Amino acid sequence of the heat-stable inhibitor of the cAMP-dependent protein kinase from rabbit skeletal muscle
(protein kinase inhibitor/protein phosphorylation/microsequencing)

JOHN D. SCOTT*, EDMOND H. FISCHER†, KOJI TAKIO†, JACQUES G. DEMAILLE†, AND EDWIN G. KREBS*

*Howard Hughes Medical Institute and Department of Pharmacology, and †Department of Biochemistry, University of Washington, Seattle, WA 98195; and ‡Faculté de Médecine de Montpellier and Centre de Recherches de Biochimie Macromoléculaire du Centre National de la Recherche Scientifique, B.P. 5051, 34033-Montpellier, France

Contributed by Edwin G. Krebs, May 13, 1985

ABSTRACT The amino acid sequence of the heat-stable inhibitor of the cAMP-dependent protein kinase from rabbit skeletal muscle has been determined by microsequencing techniques. The amino terminus of the inhibitor is blocked by a unidentified group. The amino-terminal region of the molecule contains the kinase inhibitory domain, and synthetic peptides based on the sequence of residues 11–30 are potent competitive inhibitors of the cAMP-dependent protein kinase. The amino-terminal amino acid sequence of any protein.

The heat-stable inhibitor of the cAMP-dependent protein kinase, PKI, interacts specifically with the free catalytic subunit (C) of this enzyme after dissociation of the holoenzyme form (1–3). PKI, initially isolated from rabbit skeletal muscle by means involving heating at 90°C, ion-exchange chromatography, gel filtration, and acid-purification, was reported to have a molecular weight of 26,000 (2). Later, purification to a state of homogeneity was achieved by affinity chromatography on C-Sepharose; this purification made it possible to determine some of the chemical characteristics of the inhibitor (4). It was found that the amino terminus of the molecule is blocked. It was also shown that PKI is an acidic molecule (pI 4.24) lacking trypsin, proline, and sulfur-containing amino acids. The existence of three charge isoforms of the inhibitor has been reported (5, 6) on the basis of their characteristics of elution from DEAE-cellulose. Each charge isoform can exist in two apparent molecular weight forms (6–8), thus making a total of six possible isoforms.

PKI is an extremely potent competitive inhibitor (Ki of 0.5–2 nM) of C (4, 9). This value is approximately four orders of magnitude lower than the Ki for the synthetic peptide substrate Leu-Lys-Arg-Arg-Ala-Asp-Leu-Gly (Kemptide) commonly used to assay the enzyme (10). Several investigators have established that basic residues, in particular arginine side chains preceding the serine or threonine susceptible to phosphorylation, are essential recognition sites for the PKI molecule. The amino terminus of the inhibitor is blocked by an unidentified group. The amino-terminal region of the molecule contains the kinase inhibitory domain, and synthetic peptides based on the sequence of residues 11–30 are potent competitive inhibitors of the cAMP-dependent protein kinase. The amino-terminal amino acid sequence of any protein.

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PKI can be isolated only in extremely small amounts, and sequence determination was not feasible until the development of microsequencing techniques (18) and amino acid analysis in the picomolar range (19). This report documents the determination of the amino acid sequence of rabbit skeletal muscle PKI, using >200 μg of purified protein.

MATERIALS AND METHODS

Materials. Highly purified PKI was obtained by use of a new procedure involving reversed-phase HPLC (unpublished observation). All standard chemicals were purchased from Sigma. Proteinases were obtained from Worthington. HPLC solvents were supplied by Burdick and Jackson (Muskegon, MI). All sequenator chemicals and reagents were from Applied Biosystems (Foster City, CA). Kimax hydrolysing ammles for amino acid analysis were purchased from Kimble (Toledo, OH).

Selective Enzymic Cleavage. Intact PKI was digested with various proteinases that generated the fragments required to prove the structure of the molecule. Arginyl and lysyl bonds were cleaved by digestion with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin; PKI (10 nmol) dissolved in 1% (wt/vol) NH4HCO3 (pH 7.8) was treated with 1 μg of trypsin for 1 hr at 37°C. The reaction was stopped by freezing prior to drying. Hydrolysis of tyrosyl, phenylalaninyl, and leucyl bonds was obtained with chymotrypsin; PKI (3 nmol) dissolved in 1% (wt/vol) NH4HCO3/10 mM p-amino benzamidine, pH 7.8 was incubated with 600 ng of chymotrypsin for 6 hr at 37°C. The reaction mixture was frozen prior to drying. Digestion of tryptic fragment T1 (5 nmol) was carried out with chymotrypsin (250 pmol) as described above. Glutamyl and some aspartyl bonds were cleaved with Staphylococcus aureus V8 proteinase. Intact PKI (5 nmol) dissolved in 0.1 M NH4HCO3/2 M urea was incubated with 1 μg of proteinase for 18 hr at 37°C, the pH was adjusted to 2.5 with 10% trifluoroacetic acid, and the acidified digest was applied to the HPLC column for separation of fragments.

Abbreviations: PKI, the heat-stable inhibitor of the cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; RI and RII, types I and II regulatory subunits of the cAMP-dependent protein kinase.
Decylation of the Amino Terminus. This was achieved by acid treatment. Peptide T,C (1 nmol) was dissolved in 12 M HCl and incubated at room temperature for 16 hr. The resulting peptide mixture was sequenced. It is assumed that the reaction proceeds through the N → O migration of the blocking group from the α-amino group of threonine-1 to the hydroxyl.

Peptide Separation. All enzymic digestion mixtures were fractionated by reversed-phase HPLC with a Varian 5000 liquid chromatograph using a SynChropak RP-P (SynChrom, Linden, IN) C18 column. The flow rates were 2 ml/min and elution was by gradients of acetonitrile in aqueous trifluoroacetic acid (20). Peptide elution was monitored at 206 and 275 nm.

Handling of Peptides. Since only minute quantities of peptides were generated, special precautions were implemented to prevent contamination and loss of material during experimental manipulations. All enzymic cleavages, collections of fractionated peptides, and amino acid analyses were carried out in tubes that had been heated to 500°C in a furnace for 8 hr prior to use. To minimize loss of material when drying samples, a Savant Speed Vac concentrator centrifuge was used.

Amino Acid Analysis. The compositions of peptides were determined by HPLC using the Waters PICO-TAG system (19). Approximately 100 pmol of each peptide was hydrolyzed in evacuated Kimax hydrolysis ampules with 6 M HCl for 16 hr at 110°C. The free amino acids were derivatized with an excess of phenylisothiocyanate to yield phenylthiocarbamoyl derivatives. The phenylthiocarbamoyl amino acids were resolved on a Waters PICO-TAG column equilibrated in 13 mM sodium acetate/0.05% (vol/vol) triethylamine/6% (vol/vol) acetonitrile, pH 6.4, and elution was achieved by increasing the acetonitrile concentration. Phenylthiocarbamoyl amino acids were detected by absorbance at 254 nm.

Sequence Determination. Peptide sequences were determined with an Applied Biosystems AB 50 gas-phase sequenator by the method of Hewick et al. (18). Identification of phenylthiohydantoin amino acids was carried out on complementary HPLC systems as described (21, 22) and was semiquantitative.

RESULTS

General Strategy for Sequence Analysis. Proof of the structure of PKI was based upon a sequence obtained from a complete set of nonoverlapping tryptic fragments. This was confirmed with fragments obtained from digestions with chymotrypsin and S. aureus V8 proteinase. This strategy is illustrated in Fig. 1, which displays the location of the peptides within the molecule. Fig. 1 also indicates the location of peptide MCP1, which represents a biologically active fragment capable of potent inhibition of the catalytic subunit of the cAMP-dependent protein kinase. Details of the isolation and partial sequence of MCP1 have been presented (17).

Cleavage with Trypsin. Fig. 2 represents the separation of
Table 1. Amino acid composition of peptides isolated from PKI

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Column headings give peptide designations (see Fig. 1) and residues included within each. The one-letter abbreviation for each amino acid is given in parentheses after the three-letter representation. Numbers in parentheses represent the number of residues identified in sequence analysis.

*Not determined.

products obtained from tryptic digestion of PKI. All peptides were completely separated; their amino acid compositions are presented in Table 1. Peptide T1 was found to be blocked at the amino terminus and was assumed to represent the amino-terminal 15 residues, since the native molecule had been shown to be blocked by Demallie et al. (4). Incomplete enzymic degradation was observed in peptides T2 and T3; in both cases, tryptophan residues were not cleaved by trypsin. This may be accounted for by neighboring acidic residues surrounding both lysines, which can limit the effectiveness of trypsin (23).

Incomplete cleavage of arginine 18 and 19 yielded a minor peptide (T3a), corresponding to peptide T5 with an additional arginyl group (Fig. 3), as shown by its amino acid composition (Table 1).

The amino acid analysis of peptides T1 to T5 (Table 1) were reasonably consistent with their observed sequences. Sequenator analysis of peptides T2–T3 placed 60 residues (Fig. 3).

Sequence Determination of Residues 1–7. Since the tryptic fragment T1 was blocked, further digestion and treatment was required to obtain the amino-terminal sequence. The location of the sole tyrosine in the molecule (Fig. 3) close to the amino terminus was advantageous for two reasons: (i) peptide elution could be monitored at 275 nm and (ii) chymotryptic, which cleaves readily by tyrosyl residues, could be used to generate heptapeptide T1C2 (residues 1–7). The isolation of T1C2 is illustrated in Fig. 2 Inset. Identification of the fragment was made by its absorbance at 275 nm and its amino acid composition (Table 1). Acid treatment of T1C2 deblocked the a-amino group and allowed a successful sequence determination of residues 1–7. This precluded the need for mass spectrometry to determine the sequence, a process that would have required an amount of PKI well in excess of that used to obtain the complete sequence of the molecule.

Chymotryptic Digestion. PKI (22 µg) was digested with chymotrypsin and fractionated on a SynChropak C18 reversed-phase column (Fig. 4). The amino acid composition of

Fig. 3. Summary of the proof of the structure of PKI. The sequences of specific peptides (named in italics) are given below the overall sequence (bold type). Prefixes T, C, E, and MCP denote peptides generated by cleavage with trypsin, chymotrypsin, S. aureus V8 proteinase, and mast cell proteinase II, respectively. T1C2 denotes a subpeptide of T1 which was refractory to Edman degradation; X represents an unidentified group attached to the a-amino group of threonine-1. The single-letter amino acid abbreviations are used. Amino acid residues represented by uppercase letters were identified by Edman degradation; those in lowercase were only tentatively identified. The portion of the MCP, sequence in boldface (residues 11–30) was published previously (17).
each peptide was determined and fragments C₁ and C₂ were sequenced. Peptide C₁ (residues 8–10) provided primary identification of a region contained in the blocked fragment T₁, while C₂ (residues 45–75) confirmed sequences obtained from fragments T₄ and T₅.

S. aureus V8 Proteinase Digestion. PKI (40 μg) was digested with V8 proteinase and the products were fractionated by reversed-phase HPLC (Fig. 5). Several major and minor cleavages at glutamyl and aspartyl residues were obtained and the amino acid compositions of two peptides, E₁ and E₂, are presented in Table 1.

The sequences of both of these peptides gave essential overlaps to complete the proof of sequence. Peptide E₁ (residues 5–24) provided several overlaps (Fig. 1) to firmly establish the structure of the amino-terminal regions of the molecule, whereas the sequence of E₂ confirmed information obtained from fragments T₃, T₄, and C₂.

The Carboxyl Terminus. Identification of the carboxyl-terminal residue (serine-75) was provided by peptides T₁ and C₂, on the basis of the amino acid composition of the intact molecule. Peptide T₃ was identified as the carboxyl-terminal fragment because all 18 residues predicted from the amino acid composition (Table 1) were identified when T₃ was sequenced (Fig. 3). Moreover the final residue, serine-75, does not represent a known cleavage site for trypsin. A similar argument is presented for C₂, although identification of the final four residues was only tentative.

All 75 amino acid residues identified by amino acid analysis (Table 1) could be placed in a unique sequence for the polypeptide chain (Fig. 3), thus confirming that serine-75 represents the carboxyl terminus.

**DISCUSSION**

The primary structure of the heat-stable inhibitor of the cAMP-dependent protein kinase was established mainly on the basis of a complete set of tryptic peptides. Complementary fragments were obtained by digestion with chymotrypsin, S. aureus V8 proteinase, and mast cell proteinase II. Proof for the structure relied primarily upon replicate identifications of phenylthiohydantoin derivatives and was confirmed by amino acid composition. Eighty-nine percent of the amino acid residues were clearly identified from the sequence determination of more than one peptide. In all cases, identification of each phenylthiohydantoin amino acid was performed on two complementary HPLC systems. Determination of the complete amino acid sequence required ~200 μg of purified PKI. The two least-confirmed parts in the sequence are the amino- and carboxyl-terminal regions, residues 1–4 and 71–75, respectively.

The amino terminus of PKI is blocked by a group as yet unidentified. Therefore sequence determination of residues 1–4 (Thr-Asp-Val-Glu) involved both subdigestion and deacylation of the α-amino group. Decylation by acid treatment of T₁C₂, probably causes a N → O shift of the blocking group from the α-amino to the hydroxyl of threonine-1 (24), produced some molecules with free amino termini and allowed the determination of residues 1–7 by Edman degradation (Fig. 3).

Amino-terminal blocking groups are common in many intracellular proteins and mass spectrometry is a preferred procedure of identification (25). However, identification of the blocking group and adjoining amino acid sequence by this procedure requires a minimum of 20 nmol of purified methylated peptide (26). The limited availability of PKI virtually precluded this approach.

The amino-terminal residues of both subunits of the cAMP-dependent protein kinase are subject to posttranslational modifications (27–29). The catalytic subunit (C) has covalently bound myristate (30), whereas both types of regulatory subunit (RI and RII) are acetylated (28, 29). The nature of the acyl group attached to threonine-1 of PKI has not been determined but analysis of partially purified PKI for the presence of long-chain fatty acids was negative. The elution of peptide T₁C₂ from a reversed-phase column at 15% acetonitrile is consistent with its amino acid composition, indicating that the blocking group cannot be a long-chain fatty acid. Peptides modified by long-chain fatty acids elute at a concentration of acetonitrile higher than would be predicted from their amino acid composition (31). Therefore the blocking group is likely to be a short-chain acyl group such as formyl or acetyl.

The carboxyl-terminal four residues were identified primarily in peptide T₁ and were consistent with the sequence of C₂. Carboxypeptidase treatment of PKI was not carried out since methods for detection of released amino acids are not sensitive enough to work in the subnanomolar range dictated by the amounts of inhibitor available. Thus, we have not excluded the possibility that the carboxyl terminus is amidated.

Computer-aided searches for homology in the data bank of the National Biomedical Research Foundation (2898 sequences, February 1985) with the SEARCH program of Dayhoff (32) showed no similarities between PKI and other protein sequences. This suggests that PKI is a unique gene product. PKI is a potent competitive inhibitor of the free catalytic subunit of the cAMP-dependent protein kinase (9); however, it would be anticipated that the amino acid se-
sequence would be similar to at least a portion of both regulatory subunits, which also inhibit C (33). A direct comparison of the PKI sequence to the "hinge regions" of RI and RII showed that indeed a 9 amino acid segment of the inhibitor closely resembled these regions (residues 14–22). The sequence Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile represents a "pseudosubstrate site" for C and shows 66% identity with the hinge region of RII (34). We have shown previously that this pseudosubstrate site of the molecule is biologically active and synthesis of peptides derived from this region are potent competitive inhibitors of C (16, 17).

PKI, RI, and RII are inhibitors of C containing a hinge region which directly interacts with the active site of the kinase (16, 17, 35). Both regulatory subunits contain an additional 325 residues which form two internally homologous cAMP-binding domains (28, 29). These regions determine holoenzyme dissociation depending upon their occupancy by cyclic nucleotide (28). PKI may be thought of as a cyclic nucleotide-independent modulator of C lacking a cAMP-binding domain.

The hinge regions of RI and RII are the proposed sites of interaction with C within the holoenzyme complex (34–36). Weldon and Taylor (35) have confirmed this by an elegant study involving anti-RI monoclonal antibodies and RII fragments. They showed that arginine residues in the hinge region not only confer specificity for phosphorylation but also stabilize the holoenzyme complex. The presence of a similar hinge region in PKI that is essential for inhibition (16, 17) is further compelling evidence that multiple arginines are essential for recognition at the active site of C.

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