The frayed *N*-terminal of the inhibitor protein of bovine mitochondrial F₁-ATPase

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The *N*-terminal region of the bovine mitochondrial F₁-ATPase inhibitor protein is frayed. In three independently isolated samples about 62% of chains start at glycine-1. A further 22% of chains start at residue 2, serine, and the remainder at residue 3, glutamic acid. No evidence can be found for α -*N*-formylglycine reported previously. The fraying may be a consequence of proteolytic processing of the precursor of the inhibitor protein during entry into the mitochondrion.

INTRODUCTION

The bovine mitochondrial inhibitor protein inhibits the activity of free and membrane-bound F₁-ATPase (Pullman & Monroy, 1963; Horstman & Racker, 1970). Its physiological function is not yet established, but a regulatory role has been proposed (Pullman & Monroy, 1963; Asami *et al.*, 1970; Ernster *et al.*, 1973; van de Stadt & van Dam, 1974). The protein is 84 amino acid residues long and its amino acid sequence has been reported by Frangione *et al.* (1981). They found that the protein has a free *N*-terminus. In contrast, Dianoux *et al.* (1984) proposed that the *N*-terminal residue, glycine, is *N*-formylated, a post-translational modification described in only two other eukaryotic proteins (Fujiki *et al.*, 1970; Lubke *et al.*, 1971). In common with other mitochondrial proteins encoded in nuclear DNA, the inhibitor protein is synthesized on cytoplasmic ribosomes as a longer precursor with an *N*-terminal extension (Schatz & Butow, 1983; Hay *et al.*, 1984). This extension directs the protein into the mitochondrion and is removed in the import process. Were the mature protein to be α -*N*-formylated, this modification would take place subsequent to processing and might be an integral part of import. We have re-investigated the structure of the *N*-terminal region of the inhibitor. We find in three independently isolated samples that the *N*-terminus of the protein is frayed, with sequences commencing at residues 1, 2 and 3. These various forms are sufficient to account for all chains in the preparations; no evidence can be found for an α -*N*-formylated protein. This is the fourth of seven imported bovine mitochondrial ATP synthase subunits so far investigated that has a frayed *N*-terminus (Walker *et al.*, 1985).

MATERIALS AND METHODS

Chemicals and reagents

Clostripain was purchased from Sigma Chemical Co., Poole, Dorset, U.K. The sources of other chemicals and reagents have been given previously (Walker *et al.*, 1985).

Preparation of the inhibitor

The inhibitor protein was isolated from bovine heart mitochondria as described by Frangione *et al.* (1981). A second sample, kindly donated by Dr. H. S. Penefsky, was isolated independently by the same procedure. A third sample, generously given by Dr. D. A. Harris, was isolated by a different method (Gomez-Fernandez & Harris, 1978).

Protein chemical methods

Inhibitor (20 nmol) was hydrolysed with clostripain for 3 h at room temperature with an enzyme/substrate ratio of 1:70 (w/w). A buffer containing 45 mM-Tris (pH 7.4), 1 mM-CaCl₂ and 2.5 mM-dithiothreitol was used both to activate the enzyme (2 h, 75 °C) and for hydrolysis (Mitchell, 1977). Conditions for trypsin digestion, subsequent peptide isolation by h.p.l.c., amino acid

Table 1. Sequence of inhibitor protein from bovine mitochondria in a spinning-cup sequencer

A 8.3 nmol sample of protein was employed in this experiment. Abbreviation: PTH, phenylthiohydantoin derivative

Cycle no.	PTH released and yield (nmol)	PTH released and yield (nmol)	PTH released and yield (nmol)
1	Gly* 2.2	Ser† + ve	Glu 1.5
2	Ser + ve	Glu 1.8	Ser + ve
3	Glu 4.4	Ser + ve	Gly 1.0
4	Ser + ve	Gly 0.9	Asp 1.5
5	Gly 1.9	Asp 1.7	Asn 1.1
6	Asp 3.5	Asn 1.6	Val 0.7

* The conversion of the anilinothiazolinone of glycine into the corresponding phenylthiohydantoin proceeds to about 50% completion under the standard conditions employed for autoconversion in the spinning-cup sequencer.

† The phenylthiohydantoin derivative of serine decomposes readily. Its yield cannot be accurately quantified.

Abbreviation used: f.a.b. m.s., fast-atom-bombardment mass spectrometry.

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      10              20              30
G S E S G D N V R S S A G A V R D A G G A F G K R E Q A E E

      40              50              60
E R Y F R A R A K E Q L A A L K K H H E N E I S H H A K E I

      70              80
E R L Q K E I E R H K Q S I K K L K Q S E D D D

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Fig. 1. Amino acid sequence of the F_1 -ATPase inhibitor protein from bovine mitochondria

The two versions of the sequence reported by Frangione *et al.* (1981) and Dianoux *et al.* (1984) differ in amide assignment at positions 29, 30 and 61. The former group reported glutamic acid, glutamine and glutamic acid respectively. DNA sequencing of a cDNA clone indicates the assignments shown here (J. E. Walker, N. J. Gay & S. J. Powell, unpublished work).

Fig. 2. Fractionation of a tryptic digest of F_1 -ATPase inhibitor protein from bovine mitochondria

This was carried out on a C18-Nucleosil column (25 cm $\times$ 0.7 cm; Macherey and Nagel) with a linear gradient of acetonitrile in aq. 0.1% trifluoroacetic acid (-----) at a flow rate of 2 ml/min.

analysis and sequence analysis in a spinning-cup sequencer have been described previously (Walker *et al.*, 1985). The initial yield of the *N*-terminal of proteins in a spinning-cup sequencer was determined to be 85% with a sample (5 nmol) of sperm-whale myoglobin. [Initial

yield = $100 \times (\text{yield of amino acid phenylthiohydantoin derivative in step 1 of Edman degradation}) / (\text{amount of protein sample})\%$.] Sequence analysis experiments of the myoglobin standard were performed before and after experiments to sequence the inhibitor protein.

Table 2. Analysis of some tryptic peptides isolated from a digest of the inhibitor protein (see Fig. 2)

Peak	MH ⁺ (<i>M_r</i>) by f.a.b. m.s.	Sequence by Edman degradation	Identity (residue nos.)
10*	776 (775)	ESGDNVR	3–9
10*	647 (646)	SSAGAVR	10–16
11*	—	EQAEER	26–34
11*	949 (948)	LKQSEDDD	77–84
11†	863 (862)	SESGDVNR	2–9
12‡	920 (919)	GSESGDVNR	1–9
12‡	—	EIER	59–62, 66–69

* Pure peptide (see the text).
† As a mixture with LKQSEDDD.
‡ Mixed together.

Mass spectrometry

Samples were dissolved in 5% (v/v) acetic acid and dried *in vacuo* on the tip of a copper probe. Approx. 1–2 µl of thioglycerol/diglycerol (1:1, v/v) was added and mixed thoroughly with the sample. The probe was inserted into the source of a Kratos MS 50 mass spectrometer fitted with an Ion Tech FAB gun operating with xenon at 8 kV and 30–40 µA. Mass spectra were taken at a scan speed of 100 s/decade (Bradley *et al.*, 1982).

RESULTS

Sequence analysis of intact inhibitor protein

N-Terminal sequence analysis in a spinning-cup sequencer, summarized in Table 1, showed that the protein sample is a mixture of chains starting at residues 1, 2 and 3. These three forms were present in this sample in the approximate proportions 4:1:1 respectively. The yields of the chains corrected for losses in the initial yield are calculated to be 5.2, 1.8 and 1.3 nmol respectively. This accounts exactly for the total protein employed in the experiment (8.3 nmol by amino acid analysis) and argues strongly against a major form with a blocked N-terminal.

The experiment was repeated with two samples donated by Dr. Penefsky and Dr. Harris. Essentially the same result was obtained.

Characterization of the N-terminal peptides

In order to seek confirmation of this result and to search again for evidence for blocked forms of the protein, trypsin and clostripain digests of the inhibitor were fractionated by h.p.l.c. The resulting peptides in both digests were identified by amino acid analysis, by Edman degradation and by f.a.b. m.s.

In the fractionated tryptic digest a number of peptides were homogeneous and could be identified unambiguously from their amino acid compositions and sequences (see Fig. 2). Others were simple mixtures. The components of the mixtures could be identified, as shown in Fig. 2, by sequence analysis. One region of the fractionated digest, that eluted at about 9% acetonitrile (peaks 10–12 in Fig. 2), contained a number of peptides. These peaks were individually re-chromatographed at pH 7.8 with 50 mM-

Fig. 3. Partial h.p.l.c. chromatogram of the clostripain digest of the inhibitor protein

Peaks 3–9 gave the following MH⁺ ions by f.a.b. m.s. that matched the following peptides: peak 3 (no MH⁺ observed); peak 4 (MH⁺ *M_r* 890; AQAEEER), peak 5 (MH⁺ *M_r* 949 and 776; LKQSEDDD and ESGDNVR), peak 6 (MH⁺ *M_r* 920 and 863; GSESGDVNR and SESGDVNR), peak 7 (MH⁺ *M_r* 647; SSAGAVR), peak 8 (MH⁺ *M_r* 915; LQKEIER) and peak 9 (MH⁺ *M_r* 878; DAGGAFGKR). The fractionation was performed on a Spherisorb ODS2 column with solvent A being aq. 0.1% trifluoroacetic acid and solvent B being 0.1% trifluoroacetic acid in acetonitrile (-----).

ammonium bicarbonate as starting buffer under conditions otherwise identical with those of the first fractionation. From peak 10 the peptides ESGDNVR (residues 3–9 of the protein) and SSAGAVR (residues 10–16) were obtained as homogeneous peptides (for positions of peptides in protein refer to Fig. 1). The identity of the former was established by Edman degradation and f.a.b. m.s. (see Table 2). From peak 11 the peptides EQAEER (residues 26–34) and LKQSEDDD (residues 77–84) were obtained pure, and in addition the latter peptide was obtained in a mixture with a third peptide. Sequence analysis showed unambiguously that

the third peptide was SESGDVNR (residues 2–9). Confirmation of this result was again obtained by f.a.b. m.s. (Table 2). Re-chromatography of the third peak (number 12 in Fig. 2) failed to resolve the two components. However, once again they could both be identified unambiguously by Edman degradation and by f.a.b. m.s. (Table 2). It should be noted that the peptides isolated cover the entire sequence of the inhibitor protein. Moreover, no other peptides were isolated from the digest.

The fractionated clostripain digest was similarly analysed (Fig. 3). It was found that cleavage had not been restricted to the C-terminal side of arginine residues, but peptide bonds following some lysine residues also had

been hydrolysed. As in the tryptic digest, the components of all peaks could be assigned to specific regions of the inhibitor protein; again three peptides were identified, corresponding to residues 1–9, 2–9 and 3–9. Under the conditions employed in this experiment, these were eluted as a partially resolved group at about 20% acetonitrile (see Fig. 3). The peaks labelled 5–7 were collected in a single fraction, and the f.a.b. m.s. spectrum shown in Fig. 4 shows the presence of at least five ion clusters, with their most abundant ions at m/z 949, 920, 863, 776 and 647. When these peaks were collected in partially resolved fractions and run separately by f.a.b. m.s., the spectra (Fig. 5) showed that peak 5 contained protonated molecular ions (MH^+) at m/z 949 and 776, peak 6 MH^+

Fig. 4. Partial f.a.b. m.s. spectrum of h.p.l.c. peaks 5–7 from the clostripain digest

The major ion clusters are labelled according to their most abundant isotopes, i.e. m/z 949, 920, 863, 776 and 647.

Fig. 5. Spectra obtained by f.a.b. m.s. of the partially resolved h.p.l.c. peaks 5–7 and their corresponding clostripain peptide fragments: (a) peak 5; (b) peak 6; (c) peak 7

ions at m/z 920 and 863 and peak 7 an MH^+ ion at m/z 647. This separation clearly shows that these are not fragment ions but a series of peptides that match the molecular masses for the peptides previously identified in a tryptic digest by Edman degradation and f.a.b. m.s. (Table 1).

DISCUSSION

Two independent sets of sequence analysis experiments, carried out on the intact inhibitor protein and on peptides obtained from two separate enzyme digestions, show that the ATPase inhibitor protein from bovine mitochondria has a frayed *N*-terminus. Chains starting at residues 1, 2 and 3 were identified in three independently isolated samples of the protein. The major form, with free *N*-terminal glycine, has been observed previously (Frangione *et al.*, 1981). Examination of two enzyme digests of the protein failed to provide any evidence for a form with an α -*N*-formyl group. All peptides could be identified as components of the known sequence. Three related peptides represent residues 1–9, 2–9 and 3–9. All have free α -amino groups. The identification of α -*N*-formyl-peptide (residues 1–9) reported previously depended upon an f.a.b. m.s. spectrum (Dianoux *et al.*, 1984). This contained peaks at m/z 948, 920, 863, 776 and 647. The peak at 948 was attributed to the MH^+ of α -*N*-formyl-(residues 1–9) and the other peaks as fragment ions. In the present work a similar spectrum has been obtained with peaks with the same m/z except that a peak at m/z 949 was observed rather than at 948. This peak is the MH^+ of the *C*-terminal peptide. The peaks at m/z 920, 863 and 776 are the MH^+ ions of residues 1–9, 2–9 and 3–9 respectively, and not fragment ions, as shown by further h.p.l.c. separations of both a tryptic digest and a clostripain digest. The MH^+ with M_r 647 could correspond to either residue 4–9 or the second tryptic fragment (residues 10–16). However, this peptide was clearly identified by Edman degradation to be the second tryptic fragment. This was confirmed by esterification of this peptide with methanolic 0.5 M-HCl. The MH^+ M_r was shifted to 661 and not 675, indicating the presence of one carboxy group, as would be expected for the second tryptic fragment. These results thus limit the *N*-terminal starting points of the inhibitor protein to positions 1, 2 and 3.

Frayed *N*-terminals have also been observed in the α -

β - and δ -subunits of the bovine mitochondrial F_1 -ATPase complex (Walker *et al.*, 1985). Two explanations can be advanced to explain their origin. Firstly, they may arise as artifacts by proteolysis during protein isolation or, secondly, they may be produced by inexact proteolytic processing during import of the proteins into the mitochondrion. That samples of F_1 -ATPase and inhibitor isolated in different laboratories and by different procedures are similarly frayed tends to argue against the first proposal. Closer study of the processing event is required to test the alternative hypothesis.

We are grateful to Dr. D. A. Harris and Dr. H. S. Penefsky for gifts of inhibitor protein. B.W.G. was supported by a Science and Engineering Research Council postdoctoral fellowship.

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Received 17 September 1985/23 October 1985; accepted 13 December 1985