The complete primary structure of GTP:AMP phosphotransferase from beef heart mitochondria

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To complete the amino acid sequence of GTP:AMP phosphotransferase (MgGTP+AMP ⇌ MgGDP+ADP) from beef heart mitochondria it was necessary to sequence an intermediate region of about 33 residues after position 102 [(1984) Eur. J. Biochem. 143, 331–339] and find a suitable overlap with the rest of the protein. The required peptides were obtained by cleaving the enzyme with endoproteinase Lys-C. One peptide, covering the region from residue 79 to 144, was sequenced up to residue 124. Another peptide, extending from residue 79 to 169, was subcleaved with *Staphylococcus aureus* V8 protease and provided the fragment from residue 99 to 139 which was sequenced. Several other peptides from endoproteinase Lys-C cleavage were used to check large sections of the previously published sequence work. The complete sequence contains 225 amino acids and has an M_r of 25 469

GTP:AMP phosphotransferase *Mitochondria* *HPLC* *Amino acid sequence* *Gas-phase sequencer* *Adenylate kinase*

1. INTRODUCTION

GTP:AMP phosphotransferase (AK3, MgGTP + AMP ⇌ MgGDP + ADP) is an enzyme homologous to adenylate kinase (ATP:AMP phosphotransferase) [1,2]. However, the two enzymes differ with respect to their specificity for the nucleoside triphosphate. AK3 is specific for GTP (ITP) [3,4], while adenylate kinase uses ATP and, to some extent, other nucleoside triphosphates [5–7]. Elucidation of the structures of these kinases is essential to solve their catalytic mechanism. The primary structures of several ATP:AMP phosphotransferases have been reported [8–13] and crystals from porcine AK1 allowed the construction of a three-dimensional model at 0.3 nM resolution. Crystallographic studies on AK3 [14] as well as its partial primary structure [1] have been reported. The present work was undertaken to complete the amino acid sequence of AK3 and thus facilitate further X-ray analyses.

2. MATERIALS AND METHODS

Endoproteinase Lys-C from Lysobacter enzymogenes (Boehringer Mannheim) and *Staphylococcus aureus* V8 protease (Miles Laboratories) were used for proteolytic cleavage. Reagents and solvents for the gas-phase sequencer were from Applied Biosystems. All other chemicals were of analytical grade.

2.1. *Protein preparation and proteolytic cleavages*

AK3 was prepared and assayed according to Tomasselli et al. [2]. The enzyme used here was homogeneous by SDS-polyacrylamide gel electrophoresis and had a specific activity of 270–300 U/mg.

2.2. *Endoproteinase Lys-C cleavage*

Two different procedures were used: (i) 433 nmol AK3 was incubated with 56 μg (12 U/mg) endoproteinase Lys-C in 3.6 ml of 60 mM NH_4HCO_3, pH 8.2, at 37°C for 165 min.

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Then, a further 40 μg endoproteinase Lys-C was added to the incubation mixture and the digestion continued for another 165 min. 100 μl incubation mixture was withdrawn, boiled for 10 min, dried, taken up with 60% acetonitrile in 0.1% TFA (solution B) and analyzed by HPLC. Since the proteolytic cleavage was poor, the main incubation mixture was made 0.1% in SDS and allowed to react for another 15 h. The reaction was stopped by boiling for 10 min. It was dried and taken up with solution B for HPLC separation. (ii) 100 nmol AK3 in 1.0 ml of 60 mM NH_4HCO_3, pH 8.2, was warmed to 60°C for 15 min to unfold the protein. It was then incubated with 42 μg endoproteinase Lys-C at 37°C for 165 min. 21 μg endoproteinase Lys-C was added to the incubation mixture and the reaction continued for another 135 min. The products were analyzed by HPLC.

2.3. S. aureus *V8 protease cleavage*

S. aureus V8 protease [15] sub-digestion of K15 (fig.1) was carried out with 15 nmol peptide and 30 μg protease in 300 μl of 60 mM NH_4HCO_3, pH 7.5–8.0, at 37°C for 25 h. The reaction was stopped by boiling for 15 min and the products analyzed by HPLC.

2.4. *Sequence analysis*

Amino acid analysis with 1–5 nmol peptide, Edman degradation with 0.5–5 nmol peptide, and identification of the amino acid-phenylthiohydantoins were done as described in [12]. Here, a gas-phase sequencer (model 470A, Applied Biosystems) was used. The initial and repetitive yields were 48 ± 22 and 93 ± 5%, respectively. An amino acid-phenylthiohydantoin was accepted when the area corresponding to its peak increased more than 40% over its value in the preceding cycle. Each residue in the missing area was sequenced at least twice in different peptides.

3. RESULTS AND DISCUSSION

The sequence of AK3 was previously published with a gap of about 33 residues after residue 102 [1]. Fig.1 summarizes the strategy we have used here to fill the gap and to check large sections of the previously published sequence work. AK3 was cleaved with endoproteinase Lys-C using procedures (i) and (ii). The peptides resulting from these cleavages were separated by HPLC (fig.2a,b) and analyzed for amino acid compositions. Both K15 and K16 contained the unsequenced segment. Their amino acid compositions and HPLC yields and those of K'14 and of subcleavage products of K15 (see below) are given in table 1. Those peptides shown in fig.1 but not presented in table 1 had amino acid compositions that confirmed the previously published sequence.

K15, covering the region from residue 79 to 169, was subcleaved with *S. aureus* V8 protease. After drying, the products of the cleavage were taken up with 0.1% TFA (solution A of the HPLC), centrifuged, and the supernatant analyzed by HPLC (fig.2c). Amino acid compositions (table 1) of the well-resolved peptides of fig.2c indicated that the

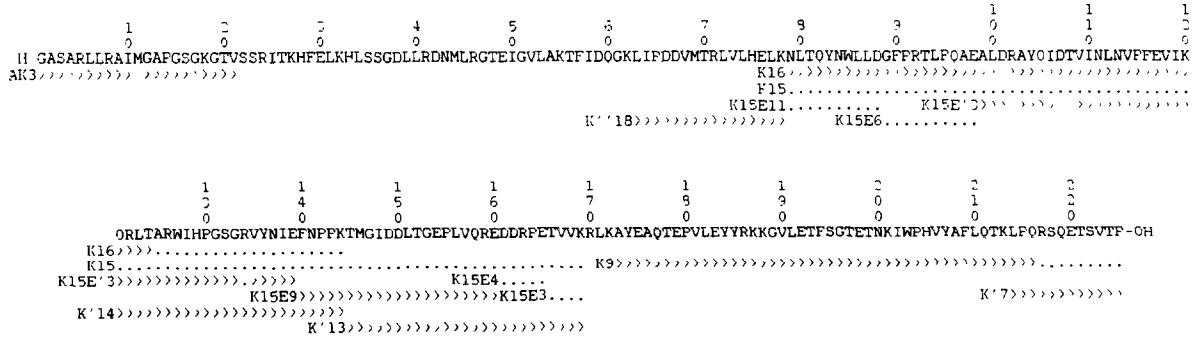

Fig.1. Summary of the proof of the GTP:AMP phosphotransferase (AK3) sequence. The nomenclature of the peptides is explained in fig.2 except for K''18, a peptide obtained from subcleavage of a cyanogen bromide fragment (called C2C5 in [1]) with endoproteinase Lys-C. The peptides underlined by > were sequenced by Edman degradation, except for the residues underlined by • which were only identified by amino acid analysis.

Table 1

Amino acid compositions of endoproteinase Lys-C peptides (K...) and *S. aureus* V8 subcleavage products (K15E...)

	K'14 121–144	K16 79–144	K15 79–169	K15E11/ E'2 79–88	K15E6 89–98	K15E'3 99–139	K15E9 140–160	K15E4 161–165	K15E3 166–169
Asx	1.7 (2)	8.6 (9)	12.2 (13)	2.7 (3)	–	5.0 (5)	3.0 (3)	1.8 (2)	–
Thr	0.9 (1)	3.9 (4)	6.8 (7)	1.1 (1)	0.7 (1)	2.0 (2)	2.1 (2)	–	1.0 (1)
Ser	1.2 (1)	1.2 (1)	1.3 (1)	–	–	1.1 (1)	–	–	–
Glx	1.7 (2)	7.3 (7)	11.1 (11)	1.0 (1)	2.2 (2)	4.3 (4)	3.3 (3)	1.1 (1)	–
Pro	2.8 (3)	5.7 (6)	7.7 (8)	–	2.1 (2)	2.1 (2)	2.8 (3)	1.0 (1)	–
Gly	1.8 (2)	3.0 (3)	4.8 (5)	–	1.1 (1)	2.1 (2)	2.0 (2)	–	–
Ala	1.0 (1)	4.0 (4)	3.9 (4)	–	1.1 (1)	2.3 (3)	–	–	–
Val	0.5 (1)	1.5 (4)	2.7 (7)	–	–	2.0 (2)	0.6 (1)	–	1.3 (2)
Met	–	–	0.9 (1)	–	–	–	0.8 (1)	–	–
Ile	1.1 (2)	2.3 (5)	3.4 (6)	–	–	2.8 (5)	0.8 (1)	–	–
Leu	1.3 (1)	7.3 (7)	9.1 (9)	3.0 (3)	0.7 (1)	3.0 (3)	2.1 (2)	–	–
Tyr	0.8 (1)	2.7 (3)	3.1 (3)	1.0 (1)	–	1.7 (2)	–	–	–
Phe	1.0 (1)	3.2 (3)	3.4 (3)	–	0.9 (1)	1.0 (1)	1.1 (1)	–	–
Lys	1.3 (1)	2.0 (2)	2.9 (3)	–	–	1.0 (1)	1.2 (1)	–	1.2 (1)
His	0.8 (1)	0.8 (1)	0.8 (1)	–	–	0.5 (1)	–	–	–
Arg	2.6 (3)	4.5 (5)	6.6 (7)	–	1.1 (1)	4.0 (4)	1.8 (2)	1.1 (1)	–
Trp	–[a] (1)	–[a] (2)	–[a] (2)	– (1)	–	– (1)	–	–	–
Total	24	66	93	10	10	39	22	5	4
yield (%)	27	17	18	54	75	30	51	78	61

[a] Tryptophan was found by Ehrlich reagent

Amino acids found in the sequence are in parentheses. (–) Values lower than 0.3

sequence spanning from residue 99 to 139 was missing. Hence, we dissolved the precipitate in solution B (of the HPLC) and analyzed it by the same HPLC procedure (fig.2d). Peptide K15E'3 was sequenced and it covered the region from residue 99 to 139 producing the needed overlap. Peptide K15E'2 had the same amino acid composition as peptide K15E11 (fig.2c). K'14 strengthened the overlap in the area under study, identified positively Arg 134 (that was unclear in the K15E'3 sequence) and confirmed the K15E'3 sequence.

The sequence of K16 revealed that a Trp in position 85 and not a Pro as previously published [1] was present. Furthermore, the sequence of K15E'3 could not confirm the sequence of a peptide called T16 in [1]. T16, that was provisionally assigned to the gap region [1], had the positions of Trp and Pro reversed and contained an additional Thr residue poorly supported by amino acid composition. The problem concerning Trp and Pro was caused by the difficulty in separating phenylthiohydantoin-Trp and phenylthiohydantoin-Pro with the HPLC column and the conditions used in [1]. All other Trp and Pro residues published in [1] were checked (fig.1) and found to be correct.

Concerning the specificity of Lys-C we have noted that, when AK3 was denatured with SDS, there was no appreciable cleavage at several Lys. For example, both the peptides K16 and K15 (obtained with 17 and 18% yield, respectively) contained Lys 120, and peptide K9 (173–225) (obtained with 38% yield) contained 4 Lys. On the other hand, when AK3 was denatured by heat, the cleavage at Lys 120 took place (recovery of peptide K'14 was 27%) and instead of K9 we obtained 4 fragments as a result of cleavage at Lys 188, 201 and 213 (Lys 187 was not cleaved).

Concerning the specificity of the *S. aureus* V8

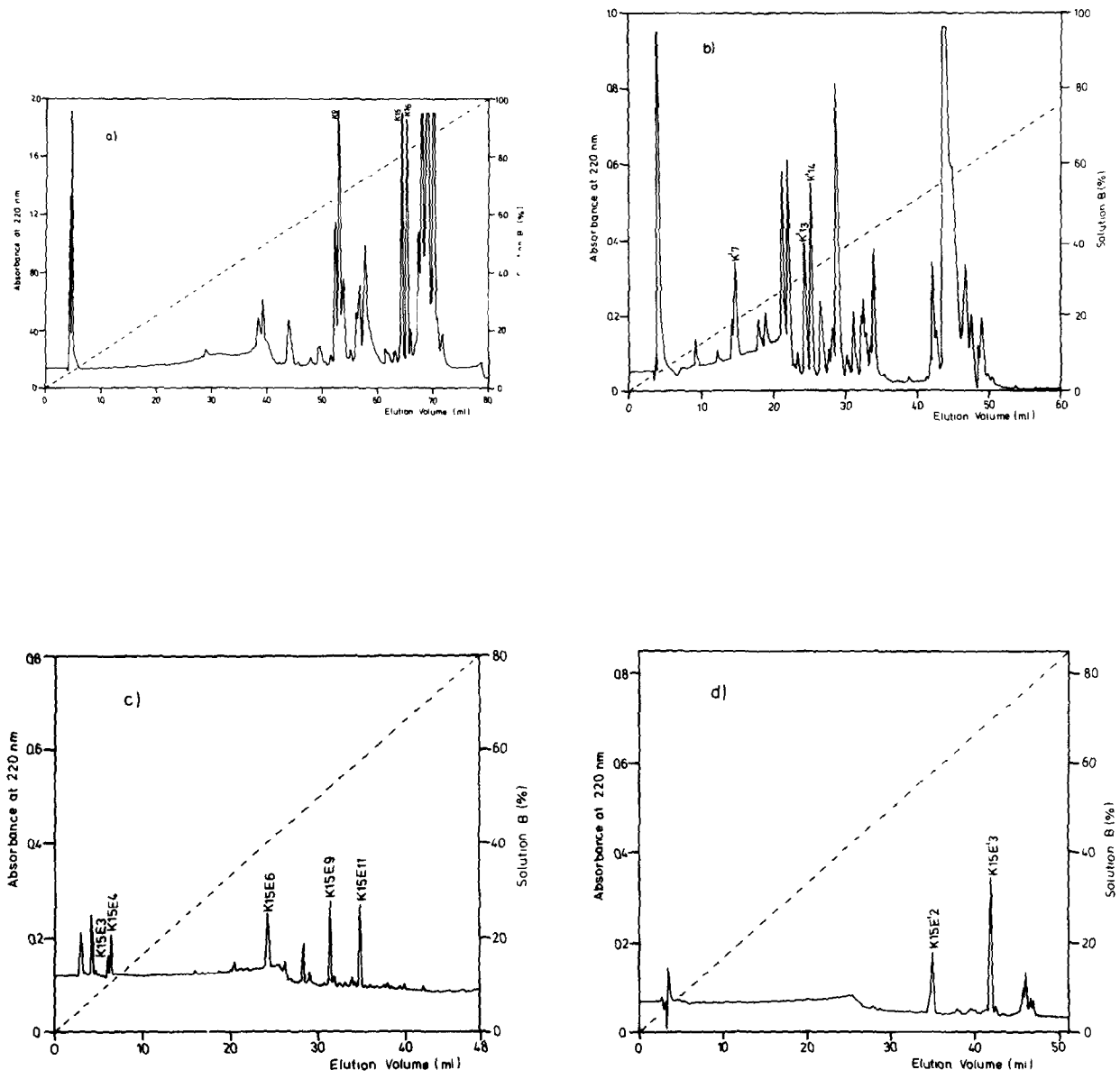

Fig.2. Separations of peptides by reverse-phase HPLC. (a,b) Cleavage of AK3 with endoproteinase Lys-C: (a) in the presence of SDS (peptides named K...), (b) in the absence of SDS (peptides named K'...). (c,d) Subcleavage of K15 with *S. aureus* V8 protease: (c) the samples were taken up in 0.1% TFA (solution A), centrifuged and the supernatant injected (peptides named K15E...), (d) the pellet of the centrifugation was taken up with 60% acetonitrile in 0.1% TFA (solution B) and injected (peptides named K15E'...). Each set of peptides was numbered according to elution from the HPLC. In all cases a gradient of 0–100% solution B vs 0.1% TFA was run through a μBondapak phenyl column (Waters) at a flow rate of 1.0 ml/min at 50°C. (a,b) 80 min gradient; (c,d) 60 min gradient. Amounts of samples injected: (a) 4.5 nmol, (b) 5.5 nmol, (c) 3.4 nmol, (d) 3.2 nmol. A DuPont 830 liquid chromatograph equipped with an 838 programmable gradient was used.

protease, we have noted that among the 7 Asp residues present in peptide K15 only the Asp-Gly linkage was cleaved at an appreciable rate, in agreement with [15]. Among the 6 Glu residues present the Glu–Val bond was resistant to cleavage as reported in [15]. We have also noted that the Glu–Pro bond was resistant to cleavage, while the Glu–Phe bond was split even though the recovery of that peptide was somewhat lower than for the other E peptides (table 1).

In the present work the N-terminus of AK3 was also found to be heterogeneous as reported [1]. We are confident that the sequence segments of a total of 44 amino acids not checked in the present work are correct, since it was obtained at least 3 times from different peptides in [1].

The complete sequence analysis shows that AK3 has 225 amino acids (M_r 25469). This M_r was used to recalculate the enzyme's amino acid composition from the data of [2]. The amino acid composition calculated here (table 2) is in good agreement with that inferred from the sequence analysis. In [1] the N-terminal residues 1–102 and the C-terminal residues 135–225 (final numbering) of AK3 were aligned to the sequence of AK1. The complete sequence of AK3 confirms the correctness of the alignment. AK3 has 31 amino acids more than AK1 [8] and 13 less than AK2 [11]. Both AK3 and AK2 have a stretch of amino acids (25 in AK3, 30 in AK2) in the middle of the chain that is also present in cytosolic AK from yeast [12] and *E. coli* [13], but absent in mammalian cytosolic AK1 [8–10]. The function of this additional sequence will be clarified when the forthcoming X-ray analysis yields the tertiary structure of the enzyme.

Table 2

Amino acid composition of GTP:AMP phosphotransferase

	A	B
Aspartic acid and asparagine	20.2	20 (12 Asp + 8 Asn)
Threonine	18.6	19
Serine	11.6	10
Glutamic acid and glutamine	25.1	25 (15 Glu + 10 Gln)
Proline	14.2	14
Glycine	15.4	16
Alanine	13.4	12
Valine	14.3	15
Methionine	3.6	4
Isoleucine	11.3	12
Leucine	27.2	26
Tyrosine	7.0	7
Phenylalanine	6.9	7
Histidine	4.6	5
Lysine	14.0	14
Arginine	15.9	16
Tryptophan	2.0	3
Total residues		225

(A) Calculated from the data of [2] assuming M_r 25469; (B) from sequence data

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REFERENCES

[1] Wieland, B., Tomasselli, A.G., Noda, L.H., Frank, R. and Schulz, G.E. (1984) Eur. J. Biochem. 143, 331–339.
[2] Tomasselli, A.G., Schirmer, R.H. and Noda, L.H. (1979) Eur. J. Biochem. 93, 257–262.
[3] Chiga, M., Rogers, A.E. and Plaut, G.W.E. (1961) J. Biol. Chem. 236, 1800–1805.
[4] Tomasselli, A.G. and Noda, L.H. (1979) Eur. J. Biochem. 93, 263–270.
[5] O'Sullivan, W.J. and Noda, L.H. (1968) J. Biol. Chem. 243, 1424–1433.
[6] Ito, Y., Tomasselli, A.G. and Noda, L.H. (1980) Eur. J. Biochem. 105, 85–92.
[7] Tomasselli, A.G. and Noda, L.H. (1980) Eur. J. Biochem. 103, 481–491.
[8] Heil, A., Müller, G., Noda, L.H., Pinder, T., Schirmer, R.H., Schirmer, I. and Von Zabern, I. (1974) Eur. J. Biochem. 43, 131–144.
[9] Von Zabern, I., Wittmann-Liebold, B., Untucht-Grau, R., Schirmer, R.H. and Pai, E.F. (1976) Eur. J. Biochem. 68, 281–290.
[10] Kuby, S.A., Palmieri, R.H., Frischat, A., Fischer, A.H., Wu, L.H., Maland, L. and Manship, M. (1984) Biochemistry 23, 2392–2399.

[11] Frank, R., Trosin, M., Tomasselli, A.G., Noda, L.H., Krauth-Siegel, L. and Schirmer, R.H. (1986) Eur. J. Biochem. 154, 205–211.
[12] Tomasselli, A.G., Mast, E., Janes, W. and Schiltz, E. (1986) Eur. J. Biochem., in press.
[13] Brune, M., Schumann, R. and Wittinghofer, F. (1985) Nucleic Acids Res. 13, 7139–7151.
[14] Pai, E.F., Schulz, G.E., Tomasselli, A.G. and Noda, L.H. (1983) J. Mol. Biol. 164, 347–350.
[15] Houmard, J. and Drapeau, G.R. (1972) Proc. Natl. Acad. Sci. USA 69, 3506–3509.