF_1 interacts with the β-subunit of F_1; the binding of one molecule of IF_1 to one molecule of F_1 is sufficient to bring about full inhibition of the ATPase activity despite F_1 possessing 2 or perhaps 3 β-subunits [4]. Progress in the understanding of the interaction of F_1 with IF_1 requires detailed knowledge of the structure of the two interacting peptides. We have thus undertaken to determine the amino acid sequence of IF_1 purified from beef heart mitochondria. Here, we describe the limited proteolytic fragmentation of IF_1 and the isolation in pure state of tryptic and chymotryptic fragments. Partial proteolysis by trypsin resulted in the accumulation of a tryptic peptide (T_1) of M_r 8000, still capable of inhibiting the ATPase activity of F_1. However, partial proteolysis by chymotrypsin resulted in a peptide (C_1) of M_r 7000, which was devoid of inhibitory activity. Both T_1 and C_1 shared with IF_1 the carboxyl-terminal amino acids. The T_1 peptide differed from C_1 by the presence of about a dozen amino acids at the amino-terminus of T_1; this short sequence, reported here, appears to be essential for the inhibitory activity of T_1 either directly or indirectly and may therefore play a strategic role in the interaction of IF_1 and F_1.

Abbreviations: TEMD, *N,N,N'N'*-tetramethylethylenediamine; SDS, sodium dodecylsulfate; TPCK, L-(1-tosylamido-2-phenylethyl-chloromethyl ketone); ATPase, EC 3.6.1.3; AS particles, submitochondrial particles depleted of natural inhibitor

2. Materials and methods

Acrylamide, bis-acrylamide and TEMD were purchased from Eastman Kodak, and SDS from Serva. TPCK-treated trypsin was obtained from Worthington Biochemicals and α-chymotrypsin from Miles Labs. The soybean trypsin inhibitor and the bovine pancreas trypsin inhibitor, which also inhibits chymotrypsin, were from Sigma. Carboxypeptidase P was obtained from Takara-Shuzo, Kyoto. The molecular mass markers were myoglobin and its CNBr cleavage fragments; they were purchased from BDH Labs. Beef heart mitochondria were prepared according to [5] and ATPase inhibitor (IF_1) was purified following [6], as modified [7]. The inhibitory activity of IF_1 on ATPase was assayed with AS particles [8]. The protein concentration was estimated as in [9], using bovine serum albumin as standard. Polyacrylamide slab gel electrophoresis in presence of SDS was done as in [10], with a 10% stacking gel and a 20% separating gel. Electrophoresis was run for 16 h at 125 V constant voltage.

Protein hydrolysis was carried out in an acid mixture made of pure trifluoroacetic acid and 11.5 N HCl (1:2, v/v), containing 0.003% phenol, at 166°C under vacuum for 25 and 50 min [11]. Amino acid analysis was performed on a Durrum D500 amino acid analyzer according to the Operation Manual. The amino terminal amino acids were determined by manual oper-

ation [12] or by automatic Edman degradation [13] with a Beckman sequanator 890-C, equipped with an automatic converter (P_6 from Sequamat). The resulting phenylthiohydantoin amino acids were analyzed by high-pressure liquid chromatography (Pye Unicam LC3) with a Partisil 10 column (Whatman) and solvent systems as in [14], and also by thin-layer chromatography (F1700 Schleicher and Schüll), using 0.75% pyridine–acetate buffer (pH 4.25) as solvent [13]. Digestion of IF_1 and IF_1 fragments by carboxypeptidase P was done in 0.1 N pyridine–formate buffer (pH 2.5) at 37°C for 6 h. The enzyme to substrate ratio was 1/50 (w/w). The released amino acids were analyzed directly by amino acid analyzer [15].

Digestion of IF_1 by trypsin was carried out in 1% NH_4 bicarbonate and 50 mM NH_4 acetate (pH 7.8) at 10°C for 7 min; the trypsin/IF_1 ratio was 1/100 (w/w). Trypsin action was stopped by addition of an amount of soybean trypsin inhibitor corresponding in weight to 3-times that of trypsin. Digestion of IF_1 by chymotrypsin was done in the same medium as that described for trypsin, at 20°C for 3 min, using a ratio of chymotrypsin to IF_1 of 1/200 (w/w). The chymotrypsin action was stopped by addition of an excess of beef pancreas inhibitor (the inhibitor/chymotrypsin ratio was 6/1 (w/w)). This inhibitor-stop procedure was also used when the effect of chymotrypsin or trypsin on the activity of IF_1 was analyzed. The two inhibitors had no effect on the ATPase activity or the inhibitory activity of IF_1.

3. Results and discussion

3.1. *Products of limited proteolysis of IF_1 by trypsin and chymotrypsin*

Native IF_1 was subjected to limited tryptic and chymotryptic digestions as in section 2. The products were separated by electrophoresis on a 20% acrylamide–SDS gel, and then stained by Coomassie blue. The rates of migration of the 2 major products corresponded to $M_r \sim 8000$ (T_1) from the tryptic digest and 7000 (C_1) from the chymotryptic digest. Accumulation of T_1 and C_1 from the 10 000 M_r IF_1 was obviously concomitant with the release of short peptide(s) that could not be fixed and visualized on the stained gels. The recoveries of T_1 and C_1 were determined by scanning the gel at 600 nm. The inhibitory activity of the whole digest was assayed on the ATPase activity of AS particles (section 2). As shown in table 1, a mixture of IF_1 (20%) and T_1 (80%) exhibited the same specific inhibitory activity as native IF_1, indicating that T_1 was still fully active. However, a mixture of IF_1 (50%) and C_1 (50%), arising from the action of chymotrypsin on IF_1 had only half the spe-

Table 1
Products obtained by limited proteolysis of IF_1 by trypsin and chymotrypsin

Proteolytic enzyme	Components[a] of the digest		Inhibitory[c] efficiency of the digest
TPCK-trypsin	native IF_1	(20%)[b]	100%
	T_1	(80%)	
α-Chymotrypsin	native IF_1	(50%)	50%
	C_1	(50%)	

[a] The products were identified by SDS–polyacrylamide gel electrophoresis

[b] Percentage (into brackets) was measured by Coomassie blue absorption at 600 nm

[c] The inhibitory activity of the cleavage products of IF_1 on ATPase was determined with AS particles (section 2)

Experimental conditions of incubation are given in section 2

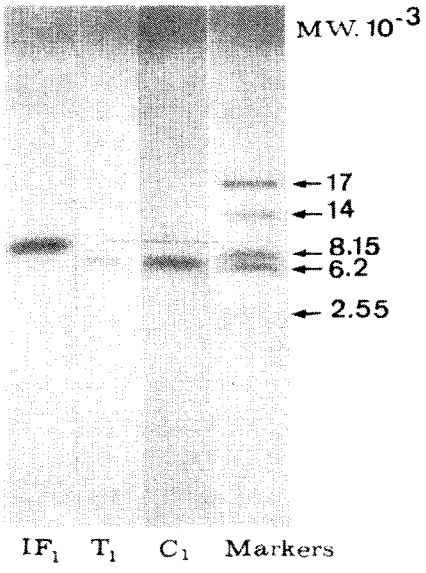

Fig.1. SDS–polyacrylamide gel electrophoresis of purified IF_1, T_1 and C_1. T_1 and C_1 were isolated from polyacrylamide gel electrophoresis by the technique described here. They were rerun on a 20% acrylamide–SDS gel in parallel with native IF_1 and molecular markers made of CNBr cleavage products of myoglobin, and then stained by Coomassie blue.

Table 2

Amino acid	IF_1	T_1	C_1	T_1–C_1
Asp	8	5	4	1
Thr	0	0	0	0
Ser	7	5	4–5	0–1
Glu	20	16	16	0
Pro	0	0	0	0
Gly	6–7	6	3	3
Ala	11	9	6	3
Cys/2	0	0	0	0
Val	2–3	2	0–1	1
Met	0	0	0	0
Ile	4	4	4	0
Leu	5–6	4	4	0–1
Tyr	1	1	1	0
Phe	2	2	1	1
His	4–5	4	4–5	0
Lys	11	9	9	0
Arg	8	6	5–6	1
Trp	0	0	0	0
No. residues	89–93	73	61–65	10–12
Calc. M_r	10 100–10 328	8244	7155–7635	902–1102

The values are integrated numbers from the results obtained after total hydrolysis for 25 and 50 min (section 2)

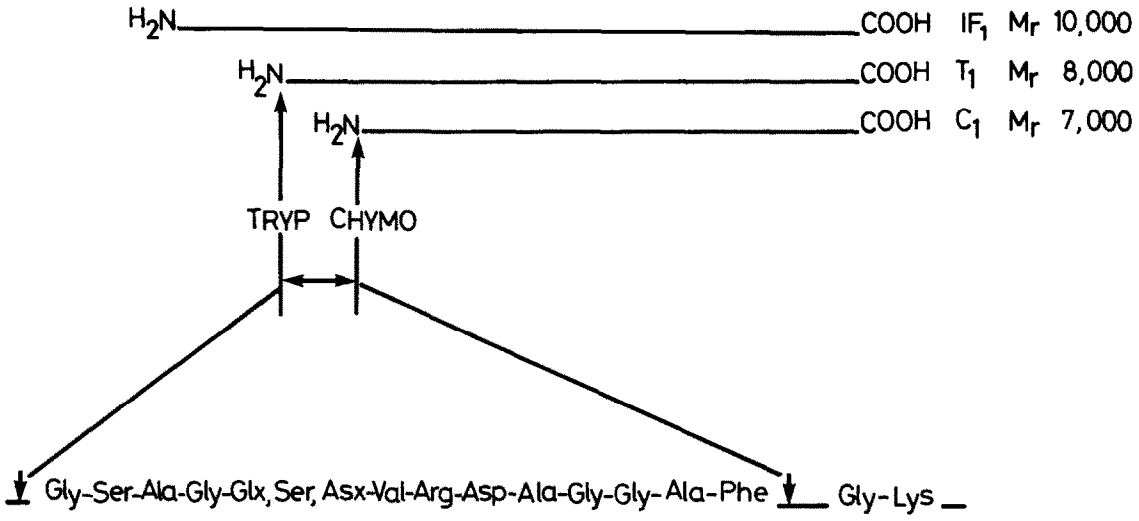

Fig.2. Localization of cleavage points of IF_1 by limited trypsin and chymotrypsin digestion. The amino acid sequence of the amino-terminal protein of T_1 is given.

cific activity of native IF_1; it was further shown that extracts containing a higher percentage of C_1 exhibited a noticeable decrease of inhibitory activity. The latter data clearly indicated that C_1 was inactive.

3.2. *Purification of T_1 and C_1*

After limited proteolysis of IF_1 by trypsin and chymotrypsin as above, the digests were lyophilized, dissolved in a minimal volume of 70% formic acid and 10% *n*-propanol in water, and then chromatographed on a Biogel P-30 column (90 × 9 cm) equilibrated with 70% formic acid and 10% *n*-propanol in water. The fractions corresponding to the mixtures of IF_1 and T_1 (tryptic digestion), and IF_1 and C_1 (chymotryptic digestion) were respectively pooled, concentrated and then separated by SDS–polyacrylamide gel electrophoresis. After staining, the colored bands were cut out, sliced into small pieces, and extracted by soaking in 70% formic acid under constant stirring for 24 h at 4°C. After clarification by centrifugation, the extraction fluid was sucked off with a Pasteur pipette, placed in a centrifuge conical tube and dried under vacuum. The dried samples containing T_1 or C_1 plus Tris, glycine, SDS and Coomassie blue were dissolved in 50 μl 70% formic acid and applied to a 1 ml column of Biogel P-4 (100-200 mesh) equilibrated with 70% formic acid. Elution was performed with the same acid. T_1 and C_1 were excluded in the void volume, well separated from the other reagents. The purity of the samples was tested by SDS–polyacrylamide gel electrophoresis (fig.1) and by amino acid analysis. The amino acid compositions of IF_1, T_1 and C_1, together with the difference in residues between T_1 and C_1, are given in table 2. Integral numbers of amino acids were calculated using the known absolute values of phenylalanine and tyrosine from sequence data of IF_1. The difference in molecular weights of T_1 and C_1 agreed with the difference in number of amino acid residues in T_1 and C_1.

3.3. *End group analysis of proteolytic fragments*

Amino acids from the carboxyl-terminal end of IF_1, T_1 and C_1 were released by carboxypeptidase P digestion under the same conditions (section 2). The released amino acids were similar for IF_1, T_1 and C_1, indicating that the 3 peptides possess a common carboxyl-terminal sequence (table 3).

The amino-terminal amino acids of T_1 and C_1 released by the Edman degradation were identified as Gly–Ser for T_1 and Gly–Lys for C_1. For 3 different preparations of IF_1, no amino acid was released even after 20 cycles of degradation in liquid phase sequencing, suggesting that the amino-terminus of IF_1 is blocked. These data indicated that IF_1 differs from T_1 as well as from C_1 by the amino-terminal sequence.

Table 3
Amino acids released from the C-terminal end of IF_1, T_1 and C_1 by carboxypeptidase P

Amino acid	IF_1	T_1	C_1
	(mol/mol fragment or protein)		
Asp	2.5	2.1	2.1
AAn[a] + Ser	2.7	3.1	2.9
Glu	3.7	4.2	4.4
Gly	0.35	0.83	0.58
Ala	1.0	1.5	1.5
Ile	0.90	0.89	0.95
Leu	0.80	0.70	0.77
Tyr	0.12	0.18	0.15
Phe	0.19	0.23	0.19
His	0.39	0.38	0.3
Lys	3.2	3.0	2.7
Arg	2.4	2.7	2.5

[a] AAn: Gln and Asn

Digestion was done as in section 2

Using some established data from the sequence work of IF_1 (to be published elsewhere) and manual Edman degradation of T_1 and C_1, the terminal amino acid sequence by which T_1 differed from C_1 was determined, and is given in fig.2. The enzymatic cleavages of IF_1 by trypsin and chymotrypsin are summarized in the same figure. The fact that T_1 is characterized by full inhibitory activity whereas C_1 is inactive is probably related to the additional sequence of the dozen of amino acids placed at the amino-terminal end of T_1.

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