Isolation and Properties of the Rabbit Skeletal Muscle Protein Inhibitor of Adenosine 3′,5′-Monophosphate Dependent Protein Kinases†

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ABSTRACT: The heat-stable protein inhibitor (Walsh, D. A., et al. (1971), *J. Biol. Chem. 246,* 1977–1985) of the cyclic adenosine 3′,5′-monophosphate dependent protein kinases has been isolated in pure form from rabbit skeletal muscle after a 430 000-fold purification with a 47% yield. The four-step procedure involves sequentially a heat treatment, batchwise anion and cation exchange, and affinity chromatography on protein kinase catalytic subunit covalently coupled to Sepharose 4B. The inhibitor is an acidic protein (p*I* = 4.24) of molecular weight 11 300. It contains 98 amino acid residues none of which contains sulfur and only 2 (phenylalanine and tyrosine) are aromatic. The NH_2-terminus is blocked. The muscle content is ca. 0.6 mg of inhibitor per L of intracellular water. The inhibitor is tightly bound to the catalytic subunit of protein kinase ($K_i \simeq 2 \times 10^{-9}$ M) and acts competitively with respect to the protein substrates. Protein kinase recognizes a short stretch of the inhibitor sequence, in which arginyl side chains play a crucial role. A study of various competitive inhibitors of the kinase confirms the importance of guanidino groups and hydrophobic side chains in the specific interaction with the substrate binding site.

It is now well accepted that the action of many hormones, particularly catecholamines and peptide hormones, is expressed through the synthesis of cAMP[1] (Robison et al., 1971) by the membrane-bound adenylate cyclase; the cyclic nucleotide then binds to the regulatory subunit (R) of cAMP-dependent protein kinases (EC 2.7.1.37; ATP:protein phosphotransferase) which dissociate (Hofmann et al., 1975) into two catalytically active subunits (C) and a dimeric, cAMP-regulatory subunit complex $(R\text{-}cAMP)_2$ (for review, see Walsh and Krebs, 1973; Rubin and Rosen, 1975).

The cAMP-dependent protein kinase is inhibited by a heat-stable protein, which was partially purified by Walsh et al. (1971) and shown to interact only with the free C subunits, not with the holoenzyme (Ashby and Walsh, 1972, 1973). It was said to act noncompetitively toward both substrates of the kinase, i.e., proteins and MgATP. The concentration of the protein kinase inhibitor (PKI) would allow for the inhibition of ca. 20% of C subunit released upon total dissociation of the holoenzyme (Walsh and Ashby, 1973); this would provide a mechanism by which basal levels of cAMP would not activate protein kinase (Beavo et al., 1974b).

The specificity of PKI for the cAMP-dependent protein kinase and its high affinity for this enzyme made particularly attractive the hypothesis (Walsh and Ashby, 1973) that intracellular levels of PKI might be under insulin control. This manuscript describes the isolation of PKI in homogeneous form and some of its physical, chemical, and enzymatic properties.

Materials and Methods

Histone IIA mixture, poly(L-arginine·HCl) type IIB (mol wt 58 000), and phosphocellulose (coarse) were obtained from Sigma; DEAE-cellulose (Whatman DE 52) was from Reeve-Angel, and subtilisin was from Teikoku Chemical Industry. ^{32}P and ^{125}I (both carrier-free) were from New England Nuclear; Bio-Gel P2, 200–400 mesh, was from Bio-Rad; CM-Sephadex C-50, Sephacryl S-200, and CNBr-activated Sepharose 4B were from Pharmacia. Cibacron blue 3G-A and MDPF (2-methoxy-2,4-diphenyl-3(2*H*)-furanone), a heat-stable fluorophor, were generous gifts from Ciba-Geigy and from Hoffmann-La Roche, respectively. Cibacron blue was coupled to epoxy-activated Sepharose 6B (Pharmacia) in 0.2 M sodium carbonate, pH 11. Protamine was coupled to glutaraldehyde-activated Ultrogel AcA 34 from LKB (1.8 mg of protein/mL of gel) according to Ternynck and Avrameas (1972). [γ-^{32}P]ATP, ca. 620 mCi/mmol, was prepared by the procedure of Glynn and Chappell (1964). Buffer A consisted of 30 mM KH_2PO_4–0.1 mM EDTA–15 mM 2-mercaptoethanol, adjusted to the desired pH with KOH.

Protein was determined by the biuret (Beisenherz et al., 1953) or Lowry (Lowry et al., 1951) procedures. Isoelectric focusing was performed in the 440-mL LKB electrofocusing column, at 2 °C using 1% Ampholine pH 3–6 (LKB). Polyacrylamide (12.5%) gel electrophoresis was carried out according to Pechère et al. (1971). Since protein kinase inhibitor was poorly stained by Coomassie brilliant blue R, the following analyses were performed on labeled inhibitor molecules: cellulose acetate electrophoresis was carried out in a Beckman Microzone System R-101 on PKI labeled with ^{125}I, using an 80 mM 2-(*N*-morpholino)ethanesulfonate buffer, adjusted to pH 5.0 or 6.5 with pyridine. PKI was then visualized by autoradiography. Dodecyl sulfate (0.1%)–15% polyacrylamide slab gel electrophoresis was carried out in a discontinuous Tris–glycine buffer (Laemmli, 1970). The protein markers and

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[1] Abbreviations used: cAMP, cyclic adenosine 3′,5′-monophosphate; PKI, protein kinase inhibitor; C and R, the catalytic and regulatory subunits of the cAMP-dependent protein kinase, respectively; MDPF, 2-methoxy-2,4-diphenyl-3(2*H*)-furanone; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; CM, carboxymethyl; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; P_i, inorganic phosphate; Gdn·HCl, guanidine hydrochloride; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

the unknown samples (1–10 μg) were labeled in 10 μL of 10 mM borate buffer (pH 9.5) containing 2 μg of MDPF (Barger et al., 1976). After 2 h at 0 °C, 10 μL of the sample buffer was added and the mixture was left overnight at room temperature. Following electrophoresis, the gel was immersed for 18 h in methanol–acetic acid–water (5:1:5) and photographed under UV light. The molecular size was measured by gel permeation on Sephadex G-75 (2.5 × 95 cm column); elution was carried out with 10 mM ammonium acetate pH 7.0, at a flow rate of 20 mL/h. V_0 was determined with Blue dextran 2000, and V_{tot} with $^{45}CaCl_2$. The values of the Stokes radii of standard proteins were taken from Andrews (1970).

PKI (6 μg) was labeled with $Na^{125}I$ (2 mCi) using the Chloramine-T procedure of Hunter and Greenwood (1962); unreacted iodine was separated on Bio-Gel P2 (0.9 × 35 cm) in 10 mM ammonium acetate. The specific radioactivity of [^{125}I]PKI was 15 μCi/μg. Binding of [^{125}I]PKI to the C subunit of protein kinase was followed qualitatively by the gel permeation technique of Hummel and Dreyer (1962) on a 0.9 × 27 cm column of Sephacryl S-200 equilibrated with [^{125}I]PKI (0.33 nM) in buffer A, pH 6.7, containing 0.1% bovine serum albumin and 0.05% sodium azide. The column was eluted at 2.8 mL/h, and fractions of 0.3 mL were collected and counted. The binding equilibria were too slow for establishment of a plateau so that no calculation of binding ratio was attempted.

Amino acid analyses were performed according to Moore and Stein (1963) after 24, 48, and 72 h of hydrolysis in 5.7 N HCl at 110 °C, in a Durrum analyzer (Model D500). Cysteine was determined as cysteic acid (Hirs, 1967) and tryptophan according to Penke et al. (1974). The NH_2 terminus of the molecule was studied in the sequencer as described by Hermodson et al. (1972). Arginyl side chains were modified by 0.15 M cyclohexanedione (75% substitution after 3 h at 38 °C in 0.25 M borate buffer, pH 9.0) and completely regenerated within 5 h at pH 7.0 and 40 °C by 0.5 M hydroxylamine according to Patthy and Smith (1975). N → O acyl rearrangement of protamine was performed in concentrated H_2SO_4, and the O-peptide linkages were hydrolyzed by 6 N HCl at 20 °C (Iwai and Ando, 1967). The newly generated COOH termini were substituted with glycinamide or octylamine at pH 4.75, using ethyldimethylaminopropylcarbodiimide (Carraway and Koshland, 1972).

Assays. The catalytic subunit of protein kinase was assayed by the filter paper method (Corbin and Reimann, 1974), after addition of 20 μL of the enzyme solution to 50 μL of a reaction mixture containing 0.5 mg of histone II A mixture, 0.83 μmol of potassium phosphate (pH 7.0), 0.3 μmol of magnesium acetate, 17 nmol of [γ-^{32}P]ATP (100–200 cpm/pmol), and 50 nmol of dithiothreitol. Since muscle contains also cAMP-independent protein kinases which are not inhibited by PKI, measurement of catalytic subunit activity in the course of purification was taken as the difference between two assays containing both 5 μM cAMP, but differing only in that one of them contained an excess (50 units) of PKI. The specific activity of the C subunit at any stage of its purification increased considerably upon dilution to reach a plateau value that was used in subsequent calculations. This value represents 0.75 V_{max} since the protein substrate concentration in the assay mixture is only three times above K_m (K_m = 2.4 mg/mL).

Protein kinase inhibitor was assayed in 20-μL samples using twofold serial dilutions in buffer A, pH 7.0, containing 0.1% bovine serum albumin. After addition of 50 μL of the histone–Mg–ATP mixture described above, the reaction was initiated by addition of 20 μL of catalytic subunit from either rabbit skeletal muscle (peak I protein kinase) or bovine heart (peak II enzyme) dissolved in buffer A, pH 7.0, containing 0.1% bovine serum albumin. A 10 nM final concentration of C subunit was introduced, which, under these assay conditions, catalyzed the incorporation of 30 pmol of P_i min^{-1}. One unit of inhibitory activity is defined as the amount of PKI capable of inhibiting this incorporation by 50%.

Preparation of Protein Kinase Catalytic Subunit. The catalytic subunit of protein kinase (peak I) was purified from rabbit skeletal muscle according to the diagonal procedure of Beavo et al. (1974a). When large quantities of catalytic subunit were needed, it was found more practical to use bovine heart as starting material, and to prepare the C subunit from the peak II enzyme (Rubin et al., 1974). However, due to the large concentration of myoglobin in this extract, the following procedure, which combines features from these two methods, was routinely used.

Fresh bovine hearts were dissected from fat and connective tissue; 10 kg of ventricular muscle was ground and homogenized in a Waring blender for 1 min at full speed with 2.5 vol of 4 mM EDTA–15 mM 2-mercaptoethanol, pH 7.0. The homogenate was centrifuged at 7000*g* for 30 min and the supernatant was filtered through glass wool and then through Whatman 114 paper. The pH was adjusted to 7.6 with 6 N NH_4OH and 5 L of packed DE-52 cellulose, previously equilibrated with 50 mM Tris–10 mM NaCl–4 mM EDTA–15 mM 2-mercaptoethanol adjusted to pH 7.6 with HCl, was added. After stirring for 30 min, the suspension was allowed to settle overnight and most of the supernatant was siphoned off. The cellulose was washed on a Buchner funnel with 15 L of the Tris buffer and then eluted with two 5-L portions of the same buffer containing 0.5 M NaCl. The eluate was brought to 2.9 M with solid ammonium sulfate; after stirring for 1 h, the precipitate was collected by centrifugation at 14 000*g* for 45 min, redissolved in ca. 500 mL of buffer A, pH 6.1, and dialyzed overnight vs. 40 L of this buffer. Successive batch adsorptions on CM-Sephadex C50 at pH 6.1 and 6.7 (Beavo et al., 1974a), each using 500 mL of gel, were followed by addition of 10 μM cAMP. CM-Sephadex C-50 (200 mL in buffer A, pH 6.7) was then added; the suspension was stirred for 15 min, and the gel was collected on a Buchner funnel, washed with 12 vol of buffer A, pH 6.7, and finally poured into a column. The catalytic subunit was eluted with 1 M KCl in buffer A, pH 6.7.

Final purification was provided by adsorbing the enzyme, predialyzed vs. buffer A containing 10 mM magnesium acetate, pH 6.7, on a 50-mL Cibacron blue Sepharose 6B column to which the kinase binds through its ATP binding site (Witt and Roskoski, 1975; Thompson and Stellwagen, 1976). After washing with 100 mL of buffer, the C subunit was eluted with 5 mM ATP, 20 mM magnesium acetate, 1 M KCl in buffer A, pH 6.7. The active fraction (ca. 60 mL) was concentrated to approximately 2 mg/mL by ultrafiltration on a B12 collodion membrane (Arthur Thomas). The 1500-fold purified C subunit ($A_{1cm,280nm}^{1\%}$ = 14.9; V_{max} = 1.16 μmol min^{-1} mg^{-1}) was obtained in a ca. 12% yield; it was homogeneous in dodecyl sulfate–polyacrylamide gel electrophoresis.

Preparation of Immobilized Catalytic Subunit. C subunit (10 mL of a 47 μM solution in 0.1 M $NaHCO_3$–0.5 M NaCl–5 mM EDTA (pH 8.3)) was mixed with 2 g of CNBr-activated Sepharose 4B previously washed at 0 °C with 400 mL of 1 mM HCl followed by 15 mL of the above buffer. After mixing overnight at 4 °C, the gel (ca. 6 mL) was collected on a sintered glass funnel, washed with 30 mL of coupling buffer, suspended in 0.5 M ethanolamine, 15 mM 2-mercaptoethanol, pH 8.3 at

TABLE I: Purification of Protein Kinase Inhibitor (from 10 Kilograms of Rabbit Skeletal Muscle).

Step	Total units $\times 10^{-3}$	Total protein (mg)	Spec act. (units/mg)	Purification[a]	Yield[a] (%)
Crude extract[a]	351	605 000	0.58		
Heat step filtrate	351	9 560	36.7	63	100
DEAE-cellulose eluate	211	1 170	180	310	60
P-cellulose eluate	175	254	690	1 200	50
Sepharose-catalytic subunit eluate	165	0.66	250 000	430 000	47

[a] Assuming that PKI present in the crude extract is entirely recovered in the filtrate after the heat step.

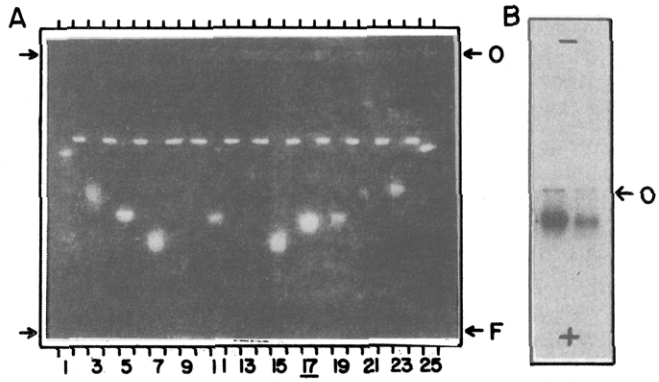

FIGURE 1: (A) Dodecyl sulfate (0.1%)–15% polyacrylamide gel electrophoresis of the protein kinase inhibitor (slot 17). PKI (1 nmol) and the protein markers were labeled by MDPF prior to electrophoresis. The gel was photographed under UV light. Markers (1 μg each) included: all even numbers, carbonic anhydrase (mol wt 29 000); 1, trypsin (mol wt 23 300); 3, myoglobin (mol wt 17 000); 5, lysozyme (mol wt 14 300); 7 and 15, parvalbumin (mol wt 12 000); 11 and 19, cytochrome *c* (mol wt 11 700); 21, ovalbumin (mol wt 43 000); 23, β-lactoglobulin (mol wt 17 500); 25, chymotrypsinogen (mol wt 25 600). (O) Origin; (F) dye front. (B) Cellulose acetate electrophoresis of ^{125}I-labeled protein kinase inhibitor (5 and 10 pmol). Migration was at pH 6.5 for 20 min, 250 V. The x-ray film was exposed for 48 h. (O) Origin.

4 °C for 5 h, and finally washed sequentially with buffer A, pH 6.7, containing 1 M NaCl, then buffer A pH 6.7. For storage, 0.1% bovine serum albumin and 0.05% sodium azide were added. The coupling efficiency was 80% and the specific activity of the bound kinase (62 μM in the gel phase, or 2.4 mg/mL) was ca. 20% that of the soluble enzyme.

Results

Purification of the Protein Kinase Inhibitor. Extraction and Heat Treatment. Rabbit skeletal muscle (13.6 kg or 30 lb) was ground and homogenized with 2.5 vol (34 L) of 4 mM EDTA, pH 7.0. After centrifugation at 7000*g* for 30 min, the supernatant was filtered through glass wool and the pH was adjusted to 7.0 with 6 N NH_4OH. The filtrate was brought within 10 min to 80 °C, under vigorous stirring, in the 100-L stainless steel vessel of a New Brunswick Fermacell, Model F130, then rapidly cooled. Denatured proteins were removed by passing through a refrigerated Sharples centrifuge at 15 000 rpm.

Batchwise Adsorption on Ion Exchangers. One volume of ice cold water was added to the clear supernatant and the pH was adjusted to 5.5 with HCl followed by addition of 3 L of packed DE-52 cellulose, equilibrated with 15 mM HCl–1 mM EDTA adjusted to pH 5.5 with piperazine. After stirring for 30 min at 4 °C, the cellulose was allowed to settle; most of the supernatant was siphoned off and the exchanger was washed on a Buchner funnel with 8 L of the same buffer; it was then adjusted to pH 2.5 with phosphoric acid and eluted with 3 L of 25 mN H_3PO_4. All subsequent operations were carried out at room temperature.

Phosphocellulose (500 mL) previously equilibrated with 25 mN H_3PO_4 at pH 2.5 was added. After stirring for 15 min, the cellulose was collected on a Buchner funnel, washed with, and resuspended in 1.5 L of 25 mN H_3PO_4, and then poured in a 5-cm diameter column. Elution was carried out with a linear gradient from 0.1 to 0.6 M NaCl in 25 mN H_3PO_4 at a flow rate of 150 mL/h; 10-mL fractions were collected. The inhibitory fractions were pooled, dialyzed overnight in acetylated tubing vs. water, and lyophilized. The residue was dissolved in 30 mL of buffer A, containing 0.15 M KCl, pH 6.7, and dialyzed overnight vs. the same solution.

Affinity Chromatography. The PKI solution (ca. 4.5 μM) was passed at 20 mL/h through a 1.5 × 3.4 cm column of Sepharose 4B–catalytic subunit (see Materials and Methods) preequilibrated with buffer A (pH 6.7) containing 0.15 M KCl. The column was washed with this buffer until the 254-nm absorbance of the eluate dropped to background level. PKI was then eluted with 50 mL of buffer A containing 0.3 M Gdn·HCl and 1 M KCl, pH 6.7. Direct assay of PKI was possible only for the breakthrough and wash fractions; due to the strong inhibition of protein kinase by Gdn·HCl (see below), the high-salt eluate could be assayed only after dialysis. In a typical experiment in which 30 mL of 4.5 μM PKI was passed twice through the column, 76% of the inhibitor was first removed, and then 20%. PKI was finally desalted on a 1.5 × 85 cm column of Bio-Gel P2, 200–400 mesh (V_{bed} = 150 mL, V_0 = 53.5 mL), equilibrated with 0.2 M acetic acid and lyophilized.

A summary of the purification is listed in Table I. Based only on the enzymatic assay which cannot be performed before the heat step, a 430 000-fold purification with 47% yield was obtained, assuming that PKI was entirely recovered in the filtrate after the heat treatment. In order to make a rough estimate of the yield through this step, [^{125}I]PKI was added to the muscle pulp. Only 53% of the counts was recovered after the heat treatment; it is not known whether the losses result from coprecipitation or proteolytic digestion. Assuming that this approach is valid, the total purification would be reduced to ca. 230 000-fold, and the yield to 25%.

Criteria of Purity. The final product appeared homogeneous according to the following criteria: First, it migrated as a single band on electrophoresis in dodecyl sulfate–polyacrylamide gel which separates according to size (Figure 1A), and in cellulose–acetate at pH 5.0 or 6.5, which discriminates according to charge (Figure 1B). In the high-resolution 12.5% polyacrylamide gel at pH 8.9 (Figure 2), a double band is seen with R_f values relative to bromophenol blue of 0.54 and 0.59, respectively. Since no other sign of heterogeneity was observed, this doublet was attributed to a partial deamidation of the protein perhaps resulting from the low pH or heat steps of the preparation procedure. Second, on Sephadex G-75 chromatography and dodecyl sulfate gel electrophoresis, radioactivity or fluorescence from tagged PKI coincided precisely with inhibitory activity. Third, Edman degradation performed on 100 nmol gave a negative result indicating a blocked NH_2 terminus

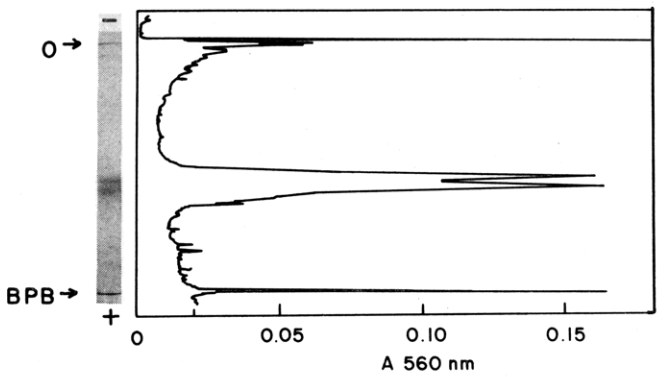

FIGURE 2: Polyacrylamide (12.5%) gel electrophoresis of the protein kinase inhibitor (1 nmol) at pH 8.9. The gel was stained with Coomassie brilliant blue R, and scanned at 560 nm. (O) Origin. (BPB) Bromophenol blue front.

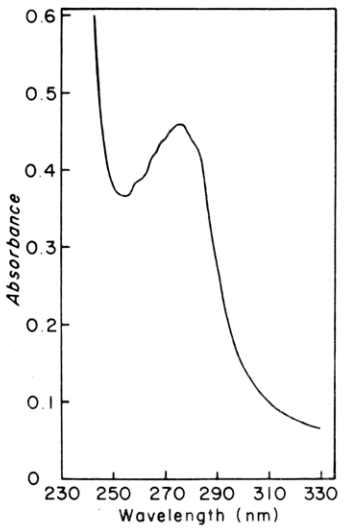

FIGURE 3: Ultraviolet spectrum of the protein kinase inhibitor; 0.32 mM protein in 50 mM ammonium bicarbonate, pH 7.8.

and the absence of reactive contaminants. Finally, the amino acid analysis showed a total absence of cysteine, methionine, and tryptophan; such unbalanced composition would be quite unlikely if one were dealing with a mixture of proteins.

From ten preparations, one unit of inhibitory activity corresponded to 6.46 ± 2.01 ng of homogeneous PKI [$\bar{X}$ ± SD, n = 10] or ca. 0.57 pmol. Therefore, in the standard assay, 6.35 ± 1.98 nM PKI caused a 50% inhibition of 10 nM C subunit. It can thus be concluded that: (a) a single molecule of PKI binds to a single C subunit; (b) apparent $K_i = K_d$ = [free PKI] $\leq 3.3 \times 10^{-9}$ M, assuming that the C–PKI complex is totally inactive, and that [free C] = [C–PKI] = 5×10^{-9} M for 50% inhibition. Therefore, PKI is a tightly bound inhibitor and K_i cannot be determined by the usual graphical procedures.

From Table I and the yield for the heat step evaluated from the isotope dilution experiment, one can calculate that rabbit skeletal muscle contains 66 000 PKI units (0.42 ± 0.14 mg) per kg, or 94 000 units (0.6 ± 0.2 mg) per L of intracellular water (assumed at 70% of wet weight; Muntwyler, 1968), which corresponds to 53 ± 17 nM.

If the maximum C subunit concentration in muscle was taken as 460 nM (Beavo et al., 1974b), the molar PKI/C ratio would be ca. 0.12, i.e., slightly less than the value of 20% previously reported (Walsh and Ashby, 1973). These figures could not be confirmed by radioimmunoassay since no antibody could be elicited as yet against PKI, either free or covalently bound to ovalbumin, hemocyanin, or microcrystalline cellulose.

Physical and Chemical Properties. Molecular Weight. Electrophoresis of MDPF-labeled PKI in dodecyl sulfate–polyacrylamide gel (Figure 1A) showed a single band corresponding to a molecular weight of 11 000–12 000, in excellent agreement with the minimum value calculated from the amino acid composition (mol wt 11 300). Gel permeation again showed a single species, with a Stokes radius of 16.5 Å, corresponding to a mol wt 11 000 for a globular protein (Andrews, 1970). The consistent values obtained by these three independent techniques leave little doubt that PKI is a globular protein made of a single polypeptide chain of ca. 98 residues.

Since a higher molecular weight of 26 000 had been previously reported for the inhibitor (Walsh et al., 1971), it was important to ascertain that the material obtained here had not resulted from the proteolytic cleavage of a larger species. To this effect, a fresh rabbit muscle extract was subjected to the mild purification procedure devised for the isolation of protein kinase (Beavo et al., 1974a). The inhibitory fractions from the first DE-52 column were pooled, dialyzed vs. 5 mM ammonium acetate, and lyophilized. The residue was dissolved in 10 mM ammonium acetate, pH 7.0, and passed through a Sephadex G-75 column. The elution profile of the inhibitory activity coincided exactly with the radioactivity of a sample of pure [^{125}I]PKI added as a tracer, indicating that the two proteins were indistinguishable.

Isoelectric Point and Absorption Spectrum. PKI is an acidic molecule with an isoelectric point of 4.24, in excellent agreement with the value previously reported (Ashby and Walsh, 1972). It has a low absorbancy index ($A_{1cm,276nm}^{1\%}$ = 1.3 at pH 7.8); its absorption spectrum (Figure 3) is closely similar to that of *N*-acetyltyrosine ethyl ester, with a maximum at 276 nm, on which some vibronic structures are superimposed around 259 nm, as expected from the amino acid composition.

The amino acid analysis shows single tyrosyl and phenylalanyl residues and no tryptophan (Table II). Another striking feature is the absence of sulfur-containing amino acids. Arginine is the most prevalent basic residue. Hydroxy and acidic amino acids account for almost half of the molecule, while alanine and the branched aliphatic side chains form another 30 hydrophobic residues, which might explain in part the remarkable stability of the molecule. Also listed in Table II is the composition of the catalytic subunit of bovine heart protein kinase (peak II) with which PKI interacts in forming the C–PKI complex. As expected from its ion-exchange behavior, it exhibits a number of basic residues.

No evidence has been obtained for the presence in PKI of phosphoserine or phosphothreonine, unusual (e.g., methylated) amino acids, carbohydrates (by concanavalin A–Sepharose chromatography), or nucleotides (from UV absorption), although no further detailed chemical analysis was performed, due to the scarcity of pure material. Inhibitory activity was not affected by addition of divalent metal ions, EDTA or EGTA. The bulk of the evidence indicates that the inhibitory activity of PKI relies mainly on the primary structure of the peptide: it is destroyed by trypsin and chymotrypsin (Walsh et al., 1971), subtilisin, and by 0.25 M acetic acid treatment at 110 °C for 18 h.

Interaction of the Inhibitor with Protein Kinase Catalytic Subunit. It has been previously reported that the inhibition of the C subunit by PKI was noncompetitive with respect to both

TABLE II: Classes of Protein Kinase Inhibitors That Are Competitive toward Protein Substrates.

Millimolar range		Micromolar range		Nanomolar range	
Compound	K_i (10^{-3} M)	Compound	K_i (10^{-6} M)	Compound	K_i (10^{-9} M)
L-Arginine	21	Protamine peptide mixture[a]		Protein kinase inhibitor	2.1
Phenethyl biguanide	6	$-COOH$[b]	44		
Agmatine	5.6	$-CONHCH_2CONH_2$[c]	19		
α-N-Benzoyl arginine ethyl ester	4.1	$-CONH(CH_2)_7CH_3$[c]	3.6		
Gdn·HCl	3.1				
Phenylguanidine	2.1	Polyarginine (mol wt 58 000)	0.76		
Guanethidine	1.6				

[a] Figures refer to the concentration of the original protamine molecule before cleavage; the equimolar mixture of the three arginine-rich peptides, obtained by acid hydrolysis following the N → O acyl shift, was assayed either as such [b] or after substitution of the COOH termini [c] as described under Materials and Methods.

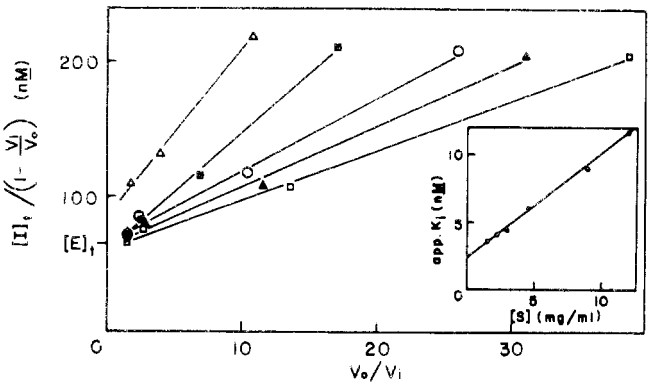

FIGURE 4: Graphical determination of the K_i of the protein kinase inhibitor according to Henderson (1972). The C subunit (66 nM) was incubated in the assay mixture with various concentrations of PKI (25–200 nM). The histone substrate concentration was 1.5 (□), 3 (▲), 6 (○), 9 (■), and 12 (△) mg/mL. $[E]_t$ and $[I]_t$ are the total concentrations of the C subunit and PKI, respectively. V_0 and V_i are the rates in the absence and presence of PKI, respectively. Inset: Slopes from Henderson plots were replotted vs. substrate concentration. Intercept yields K_i and the slope, K_i/K_m.

MgATP and protein substrates (Ashby and Walsh, 1972). While no competition with respect to ATP was indeed found here, PKI did act as a tightly bound competitive inhibitor with respect to the protein substrates.

Because a significant proportion of the total concentration of PKI is enzyme bound, the usual graphical methods cannot be applied. Therefore, data were plotted (Segel, 1975) according to the equation of Henderson (1972) for tightly bound inhibitors (Figure 4):

$$\frac{[I]_t}{\left(1 - \frac{V_i}{V_0}\right)} = K_i(\text{app})\frac{V_0}{V_i} + [E]_t$$

The apparent K_i (given by the slope) is independent of substrate concentration when the inhibitor is noncompetitive but increases with [S] for a competitive inhibition. In this instance, $K_i(\text{app}) = K_i(1 + [S]/K_m)$. Figure 4 shows that this indeed is the case. From a replot of the slopes vs. [S] (coefficient of determination $r^2 = 0.99$), K_i can be estimated from both slope and intercept at ca. 2.1 nM (Figure 4, inset).

It is now well established (Daile et al., 1975; Kemp et al., 1975, 1976a,b; Zetterqvist et al., 1976) that arginine side chains play a major role as a specific determinant common to all the local phosphorylation site sequences recognized by the cAMP-dependent protein kinases. Similarly, arginyl residues in PKI were found to play a crucial role in the binding of the inhibitor to the C subunit. (a) The richer the substrate in arginyl residues, the more successfully can it compete with PKI for the substrate binding site of the C subunit: the ineffectiveness of PKI when protamine is used as substrate is not due to a direct interaction between the two species (Ashby and Walsh, 1972) since PKI does not bind to a protamine-Ultrogel column under assay conditions. (b) Similarly, a number of guanidino compounds (Table II)—some of which prevent the binding of PKI to the C subunit such as polyarginine (Ashby and Walsh, 1972) or Gdn·HCl—are competitive inhibitors of the C subunit. Small molecules with a single guanidino group act in the millimolar range, especially when there is a hydrophobic substituent and no negative charge nearby. Polyarginine polymer or peptides are efficient in the micromolar range, particularly when the latter are substituted on their COOH terminus with a hydrophobic chain. PKI is a more potent inhibitor by almost 3 orders of magnitude. It is interesting to note that the catalytic subunit is very sensitive to inhibition by Gdn·HCl (Table II), whereas it is activated 1.7-fold by 1.33 M urea. Similarly, it retains full activity in 1 mM dodecyl sulfate, whereas the same concentration of cationic detergents such as cetyltrimethylammonium bromide or cetylpyridinium chloride totally inhibit the enzyme in a partially competitive fashion. (c) Arginine side chains of PKI are "essential", as shown by the total inactivation of PKI when 4 out of 6 arginyl residues are modified by cyclohexanedione. Partial (17%) recovery of inhibitory activity is obtained upon regeneration of arginine by hydroxylamine.

Direct demonstration of the competition between PKI, protein substrates, or competitive inhibitors for the same binding site on the C subunit was obtained by the Hummel-Dreyer gel permeation technique: the binding of [^{125}I]PKI (0.33 nM) to 2.5 nmol of C subunit was abolished when 0.1 M Gdn·HCl (a concentration 32 times above K_i) was substituted for 0.1 M NaCl (not illustrated). By contrast, binding of PKI to the C subunit was not affected in a similar experiment carried out in the presence of 100 μM Cibacron blue 3G-A, an analogue of ATP (Witt and Roskoski, 1975; Thompson and Stellwagen, 1976). Furthermore, the PKI-C complex was partly retained on Cibacron blue-Sepharose. It thus appears that PKI does not shield the ATP-binding site of the C subunit, contrary to the regulatory subunit.

Discussion

The heat treatment originally introduced by Walsh et al

(1971) was retained in the purification procedure because it brought about no noticeable denaturation of PKI or reduction in its molecular size. Furthermore, it eliminates protein kinase, which would interfere with the affinity step, and muscle proteases which would degrade the immobilized catalytic subunit.

Earlier purification procedures made use of a trichloroacetic acid precipitation (Walsh et al., 1971); this step was abandoned because parvalbumin, present at a concentration of ca. 80 μM in the sarcoplasm (Baron et al., 1975), coprecipitates and copurifies with PKI; by contrast, in this procedure, it is not adsorbed on DEAE-cellulose at pH 5.5 and can be easily separated at this step.

The greatest enrichment was provided by the affinity chromatography step (ca. 360-fold purification with 94% yield). It also provided a confirmation of the scheme: C + PKI $\rightleftharpoons$ C-PKI postulated on kinetic grounds by Ashby and Walsh (1972). The effectiveness with which Gdn·HCl competes with PKI for the C subunit prompted its use at a concentration ca. 100 times above K_i (i.e., 0.3 M) to facilitate the desorption of PKI in a reasonably small volume; this low concentration had no deleterious effect on the immobilized C subunit which could be used repeatedly over several months without appreciable loss of its binding characteristics.

Inhibition of the catalytic subunit of protein kinase by PKI is reminiscent of the interaction between the regulatory and catalytic subunits in the holoenzyme except that, of course, it is not affected by cAMP. It was, therefore, distinctly possible that PKI arose from R by a proteolytic cleavage that would eliminate the cAMP binding site. This assumption could be ruled out on a number of grounds: (a) cyanogen bromide cleavage of R did not yield any inhibitory fragment. Since PKI lacks a methionyl residue, it is resistant to CNBr cleavage and should appear in its entirety in one of the CNBr peptides derived from R. (b) The amino terminus of PKI is blocked. (c) Furthermore, when subjected to two different purification procedures, no heterogeneity in size was observed for the inhibitor as one would expect if it had resulted from partial proteolysis of R.

It is likely that R, which, in some instances, can be displaced from C by arginine-rich substrates or by polyarginine (Corbin et al., 1975; Rosen et al., 1974), shields both the protein substrate and the ATP-binding site (Witt and Roskoski, 1975), in contrast to the far smaller PKI molecule.

The main features on the protein substrates recognized by the enzyme (Zetterqvist et al., 1976; Kemp et al., 1976b) are two basic residues (one being an arginine) on the amino-terminal side of the serine to be phosphorylated, and a hydrophobic residue on the distal side. Such residues are present in a small fragment generated from PKI that retains most of its inhibitory activity (unpublished results). This peptide contains, among others, 2 arginines, 1 histidine, and 1 isoleucine, along with seryl and threonyl residues. Even though this fragment is similar to a peptide substrate, there is no evidence that phosphorylation of PKI occurs. On the other hand, there is no explanation for the very high affinity displayed by PKI for the C subunit. The tertiary structure of PKI might not be important since peptides as short as 5 residues can serve as substrates (Zetterqvist et al., 1976). Electrostatic interaction of the acidic PKI with the overall positively charged C subunit (see Table III) could perhaps help in stabilizing the C-PKI complex. By contrast, a short stretch of the PKI sequence which includes basic and hydrophobic residues, binds to a partly acidic, partly hydrophobic cleft on the C subunit.

It is of interest to note that the cGMP-dependent protein kinase, or its catalytically active proteolytic fragment, are not inhibited by PKI (Inoue et al., 1976; Takai et al., 1976; Gill et al., 1976), although they phosphorylate the same sites on histone molecules as the cAMP-dependent protein kinase (Hashimoto et al., 1976). It would thus appear that other factors must be involved in determining the specificity of PKI.

TABLE III: Amino Acid Composition of the Catalytic Subunit of Protein Kinase and of Its Inhibitor.

	Catalytic subunit[a] (residues/molecule)		Inhibitor[b] (residues/molecule)	
		Integer		Integer
Asx	31.9	32	11.6	12
Thr[c]	13.7	14	8.0	8
Ser[c]	16.3	16	11.3	11
Glx	38.8	39	13.9	14
Pro	13.7	14	0.7	1
Gly	21.1	21	9.1	9
Ala	21.9	22	14.8	15
Val[d]	19.0	19	2.8	3
Met	6.6	7	0.0	0
Ile[d]	18.9	19	5.3	5
Leu	29.5	29-30	7.1	7
Tyr	12.4	12-13	1.0	1
Phe	23.8	24	1.2	1
His	9.0	9	1.0	1
Lys	31.3	31	4.0	4
Trp[e]	7.1	7	0.0	0
Arg	14.6	14-15	5.7	6
Cys[f]	3.0	3	0.0	0
		332-335		98

[a] From peak II enzyme from bovine heart. [b] From rabbit skeletal muscle. [c] After extrapolation to zero time of hydrolysis. [d] From the 72-h hydrolysis values. [e] After hydrolysis with 3 N mercaptoethanesulfonic acid. [f] As cysteic acid.

PKI is able to neutralize a small but significant mole fraction (0.1-0.2) of the total cAMP-dependent protein kinase (see also Walsh and Ashby, 1973). Although the extent of dissociation of protein kinase in response to hormonal stimuli is not known, it is likely (Beavo et al., 1974b) that PKI buffers variations in the basal level of cAMP and prevents the activation of the kinase: its net effect would be to lower the free cAMP concentration in the cell (Walsh et al., 1971; Ashby and Walsh, 1972, 1973), whereas the cGMP-dependent protein kinase activity would remain essentially unaffected.

Isolation of PKI should provide a useful tool for the study of protein kinase both at the molecular and the pharmacological levels. In particular, it should provide an opportunity to check the hypothesis according to which the level of PKI might be under insulin control (Walsh and Ashby, 1973).

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