Expression in *Escherichia coli* and Characterization of the Heat-stable Inhibitor of the cAMP-dependent Protein Kinase*

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Pure heat-stable inhibitor of the cAMP-dependent protein kinase (PKI) has been isolated in high yield by using a bacterial expression vector constructed to synthesize the complete sequence of the rabbit muscle protein kinase inhibitor, plus an amino-terminal initiator methionine and glycine. Bacterially expressed PKI has an inhibitory activity identical to that of the protein isolated from rabbit skeletal muscle and, by gel filtration and gel electrophoresis, has the same physicochemical characteristics as the native physiological form of PKI. Fourier transformed infrared spectroscopy and CD establish that PKI has unusually large amounts of random coil and turn structures, with significantly smaller amounts of $\alpha$-helix and $\beta$ structures.

The heat-stable inhibitor of the cAMP-dependent protein kinase is a small protein which binds to the catalytic subunit of the kinase and inhibits its activity (1, 2). The effects of PKI are highly selective for the cAMP-dependent kinase: PKI does not inhibit any other serine/threonine kinase, even the closely homologous cGMP-dependent protein kinase (1, 3). While the physiological function of PKI is not yet apparent, it is clear that PKI is exceptionally potent with a $K_i$ in the subnanomolar range, and that inhibition is competitive with respect to the protein substrate (4, 5). Although PKI is present in tissues in very small amounts, it has been purified to homogeneity using conventional chromatography plus affinity chromatography on columns of the immobilized catalytic subunit (4). The complete amino acid sequence of the inhibitor from rabbit skeletal muscle has been determined (6). The finding that fragments of PKI have substantial biological activity (7) has permitted structure/function studies using synthetic peptides (8–11).

To further explore the structure of PKI and to understand the nature of its highly selective interactions with catalytic subunit, it would be desirable to obtain large amounts of the protein. While it has been possible to purify PKI from natural sources, the very low concentration of the protein has limited the availability of pure protein. Similarly, while synthetic peptides representing portions of PKI have been extremely useful, unlike intact PKI, these peptides inhibit kinases other than the cAMP-dependent protein kinase at high concentrations (3). Therefore, the complete PKI, rather than synthetic peptides, would likely be the most useful for studies addressing selective inhibition of kinase activity. In the present study, a bacterial expression vector for rabbit muscle PKI has been prepared based upon a synthetic gene that we have previously synthesized (12). PKI has been purified to apparent homogeneity in large amounts from bacteria carrying the expression vector. This has permitted the exploration of the properties of PKI that were not previously possible because of the limited amounts of material available.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Bacterial Expression of PKI**—The bacterial expression vector for the rabbit muscle PKI was constructed using a synthetic PKI coding sequence which was previously used to prepare a mammalian expression vector (15). Essentially, this involved conversion of an *Neo*I site which contained the initiation codon to an *Nde*I site (Fig. 1) and insertion of the fragment into *pT7*-7 a derivative of the *T7*-1 expression vector (14). This expression vector contains a *T7* RNA polymerase promoter upstream of a ribosome binding site and polylinker. The *Nde*I site of the vector polylinker contains an ATG which is appropriately placed relative to the ribosome binding site to facilitate translation initiation. Therefore, conversion of the *Neo*I of the PKI coding sequence to an *Nde*I site should permit high level translation of the PKI sequence. It should be mentioned that the mammalian PKI expression vector encoded both an initiator methionine and additional glycine codon at the amino terminus of PKI. The glycine was added to the PKI coding sequence to produce a protein with favorable translation initiation properties for expression in mammalian cells (22). The *Neo*I to *Nde*I conversion for preparation of the bacterial expression vector should maintain the presence of the methionine initiation codon and the additional glycine codon. The correct construction of the *T7*-7-PKI clone was assessed by restriction enzyme digestion to confirm that

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‡ The abbreviations used are: PKI, heat-stable inhibitor of the cAMP-dependent protein kinase; *T7*-7-PKI, bacterial expression vector for the heat-stable inhibitor of the cAMP-dependent protein kinase; SDS, sodium dodecyl sulfate; FTIR, Fourier transformed infrared spectroscopy.

§ Portions of this paper (including "Experimental Procedures," Table I, and Figs. 1–6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
the NdeI site had, in fact, been constructed. The T7-7-PKI DNA was then transfected into cells containing pGPI-2 in which the T7 RNA polymerase gene is controlled by λ P1. Because the cells contain a temperature-sensitive λ repressor, expression of the T7 RNA polymerase and subsequent expression of the PKI can be obtained by simply shifting the cells to the appropriate temperatures. Furthermore, because the T7 RNA polymerase is rifampicin-resistant, in contrast to the Escherichia coli enzyme, selective synthesis of PKI can be obtained by incubating the cells in rifampicin (14).

Bacteria carrying the T7-7-PKI expression vector were used to purify PKI. Analysis of crude extracts obtained from cells carrying the T7-7-PKI expression vector demonstrated the presence of material which inhibited the activity of the cAMP-dependent protein kinase (Fig. 2). Low, but detectable inhibition of kinase activity was also observed with extracts from cells which did not contain the PKI vector (data not shown). Thus, the assay of PKI activity in the crude extract may slightly overestimate the inhibitor content. Because the PKI is heat-stable, the initial step for purification of PKI involved heating the crude bacterial extract to 95 °C for 5 min. Following the heat treatment, gel electrophoresis demonstrated that the most abundant protein in the extract is a band with the appropriate mobility for PKI (Fig. 3). At this stage of purification, extracts from bacterial cells which did not contain the PKI expression vector did not inhibit cAMP-dependent protein kinase activity and thus, after this step, analysis of preparations should accurately reflect the content of PKI.

PKI was further purified by ion exchange and gel exclusion chromatography. Again, the purification was monitored by analysis of PKI activity (Fig. 2) and SDS-polyacrylamide gel electrophoresis (Fig. 3). Most of the PKI activity which could be recovered was found to elute in the 100–300 mM fraction on DEAE-chromatography, and gel electrophoresis suggested the presence of only minor contaminants at this stage of the purification. The minor contaminants were readily removed by chromatography on Sephadex G-50 yielding an apparently homogeneous preparation. The results of a typical purification are summarized in Table I. Starting from 6 liters of bacterial culture, a final preparation of 14 mg of purified PKI was obtained with an apparent overall yield of 9%. While analysis of activity suggests that only a 12.8-fold purification was necessary, this is probably an underestimate due to the presence of nonspecific inhibitory activity present in the crude extracts. The recovery was good for most steps, except for the Sephadex G-50 columns. Somewhat low recovery was encountered at this step because of the need to utilize only the peak fractions to avoid contamination with other small proteins.

Characteristics of Bacterially Expressed PKI—The bacterially expressed PKI exhibited inhibitory potency identical to that of the native skeletal muscle protein. Both equally titrate the activity of pure cAMP-dependent protein kinase and exhibit identical specific activities (Fig. 4a). Henderson analysis (23) yielded K values of 0.986 nM for the bacterial protein compared to 0.885 nM for the native protein; these values are identical within experimental error (Fig. 4b). Multiple forms of PKI, designated I and I', have been isolated from skeletal muscle (18). Of these only the I form is believed to be physiological (24). The bacterially expressed PKI exhibits characteristics identical to the physiological I form. It displays a molecular weight of 22,000 by gel exclusion chromatography (Fig. 5), quite distinct from the 11,000 apparent molecular weight characteristic of the I' form (18). Likewise, with Weber-Osborn gel electrophoresis (25), a method readily diagnostic for the two species, the bacterially expressed protein migrated identical to the I form (Fig. 6a). Both the bacterially expressed and native PKI migrated identically in either Weber-Osborn or Laemmli gel electrophoretic systems (Fig. 6b). In particular, the mixing experiment (Fig. 6b, lanes 4 and 7) demonstrates that the rabbit skeletal muscle and the recombinant PKI are indistinguishable by these two SDS-gel systems. From these combined experiments, it is clear that the bacterially expressed protein exhibits both activity and shape characteristics indistinguishable from that of the native protein.

Structural Conformation of PKI from FTIR and CD—Fig. 7 shows the buffer-subtracted FTIR spectrum of PKI and its second derivative spectrum obtained after smoothing the raw data (over 9 points where the points are separated by 1.93

![Fig. 7. FTIR Spectra of PKI. Absorbance spectra were measured for PKI in D2O. Spectra were obtained after a 25-min purge with dry N2 and were ratioed against a single-beam background collected with no cell. The spectrum of a buffer blank was subtracted from a sample spectrum to give spectra free of contributions from the buffer and cell. The lower trace is that of the second derivative (details of this procedure are described under “Experimental Procedures”).](image)

![Fig. 8. CD Spectra of PKI. CD data for PKI (0.49 mg/ml) in 10 mM phosphate buffer. The smooth line indicates a fitted spectrum obtained by fitting linear combinations of the spectra from a set of 22 basis proteins taken in groups of 19. The final fitted spectra and the values for secondary structure content are based on the average of the five 19-protein groups that best satisfy the final fitting requirements of root mean square error of less than 0.15 (details of this procedure are described under “Experimental Procedures”).](image)
The deuterium-shifted amide region, amide I', occurs between 1620 and 1700 cm\(^{-1}\) and primarily contains bands attributable to the carbonyl stretching vibrations contributed by each peptide linkage. On the basis of 21 proteins of known structure, Byler and Susi (26) have assigned 11 well-defined frequencies in the amide I' region to secondary structural elements. In the amide I' region the PKI spectrum shows a single broad peak whose maximum occurs at 1645 cm\(^{-1}\), the characteristic frequency assigned to random coil structure, indicating that this might be the dominant secondary structure in the protein. By taking the second derivative of the spectrum subtle variations in the line slope caused by contributions from multiple bands beneath the spectral envelope can be accentuated. The second derivative spectra indicate that there are bands at 1675, 1663, 1653, 1645, and 1637 cm\(^{-1}\), and possibly at 1681 and 1632 cm\(^{-1}\). The 1675–1681-cm\(^{-1}\) and 1632–1637-cm\(^{-1}\) bands can be assigned to the high and low components of extended chain structures; at 1663 cm\(^{-1}\) to turns or bends and at 1653 cm\(^{-1}\) to \(\alpha\)-helix. Resolution enhancement by Fourier deconvolution also shows evidence for a distinct band at each of these values, but the resolution of these bands is sufficiently poor that curve fitting, in order to estimate relative intensities and hence relative percentage secondary structure based on the Byler and Susi assignments, was not possible. However, it is clear from the maximum in the spectrum at 1645 cm\(^{-1}\) that a significant portion of the structure of PKI is likely to be random coil in nature.

One possible caveat to the interpretation of the FTIR data is that distorted helix structures have been observed to give rise to peaks as low as 1645 cm\(^{-1}\) (27, 28). In order to check this possibility, as well as to better quantitate the secondary structure, CD spectra were measured (Fig. 8). There is a deep minimum in this spectrum near 200 nm characteristic of a protein with a large proportion of random coil structure (29). The solid line is the CD spectrum calculated by the fitting procedure described under “Experimental Procedures.” The calculated values for secondary structure in the protein based upon the fitted spectrum are 21 ± 2% \(\alpha\)-helix, 0 ± 0% \(\beta\)-sheet, 15 ± 4% antiparallel \(\beta\)-sheet, 34 ± 2% turn, and 30 ± 2% other values including random coil. This result is in good agreement with the FTIR spectra. Thus both CD and FTIR support the conclusion that PKI contains a large proportion of random coil structure. The combined percentages of random coil and turn structures indicated by CD for this protein are unusually large.

**DISCUSSION**

Insertion of the coding sequence for rabbit muscle PKI in a bacterial expression vector has permitted the reproducible isolation of large amounts of PKI. In contrast to the isolation of the inhibitor from mammalian tissues, which requires multiple chromatographic separations and at least 10 kg of muscle tissue to isolate submilligram quantities (4), use of the bacterial expression vector permits relatively convenient and rapid isolation of 10–20-mg quantities of PKI. In several different preparations we have obtained at least 1 mg of PKI for each liter of bacterial culture. The inhibitory activity of bacterially expressed PKI is indistinguishable from that of the native skeletal muscle protein (Fig. 4).

Skeletal muscle PKI has been shown to adopt two conformations (18). One form, designated I, is the only form believed to be present physiologically (24). The other form, designated I’, appears most likely to be an artifact produced during extensive purification (4, 18, 24). The bacterially expressed PKI exhibits the characteristics of the native I conformation (Figs. 5 and 6). Past data has suggested that PKI in the native I form was unlikely to be a simple globular protein since the apparent molecular weight by gel exclusion chromatography (22,000) is markedly higher than that determined from the amino acid sequence (7829 (6)). The high resistance of PKI to conditions of both low pH and high temperature has also suggested that it may have minimal structure. The amount of material that can now be obtained by bacterial expression has allowed this to be addressed. The combined results of FTIR and CD provide strong evidence that an unusually large proportion of the PKI protein is present in random coil (Figs. 7 and 8). Also present are \(\alpha\)-helix and \(\beta\) structures, however, and this is compatible with our past data on the presence of some structure in the most active peptides derived from PKI (30). Given these data it would appear likely that the I’ form of PKI represents a more compact structure produced artifactually.

Bacterially produced PKI should provide a useful tool for studies of the catalytic subunit of the cAMP-dependent protein kinase. Natural PKI has been derivatized with fluorescein isothiocyanate and used as a probe to localize catalytic subunit in cultured cells (31, 32). Recent studies suggest that the recombinant PKI can be useful for isolation of the catalytic subunit from mammalian cell extracts (33). The ability to readily alter the PKI coding sequence should also permit further analysis of PKI structure/function. While this area has already been substantially explored using synthetic peptides (7–9), most studies to date have concentrated on the amino-terminal region which is required for biological activity of PKI. However, it seems likely that additional residues beyond this region facilitate the high affinity and selectivity of the intact PKI (10). The ability to readily alter the coding sequence of PKI coupled with the bacterial expression system should permit the functional analysis of other regions of the protein. Finally, the availability of relatively large amounts of the bacterially produced PKI may further facilitate structural studies including x-ray crystallographic studies of the inhibitor-catalytic subunit complex. Recent studies have demonstrated the expression of regulatory (34) and the catalytic (35) subunit of the cAMP-dependent protein kinase in E. coli. The regulatory subunit bacterial expression system has been used to explore structure/function questions (36–38). The ability to manipulate PKI sequences in bacterial expression vectors provides additional approaches to understanding the functions of the cAMP-dependent protein kinase.

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**SPECIAL SUPPLEMENTARY MATERIAL TO**

**Expression in E. coli and Characterization of the Heat-stable Inhibitor of the cAMP-dependent Protein Kinase**

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**EXPERIMENTAL PROCEDURES**

**Materials**

Unless otherwise stated, all reagents were analytic grade. Enzymes used in DNA manipulations were from New England Biolabs or Bethesda Research Laboratories and were where noted, were used according to the manufacturers specifications. The catalytic subunit of the cAMP-dependent protein kinase, purified to homogeneity from bovine heart according to Bechtel et al. (13) was used for characterization of the purified PKI. Initial assays during purification of PKI utilized protein kinase purchased from Sigma. Homogeneous, rabbit skeletal muscle PKI was purified as described previously (14). The synthetic peptide substrate of the cAMP-dependent protein kinase, Rossmann FKBKVL, was obtained from Peninsula Laboratories. Phosphocellulose paper. PKI was from Whittman, Zetachrom 60 DEAE ion exchange columns were obtained from CMK, Osmon, CT. Sephadex G-50 and G-75 were from Pharmacia. The bacterial expression vector p777, a derivative of p711 (15), and appropriate bacterial strains were a generous gift of Dr. Stanley Tabor (Harvard Medical School).

**Construction of PKI expression vector**

Preparation of PKI coding sequences for insertion in the T7.7 expression vector are summarized in Figure 3. The bacteriophage expression vector was prepared by modifying the previously described eskakyoto expression vector (12) which contained the complete rabbit skeletal muscle coding sequence predicted synthetically by reverse translated of the amino acid sequence, with an added methionine initiation codon and an amino terminal glycine codon. The PKI sequence of the eskakyoto vector was altered so that a Xho I site containing the translation initiation codon was converted to an Xba I site. This altered the initiation codon in the proper relationship to the ribosome binding site of the expression vector and should permit the synthesis of full-length rabbit muscle PKI with only the addition of an initiator methionine plus a glycine residue. The T7.7 PKI DNA was initially used to transform the DH-5a strain of E. coli. After characterization of the cloned DNA by restriction endonuclease digestion, DNA from an appropriate clone was used to transform strain CP1/T7-PKI for expression of PKI.

**Purification of recombinant PKI**

Litter cultures of GP1/T7.7-PKI were grown in 1-liter flasks complemented with 0.2% glucose, 50 mM potassium phosphate, pH 7.2, 100 µM ampicillin and 50 µg/ml kanamycin at 30°C with shaking until the A650 reached 1.4 to 1.6. The cultures were incubated at 42°C for 40 min and trypsin was added to 100 µg/ml and incubation was continued for another hour at 37°C. The cells were collected by centrifugation at 3000 x g for 15 min and the cell pellet was washed by reprecipitation in 20 ml of 20 mM Tris (pH 8.0), 25 mM EDTA and 10% sucrose followed by centrifugation. The cells were resuspended in 25 ml of 20 mM Tris (pH 8.0), 1 mM EDTA, 10% sucrose and frozen in an alcohol/dry ice bath. After thawing, tosylnzyme was added to a concentration of 0.2 mg/ml and the cells incubated for 45 min on ice. After two freeze/thaw cycles, cell debris was removed by centrifugation at 30,000 rpm for 40 min in a 705R rotor (Beckman). The supernatant was removed and placed in a boiling water bath. The temperature of the cell extract was monitored, so that the extract was held at approximately 95°C for 5 min. Desalted proteins were removed by centrifugation and the crude, heat-treated extract was adjusted to pH 5.0 with acetic acid. After incubation for 30 min at room temperature, precipitated proteins were removed by centrifugation and the supernatant was clarified with an equal volume of 5 mM sodium acetate, pH 5.0 and applied to a ZetaChrom 60 DEAE ion exchange column. The DEAE disk was eluted with a step gradient of 0.1 M and 0.3 M sodium acetate, pH 5.0. The proteins which eluted with the 0.3 M sodium acetate were pooled, dialyzed against a double strength of 0.3 M sodium phosphate, pH 7.4 and fractionated by chromatography on a 2.5 x 75 cm column of Sephadex G-50 in 0.01 M Tris (pH 7.3). The protein content of the various fractions was determined by spectrophotometric absorbance in 10 µl and the fractions were also examined by SDS polyacrylamide gel electrophoresis. (29)

**PFIR measurements**

PFIR spectra were measured using a Varian Matrix Alpha Constant sample setting on a 4 cm cell. To minimize the signal-to-noise ratio 1024 scans were acquired by digitization and the traces were added by Thompson et al. using a Varian Matrix Alpha Constant sample setting on a 4 cm cell. To minimize the signal-to-noise ratio 1024 scans were acquired by digitization and the traces were added by Thompson et al. using a Varian Matrix Alpha Constant sample setting on a 4 cm cell. To minimize the signal-to-noise ratio 1024 scans were acquired by digitalization and the traces were added by Thompson et al. using a Varian Matrix Alpha Constant sample setting on a 4 cm cell. To minimize the signal-to-noise ratio 1024 scans were acquired by digitalization and the traces were added by Thompson et al. using a Varian Matrix Alpha Constant sample setting on a 4 cm cell. To minimize the signal-to-noise ratio 1024 scans were acquired by digitization and the traces were added by Thompson et al. using a Varian Matrix Alpha Constant sample setting on a 4 cm cell. To minimize the signal-to-noise ratio 1024 scans were acquired by digitization and the traces were added by Thompson et al. using a Varian Matrix Alpha Constant sample setting on a 4 cm cell. To minimize the signal-to-noise ratio 1024 scans were acquired by digitization and the traces were added by Thompson et al. using a Varian Matrix Alpha Constant sample setting on a 4 cm cell. To minimize the signal-to-noise ratio 1024 scans were acquired by digitization and the traces were added by Thompson et al. using a Varian Matrix Alpha Constant sample setting on a 4 cm cell. To minimize the signal-to-noise ratio 1024 scans were acquired by digitization and the traces were added by Thompson et al. using a Varian Matrix Alpha Constant sample setting on a 4 cm cell. To minimize the signal-to-noise ratio 1024 scans were acquired by digitization and the traces were added by Thompson et al. using a Varian Matrix Alpha Constant sample setting on a 4 cm cell. To minimize the signal-to-noise ratio 1024 scans were acquired by digitization and the traces were added by Thompson et al. using a Varian Matrix Alpha Constant sample setting on a 4 cm cell. To minimize the signal-to-noise ratio 1024 scans were acquired by digitalization and the traces were added by Thompson et al. using a Varian Matrix Alpha Constant sample setting on a 4 cm cell. To minimize the signal-to-noise ratio 1024 scans were acquired by digitization and the traces were add...
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CD measurements. CD spectra were measured at room temperature on a 0.01 cm path length cell (Hellma) with a Jasco-40CS spectropolarimeter. The spectral bandwidth was 1.0 nm, the sensitivity was 50 x 10^(-4) mrad/cm, and time constants of 3 and 10 s were used. The spectra were measured for 2.5 h after a 20 min N2 purge. Samples were prepared by dilution of the protein samples prepared for FTIR measurements. Dilutions were done by weighing and the protein concentration for the spectra shown was 0.49 mg/ml in 10 mM sodium phosphate buffer, pH 7.0. Equivalent results were obtained for PKI in both phosphate and MOPS buffer systems. The CD data were collected in 0.5 nm increments over the range of 178-360 nm. The buffer data was subtracted from sample data and the resulting difference was smoothed using a Fourier smoothing routine "SMOOTH" (G1) with a window width of nine points. The secondary structure content analysis software (PURITAN 77 programs VANSELEC and SEARCH) and the 22 protein data base were obtained from W. C. Johnson (Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR). The program VANSELEC fitted the smoothed data over the range of 178-360 nm to linear combinations of the spectra from a set of 22 basis proteins taken in groups of 19 total number of groups was 1540). Following execution of VANSELEC, the output file containing the results of each of the fits to all the 20-protein groups was examined using "SEARCH" with increasing stringent requirements RMS error less than 0.18 and total secondary structure between 90 and 110%) until five groups remained. The spectra and secondary structure content from these 19-protein groups that satisfied the final search requirements were averaged to produce the fitted spectra and the values for the secondary structure content.

Table I

| Fraction      | Protein Activity | -Fold Purification | Recovery |
|---------------|------------------|---------------------|----------|
|               | (pmol/mg)        |                     | (%)      |
| Crude extract | 300₁             | 3900                | 1.0 100  |
| Heat-treated  | 369              | 9250                | 3.2 62   |
| DEAE          | 88               | 13920               | 6.7 29   |
| Sephadex G-50 | 14               | 37140               | 12.8 9    |

Figure 1. Construction of a PKI expression vector. The PKI coding sequence was isolated from the RSV-PKI mammalian expression vector and the Nde I site partially filled and digested with mung bean nuclease. The linear PKI coding sequence was isolated by further Sma I digestion and preparative gel electrophoresis and ligated into a blunt-ended Nde I and Bam HI site of the T7-7 expression vector. This procedure essentially converts the Nde I site of the PKI coding sequence to an Nde I site which contains the initiator methionine codon.

Figure 2. Analysis of PKI activity in fractions from bacterial cultures containing PKI expression vectors. Either the crude extract (A), heat-treated extract (B), eluate from DEAE-chromatography (C) or peak from Sephadex G-50 chromatography (D) were assayed for inhibition of the activity of the cAMP-dependent protein kinase.

Figure 3. Electrophoretic analysis of proteins present in fractions from bacterial cultures containing PKI expression vectors. Either 20 µg of the crude extract from cells containing only pGPI-2 which produces the T7 RNA polymerase (A) or 20 µg of crude extract (B). 20 µg of heat-treated extract (C). 10 µg of DEAE-eluate (D) or 10 µg of Sephadex G-50 peak (E) from cells carrying pGPI-2 and T7-7-PKI were analyzed by electrophoresis on an 12% containing polyacrylamide gel electrophoreses followed by staining with Coomasie brilliant blue. The position of PKI is indicated by an arrow.

Figure 4. Comparison of the inhibitory potency of bacterially-expressed PKI and native rabbit skeletal muscle PKI. The inhibitory potency was assessed through incubating increasing concentrations of PKI with purified catalytic subunit (A). Inhibitory activity was assayed as described by Whitehouse and Walsh (16). From these titrations the specific activity of the preparations was determined to be: native rabbit skeletal muscle protein, 3.98 x 10^6 units/mg; bacterially expressed protein, 3.71 x 10^6 units/mg. The potency was also assessed by Henderson (22) analysis (B). Assays were performed essentially as described by Glass et al. (19) except that the reactions contained 0.8 mM protein kinase catalytic subunit and 11.03 µM (C), 5.53 µM (D), 2.67 µM (E) or 1.38 µM (A) Keptide. The primary plot shows the data from the bacterially expressed protein and the inset plot compares the native and bacterially expressed PKIs. The K_i values determined from the replots were 0.088 nM and 0.085 nM, respectively, which are experimentally indistinguishable.
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Figure 5. Gel exclusion chromatography of bacterially-expressed PKI. PKI (3.15 μg) was applied to a 0.9 x 56 cm column of Sephadex G-75 equilibrated in 10 mM morpholineethanesulfonic acid, pH 6.8. The PKI was eluted and assayed for inhibitory activity as described by McPherson et al. (18). The elution of molecular weight standards indicated was determined separately.

Figure 6. Gel electrophoresis characterization of bacterially-expressed and native PKIs. Analysis of PKI activity by SDS gel electrophoresis (A). On separate gels, 1.95 μg of pure native skeletal muscle PKI and 1.7 μg of bacterially expressed protein were analyzed by SDS polyacrylamide gel electrophoresis according to Weber and Osborn (25). The gels were scanned, eluted and assayed for PKI activity as described by McPherson et al. (18). The elution of the I and I' forms are noted. The scale of the native protein activity has been offset by 25 units/slice for clarification. Analysis of native skeletal muscle and recombinant PKI by either Weber and Osborn (25) 12% polyacrylamide-SDS gels (lanes 1-4) or a Laemmli (26) 10-15% polyacrylamide-SDS slab gel (lanes 5-8). The samples contain either standards (lanes 1 and 6), 3.90 μg of native skeletal muscle PKI (lanes 2 and 5), 3.4 μg of the bacterially expressed PKI (lanes 3 and 4) or a mixture of 1.95 μg native skeletal muscle PKI and 1.7 μg bacterially expressed PKI (lanes 4 and 7). The molecular weight standards were: phosphorylase a, 97,400; bovine serum albumin, 66,000; ovalbumin, 42,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 22,000; hemozyme, 14,400; and apovinamin, 6,500. By each method both forms of PKI exhibit an apparent molecular weight of 16,900.