Mass spectral identification of the blocked N-terminal tryptic peptide of the ATPase inhibitor from beef heart mitochondria

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The presence of a formyl blocking group at the N-terminus of the ATPase inhibitor has been identified and the partial sequence of the N-terminal peptide has been determined by fast atom bombardment and field desorption coupled to mass spectrometry. Minor discrepancies in amino acid sequence of the inhibitor between the present and published data [(1981) Proc. Natl. Acad. Sci. USA 78, 7403–7407] are reported and its relationships with other inhbitors are briefly discussed.

ATPase inhibitor *N-formyl blocking group* *Fast atom bombardment* *Field desorption* *Amino acid sequence*

1. INTRODUCTION

The natural mitochondrial ATPase inhibitor (IF_1) is a small protein with M_r of ~10000 which inhibits the ATPase activity by binding specifically to one of the 5 subunits forming the soluble part of the enzyme [1,2]. IF_1, as well as other inhibitors of ATPases [3,4], have attracted considerable interest, and several studies have been carried out concerning their physiological role in the regulation of oxidative phosphorylation [1,5,6]. The amino acid sequence of the inhibitor from beef heart mitochondria has been determined [7], based predominantly on Edman degradation procedures. In studies aimed at establishing relationships between structure and biological activity of IF_1 [8–10], we have been working on the determination of the primary structure, and have employed several approaches including protease digestion, because the N-terminal residue was found to be undetectable by a variety of conventional methods. We report here the identification of formyl as N-terminal blocking group by direct M_r and structure determination of the terminal tryptic nonapeptide, using FAB and FD mass spectrometry. Further structural modifications concerning the localization of amide groups, and an apparent inhomogeneity at the C-terminus were found by a complete reevaluation of the sequence. Possible reasons for the discrepancy with the previously reported, unblocked structure and relations of the beef heart inhibitor to similar inhibitors from other sources are briefly discussed.

Abbreviations: IF_1, natural ATPase inhibitor; HPLC, high-performance liquid chromatography; FAB, fast atom bombardment; FD, field desorption; Cpase, carboxypeptidase; Apase, aminopeptidase

2. MATERIALS AND METHODS

Beef heart mitochondria were isolated as in [11] and stored at −20°C. Purification of the inhibitor was performed; (i) following [12], as modified in [13], employing alcohol precipitation and subsequent 3 min heating at 90°C; (ii) by replacing these

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two steps with DEAE–cellulose (DE-52) chromatography in 10 mM Tris buffer (pH 8.0) and an NaCl gradient. With both procedures, SDS-polyacrylamide gel electrophoresis of pure inhibitor [14] yielded a single band with an apparent M_r of ~10000. The ATPase inhibitory activity was assayed as in [1].

N-terminal peptide fragments were obtained by limited digestion with trypsin and thrombin in 50 mM NH_4CH_3COO/HCO_3 (pH 7.8) as in [9]. Fragments were separated either by gel electrophoresis followed by extraction [8], or reversed-phase HPLC [9]. Isolation of peptides for mass spectral analysis was performed with ~30 nmol hydrolysate on a C-18 column (30 × 0.6 cm) with a 10 mM trifluoroacetic acid/acetonitrile gradient [9]. Mass spectra were obtained with a Finnigan MAT 312 double focusing spectrometer/SS-188 data system. Details of instrumental equipment and experimental procedures for FAB and FD mass spectrometry have been described [15,16]. For the determination of FAB spectra, 1 μl peptide (~2 nmol) in 10% aqueous acetic acid mixed with 1 μl glycerol as liquid matrix was loaded on the FAB target and bombarded with a 7 keV Xe^0 primary beam. FD mass spectra were obtained with high-temperature activated carbon emitters [15] loaded with ~1 nmol peptide in 10% acetic acid. FD mass spectral analysis of aliquots from carboxypeptidase (Cpase) digestion was performed with ~1.5 nmol peptide, by accumulation of the complete desorption profile as in [17]. FAB exact mass measurements were carried out at a resolution of 6000 by peak matching [15] against the MH^+ of Des-Arg^9-bradykinin (m/z 905) as reference ion.

Complete proteolysis of the inhibitor was performed by digestion (16 h, 37°C) with trypsin and α-chymotrypsin (Worthington) at pH 8.2, and with *Staphylococcus aureus* protease V8 (Miles) at pH 6.5, using 0.1 M pyridine–collidine–acetate buffers adjusted to the appropriate pH. Digestion mixtures were separated on an SP–Sephadex C25 column (Pharmacia) with a gradient of increasing pH and ionic strength [18]. Further purification was achieved by gel filtration on Biogel P-2, P-4, P-6 and P-10 columns (Biorad) with 70% formic acid or 1% NH_4HCO_3, depending on the size and solubility of the fragments. N-terminal sequence analysis was performed by manual [19] and automatic Edman degradation with a Beckman 890 spinning cup sequenator. Phenylthiohydantoins were identified by HPLC as in [20]. N-terminal sequences were further determined by digestion with Apase M and leucine Apase (Boehringer Mannheim) in 0.1 M pyridine–collidine–acetate (pH 8.2) at 37°C. C-terminal sequences were analyzed by Cpase digestion at 37°C followed by amino acid analysis [21], with the following enzymes: Cpase A (Worthington) and B (Sigma) (pH 8.5); Cpase P (Takara Shuzo, Tokyo) in 0.1 M pyridinium formate (pH 2.5). Hydrazinolysis followed by direct amino acid analysis was carried out as in [22].

3. RESULTS

Automatic (100–200 nmol) as well as manual (30–50 nmol) Edman degradation of the inhibitor was repeatedly performed without success, yielding at most ~3% glycine from protein isolated by either purification procedure. Further evidence for the blocked N-terminus was obtained from the following experiments which were all negative to reveal a detectable N-terminal residue: dansylation followed by TLC analysis; Apase M and leucine Apase digestion; dinitrophenylation and methylamine reaction [23]; incubation with pyroglutamate aminopeptidase (Boehringer Mannheim) and with acylamino acid-releasing enzyme [24] (donated by Dr S. Tsunasawa, Osaka University). Digestion of ^{14}C-succinylated inhibitor with trypsin yielded peptide fragments, separated by Biogel P-2 chromatography [18], one of which was shown to be the terminal peptide by amino acid composition (see below), but contained no radioactivity. No free amino terminus was also found for a tripeptide with the composition, $Ser_1Glu_1Gly_1$, isolated after *S. aureus* V8 protease digestion. Limited digestion of the inhibitor with trypsin and thrombin both released a peptide identified as T(1–9) [9], with the composition, $Asp_2Ser_2Gly_2Glu_1Val_1Arg_1$, which was purified by HPLC and subjected to mass spectral analysis. While the C-terminus of this peptide was amenable to Cpase digestion, any conventional N-terminal method, i.e., Edman degradation, dansylation, dinitrophenylation and succinylation failed. Its FAB mass spectrum (fig.1a) revealed an ion of highest mass at m/z 948 which was compatible

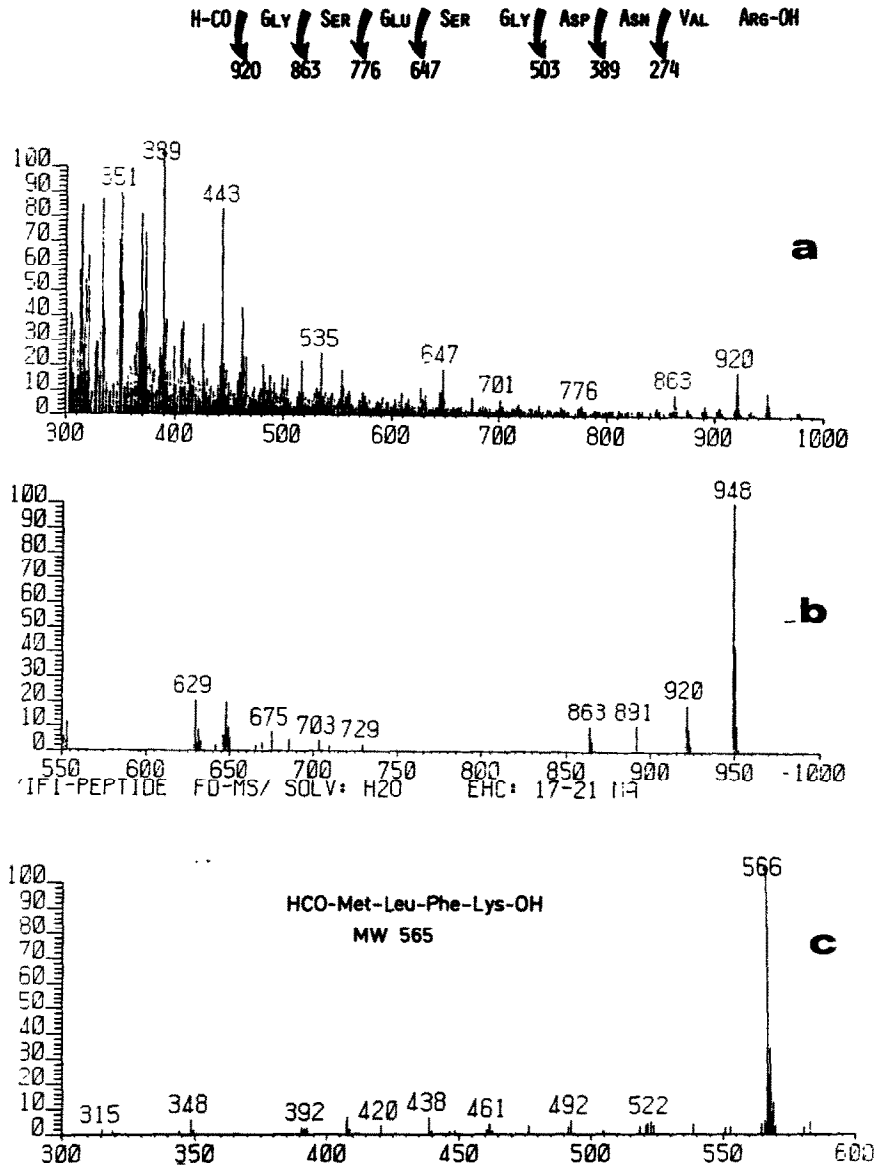

Fig.1. (a) FAB mass spectrum of the N-terminal peptide T(1–9) obtained by digestion of IF_1 with thrombin [9] and HPLC isolation; (b) FD mass spectrum of T(1–9) at 17–21 mA emitter heating current [17]; (c) FD mass spectrum of HCO-Met-Leu-Phe-Lys (M = 565) at 16–19 mA emitter heating current. For other experimental conditions see section 2.

with the protonated molecular ion, MH^+, of a formylated peptide by subtraction of the calculated M_r (919). The same, most abundant MH^+ ion was obtained in the FD mass spectrum (fig.1b). Precise mass measurement of this ion yielded the elemental composition, $C_{35}H_{58}N_{13}O_{18}$ (found: 948.4048; calc. 948.4027, δ = 2.1 mu) which, due to the absence of other possible sites of formylation, established the identity of the amino-terminal formyl residue. The sequence of this peptide was, partially, deduced directly from the FAB spectrum, which by peptide bond cleavage yields characteristic fragment ions with protonated amino end groups [15,25]. Thus, fragments at m/z 920, 863, 776, 647, 503 and 389 led to the assignment, HCO-Gly-Ser-Glu-(Ser,Gly)-Asp-Asn-

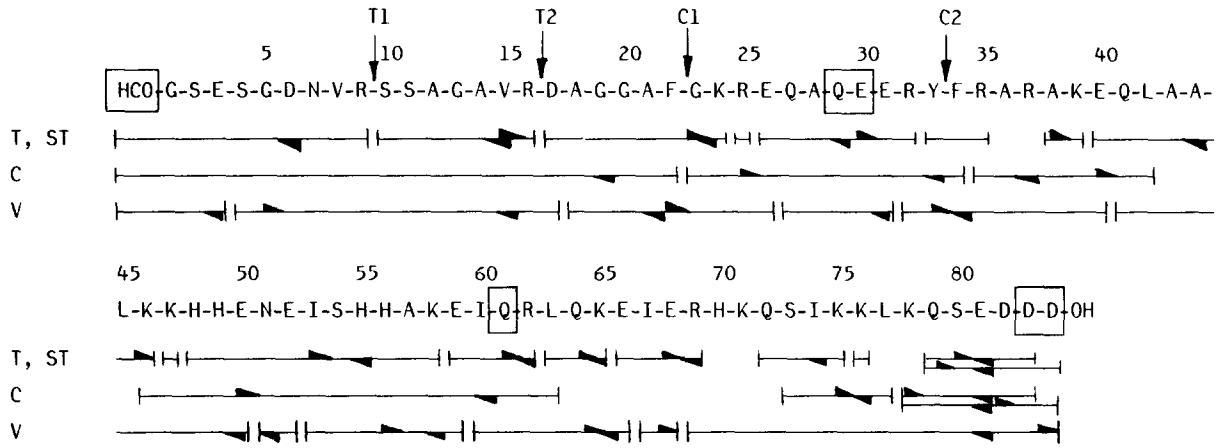

Fig.2. Complete sequence of the ATPase inhibitor from beef heart mitochondria. Sequences in brackets (⊢—⊣) indicate peptides obtained by digestion with trypsin (T), trypsin digestion of ^{14}C-succinylated inhibitor (ST), digestion with α-chymotrypsin (C) and *S. aureus* V8 protease (V). Arrows above peptide fragments (——►) denote partial sequences obtained by manual Edman degradation, lower arrows (◄——) denote partial sequences determined by carboxypeptidase A, B and/or P digestion [8,10]. Fragments T1, T2, C1 and C2 indicate tryptic/chymotryptic peptides obtained by limited proteolytic digestion. Positions 10–16 of T1, and 34–52 of C2 were determined by automatic Edman degradation. Revised sequence positions [7] are indicated by boxes.

(Val,Arg) (see scheme in fig.1). The sequence was complemented and confirmed by FD mass spectra after partial Cpase digestion [17], yielding MH^+ ions at *m/z* 792, 693 and 578, and by digestion with *S. aureus* V8 protease. Notable was the ion of low abundance at *m/z* 920 due to the deformylated peptide in the FD spectrum (fig.1b), which was obtained at conditions that minimize fragmentation [15,17]. FD mass spectra of several N-formyl oligopeptides did not show any significant deformylated fragment ions, as illustrated by the spectrum of HCO-Met-Leu-Phe-Lys (*M* = 565, fig.1c), which suggested the presence of a small amount of unblocked N-terminal tryptic peptide of IF_1.

The complete sequence of the inhibitor was determined by analysis of tryptic (T), chymotryptic (C) and *S. aureus* protease V8 (V) fragments (fig.2). Although the tryptic and chymotryptic peptides did not cover the entire protein, all peptides from the staphylococcal protease digestion were recovered and together provided sufficient overlapping for a conclusive sequence assignment. In addition to the N-terminal formyl group, modifications at 3 further sites (see boxes in fig.2) were obtained by this study, which otherwise is in accord with the previously published structure [7]. Positions 29/30 were assigned -Gln^{29}-Glu- instead of -Glu^{29}-Gln- [7] by sequence analysis of the fragment, T(26–32), which was confirmed by the *S. aureus* protease digest yielding only one peptide, V(27–31). Position 61 was shown to be Gln instead of Glu by the C-terminal sequences of fragments ST(38–62) and C(46–63), and by the staphylococcal digest which yielded only a single fragment, V(59–66) in that region. Thus, the sequences (58–62) and (65–69) are not repetitive as reported [7]. Furthermore, a detailed sequence analysis of the C-terminus revealed inhomogeneity by one Asp residue, since two peptides differing only by one terminal Asp were separated and identified both by tryptic [T(79–83), T(79–84)] and chymotryptic [C(78–83), C(78–84)] digestion.

4. DISCUSSION

The unequivocal identification of formyl as N-terminal blocking group of the beef heart mitochondrial ATPase inhibitor by low- and high-resolution FD and FAB mass spectrometry of its terminal tryptic peptide is consistent with the failure of all our previous conventional methods, but was surprising in view of the communication by authors in [7] who obtained the N-terminal sequence without reporting a specific deblocking

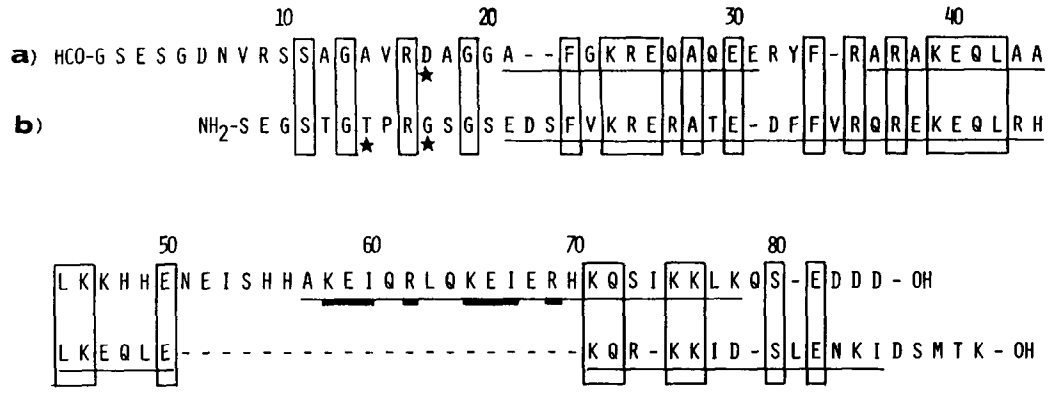

Fig.3. Comparison of the amino acid sequence of natural ATPase inhibitors from (a) beef heart and (b) yeast *S. cerevisiae* mitochondria. (▭) Common residues, (▬) repeated sequence, (——) α-helix region, (★) β-turn, absence of β-sheet [29].

procedure. The possibility of artificial formylation, upon treatment with formic acid, during isolation of the inhibitor and/or N-terminal peptides was, therefore, carefully examined and appears to have been safely excluded: (i) by identical results ($\leq 3\%$ of terminal Gly by automatic Edman degradation) obtained with two different purification procedures of the inhibitor [7,13], one of which is especially mild and avoids heat treatment; (ii) peptides repurified in the presence of formic acid here and in [8,21] were successfully subjected to up to 40 steps of Edman degradation; (iii) purification of samples of the inhibitor and its terminal peptide used for mass spectral analysis was carried out without employing formic acid. Moreover, FD mass spectra of several model oligopeptides obtained from aqueous formic acid solutions did not show any ions due to formylation products. The presence of some de-formylated protein is suggested by the small amount of terminal Gly observed, and the minor ion at *m/z* 920 due to the free peptide, T(1–9) (fig.1b). Besides possibly a different source of material, partial or complete deformylation of the inhibitor during purification procedures in [7] may then be a possible reason for the discrepancy with the previous study. Only a few examples of natural N-formyl proteins or peptides, besides proteins with formyl-Met have been described [26,27]. As shown here, FD and FAB mass spectrometry using an appropriate terminal polypeptide fragment is particularly suited for their direct structural identification.

The biological significance of the formylated N-terminal part of IF_1 is uncertain, since the fragments T1 and T2 obtained by limited tryptic digestion (see fig.2) retained most of the ATPase inhibitory activity. In contrast, the smaller chymotryptic fragments C1 and C2 are devoid of activity. However, the biological activity was substantially reduced after Cpase P digestion of approx. 10 amino acids [10]. With the use of these data, a structural comparison of the beef heart inhibitor with the shorter sequence of 63 residues of the ATPase inhibitor from the yeast *Saccharomyces cerevisiae* [3] revealed extensive homology with 26 common positions both at the central and the C-terminal region of the two proteins, suggesting the critical role of these areas for the biological function (fig.3). In comparison with the mammalian and yeast inhibitor, ATPase ϵ-subunits from bacteria and chloroplasts [4,28] do not show any apparent structural similarity.

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