Differential and Common Recognition of the Catalytic Sites of the
cGMP-dependent and cAMP-dependent Protein Kinases by Inhibitory
Peptides Derived from the Heat-stable Inhibitor Protein*

David B. Glass‡, Heung-Chin Cheng§, Bruce E. Kemp§, and Donal A. Walsh§
From the ‡Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322, the §Department of
Biological Chemistry, School of Medicine, University of California, Davis, California 95616, and the ¶Department of Medicine,
University of Melbourne, Repatriation General Hospital, Heidelberg, Victoria 3081, Australia

Synthetic peptides corresponding to the active domain of the heat-stable inhibitor protein of cAMP-
dependent protein kinase (Cheng, H.-C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten,
S. M., and Walsh, D. A. (1986) J. Biol. Chem. 261, 989-992) were tested as inhibitors of cGMP-depend-
pent protein kinase. The peptides themselves were not substrates, cGMP-dependent protein kinase activity
was assayed using histone H2B and two synthetic peptide substrates. Consistent with previous observations
of other peptide inhibitors of this enzyme (Glass, D. B. (1983) Biochem. J. 213, 159-164), the inhibitory pepti-
des had no effect on the phosphorylation of histone H2B, but they competitively inhibited cGMP-depend-
et phosphorylation of the two peptide substrates. The parent inhibitor peptide, PKI(5-24)amide, and a series
of analogs had \( K_i \) or IC\(_{50} \) values for cGMP-dependent protein kinase in the range of 15-190 \( \mu M \). In contrast
to their effects on the cAMP-dependent protein kinase, the inhibitory peptides were substantially less potent
with cGMP-dependent protein kinase, and potency was reduced by the presence of the NH\(_2\)-terminal residues
(residues 5-13). We conclude that the two protein kinases share a recognition of the basic amino acid cluster
within the pseudosubstrate region of the peptide, but that the cGMP-dependent protein kinase does not recog-
nize additional NH\(_2\)-terminal determinants that make the inhibitor protein extremely potent toward the
cAMP-dependent enzyme. Even when tested at high concentrations and with peptide substrates, the
native inhibitor protein did not inhibit cGMP-dependent protein kinase under assay conditions in which the pepti-
des derived from it were inhibitory. Thus, the native inhibitor protein appears to have structural fea-
tures which block interaction with the cAMP-dependent protein kinase.

The cGMP-dependent and cAMP-dependent protein ki-
nases are homologous proteins (1) which have similar but not identical substrate specificities (2-4). The subunit structures and mechanisms of activation are different, however, with the cAMP-dependent enzyme being dissociated into subunits by

\[ \text{PKI(5-24)amide} \]

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1 The nomenclature of the 20-amino-acid peptide derived from the Inhibitor Protein by \( S. aureus V_q \) protease digestion has been changed to PKI(5-24) reflecting the now established amino acid sequence of the native inhibitor protein (19). This is the same peptide previously termed by us (25) as "IP\(_{ZO} \)." Synthetically, this peptide is prepared as the COOH-terminal amide and is thus termed PKI(5-24)amide. The sequences of this and related peptides are given in Table I. Other abbreviations used are: (Ala\(^{26}\)H2B(29-35), Arg-Lys-Arg-Ser-Arg-Ala-Glu (Ala\(^{26}\)H2B(29-35), Arg-Lys-Arg-Ala-Arg-Lys-Glu; Kempt-
tide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; (Ala)Kemptide, Leu-Arg-Arg-
Ala-Ala-Leu-Gly; IC\(_{50} \) concentration producing 50% inhibition.

2 PKI(5-24) and PKI(5-24)amide exhibit \( K_i \) values of 0.3 nM (23) and 2.3 nM (25), respectively. The reasons for this difference are under current investigation.
from the inhibitor protein appeared to be selective for the cAMP-dependent protein kinase and under the conditions tested had no effect on a number of other protein kinases, including the cGMP-dependent enzyme. Since the inhibitor peptide is competitive with respect to protein/peptide substrate, and the cAMP-dependent and cGMP-dependent protein kinases have similar substrate specificities, it has never been clear why the inhibitor protein appears to be absolutely selective for the cAMP-dependent enzyme. We now report more detailed studies on the effect of PKI(5-24)amide and other synthetic peptide analogs on the activity of the cGMP-dependent protein kinase. In the 10–300 μM range, these peptides are able to inhibit the cGMP-dependent protein kinase, but the interaction is substrate-dependent. The peptides, however, are clearly recognized differentially by the cGMP-dependent and cAMP-dependent protein kinases, and PKI(5-24)amide is several orders of magnitude more potent in inhibiting the cAMP-dependent enzyme. The study shows that the two enzymes do share some of the same recognition sites, but also provides insights into the structural features that contribute to the inhibitor protein’s absolute specificity for the cAMP-dependent protein kinase.

**EXPERIMENTAL PROCEDURES**

Synthetic Peptides—The 20-amino-acid peptide from the inhibitor protein of the cAMP-dependent protein kinase and its analogs were synthesized as COOH-terminal amides by solid-phase synthesis and purified as described by Cheng et al. (25). The purity of these peptides has been previously confirmed by high performance liquid chromatography, amino acid analysis, and sequencing (25, 26). The sequences of the parent peptide, PKI(6-24)amide, and the analogs used in this study are given in Table I. (Ala4)H2B(29-35), a model substrate of cGMP-dependent protein kinase based upon the phosphorylation site sequence in histone H2B, was synthesized as originally described (28) with the exception that N'-α-amyloxycarbonyl-L-arginine (N'-tosyl) was used, and the complete peptide was cleaved from the support with anhydrous HF/amine (9:1) (29). (Ala5)H2B(29-35), an analog of the substrate peptide and an inhibitor of the cGMP-dependent protein kinase, was synthesized as described previously (9). Kempetide, a synthetic peptide based upon the cAMP-dependent protein kinase phosphorylation site of porcine pyruvate kinase (30), was purchased from Peninsula Laboratories (Belmont, CA). All peptide concentrations were determined by amino acid analysis.

**Enzyme, Inhibitor Protein, and Histone Purification—** Cyclic GMP-dependent protein kinase was purified to homogeneity from bovine brain as described by Reimann et al. (31) using bovine serum albumin as standard.

Inhibitor Protein was purified by our recently described modifications (32). Purified histone H2B was prepared from calf thymus by the methods of Johns (32) and Oliver et al. (33). The concentrations of the stock solutions of both were determined by amino acid analysis.

**Phosphotransferase Assays—** Cyclic GMP-dependent protein kinase activity was measured essentially as described previously (28, 34). Assays were conducted for 2 min at 30 °C in a reaction mixture of final volume 0.08 ml containing 30 mM Tris-HCl (pH 7.4), 2 mM magnesium acetate, 1 mM cGMP, 0.2 mM [γ-32P]ATP (215–300 cpm/pmol), substrate protein or peptide as indicated below, the indicated concentrations of the various inhibitor peptides, 3 mM 2-mercaptoethanol, 0.3 mg/ml bovine serum albumin, and 2–0.4 μg/ml (1.2–2.8 μM) cGMP-dependent protein kinase. Either 1.5 μg histone H2B, 20 μM (Ala4)H2B(29-35), or 150 μM Kempetide was used as substrate. These concentrations are slightly below the K_{i} values of cGMP-dependent protein kinase for the respective substrates (3, 28). Under these assay conditions, the conversion of histone H2B, (Ala4)H2B(29-35), and Kempetide to product were approximately 55%, 18%, and 3%, respectively. Utilization of [γ-32P]ATP was approximately 2% or less in each case. When analogs of PKI(5-24)amide were tested as possible substrates of cGMP-dependent protein kinase, they were used at a final concentration of 50 μM, and the cGMP-dependent kinase was used at a concentration of 20 μg/ml. Most assays were initiated by the addition of enzyme. In some experiments, as noted, the cGMP-dependent protein kinase was preincubated with buffer, cGMP, Mg2+, [γ-32P]ATP, and inhibitor peptide for 2 min at 30 °C, after which the reaction was initiated by the addition of protein or peptide substrate. Reactions were terminated and 32P-phosphopeptide or histone was quantitated in 50–μl aliquots of reaction mixture by the phosphocellulose paper method (35) using a phosphoric acid-washing procedure (36). The highest amounts of peptide substrates and inhibitors used were well over an order of magnitude lower than the capacity of phosphocellulose paper squares for binding peptide (35).

For determination of K_{i} values and type of inhibition of selected inhibitor peptides, 8–100 μM (Ala4)H2B(29-35) was used as variable substrate with [γ-32P]ATP fixed at a concentration of 200 μM. Alternatively, 10–100 μM [γ-32P]ATP was used as variable substrate at a fixed concentration of 30 μM (Ala4)H2B(29-35). In these assays, less than 16% of the least abundant substrate was converted to product. Data from initial enzyme velocity measurements were fitted to the Michaelis-Menten equation by the method of weighted least squares (37). The purity of these peptides has now been tested as potential inhibitors of the cGMP-dependent protein kinase. (Ala4)H2B(29-35) was used as substrate at near its K_{m} concentration. The sequence of this substrate peptide is based upon the site in histone H2B phosphorylated by the cGMP-dependent protein kinase. As detailed previously, this peptide is an effective substrate for this enzyme (28, 40) and is also one with which other inhibitory peptides have been examined (9). For reference, the peptides derived from PKI(5-24) were compared to two other inhibitory peptides that have been previously described, (Ala4)H2B(29-35), in which the cGMP-dependent phosphorylation site serine residue has been replaced by alanine (9, 40), and (Ala4)Kempetide, the inhibitory peptide whose sequence is based upon the cAMP-dependent phosphorylation site in porcine pyruvate kinase (41, 42).

Several observations are apparent from the data presented in Fig. 1 and Table I. PKI(5-24)amide and four peptides tested with deletions at the NH2- or carboxyl-terminal (Table I, peptides 1–5) were effective inhibitors of the cGMP-dependent protein kinase. Inhibition was concentration-dependent, with high concentrations inhibiting by at least 80%. The IC_{50} values of peptides 1–5 were in the range of 30–120 μM. These concentrations are substantially higher than the amounts needed to inhibit the cAMP-dependent protein ki-
Inhibitory peptides were PKI(5-24)amide, PKI(10-24)amide, PKI(14-24)amide, and PKI(17-22)amide. A similar pattern of results was also observed for the peptides in which the arginine residues were replaced by an alternate basic residue lysine (Table I, peptides 6–8). These substitutions markedly reduced inhibitory potency with the CAMP-dependent protein kinase (between 8- and 800-fold), but caused only a minimal (peptides 6 and 7) or small (peptide 8) change with the cGMP-dependent protein kinase. With both enzymes, substitution of Arg° caused the biggest decrease in inhibitory activity of PKI(14-24)amide. These peptides were substantially more potent inhibitors of cGMP-dependent protein kinase, however, than (Ala)Kemptide. Overall, in comparison of all the peptides tested that were based upon the native inhibitor protein sequence, but differing in length or type of basic amino acid, while they were good inhibitors for the cGMP-dependent protein kinase, all had similar potencies. For the CAMP-dependent enzyme, the IC50 values only covered a 6.4-fold range from 30 μM for PKI(14-24)amide to 191 μM for (Lys18)PKI(14-24)amide; in contrast, inhibition of the cAMP-dependent protein kinase was much more dependent on the presence of the full native sequence and, for the same group of peptides, Kᵢ values ranged over 4 orders of magnitude from 2 nM for PKI(5-24)amide to 36 μM for (Lys18)PKI(14-24)amide. None of the peptides based upon the inhibitor protein sequence was more effective in inhibiting the cAMP-dependent protein kinase than the cAMP-dependent enzyme. For the cGMP-dependent enzyme, (Ala32)H2B(29-35) remains the most selective inhibitory peptide, being ~7-fold more potent in inhibiting it than in blocking the CAMP-dependent enzyme.

The kinetic type of inhibition of the cGMP-dependent protein kinase was determined for two representative inhibitor protein peptides. These data are shown in Fig. 2, with respect to protein substrate, and in Fig. 3, with respect to nucleotide substrate. As illustrated, both PKI(14-24)amide and PKI(5-22)amide were competitive inhibitors versus peptide substrate and exhibited mixed-type noncompetitive inhibition versus MgATP2-. These kinetics are essentially the same as have been previously reported for inhibition by (Ala32)H2B(29-35) (9, 40), and they indicate that these inhibitory peptides act by interacting at the peptide/protein sub-

### Table 1

| Peptide | IC50 (μM) | Kᵢ (μM) | Kᵢ (μM) |
|---------|-----------|----------|----------|
| PKI(14-24)amide | 30 | 15 | 0.057* |
| PKI(10-24)amide | 54 | 0.073* |
| PKI(7-22)amide | 62 | 0.027* |
| PKI(5-22)amide | 68 | 31 | 0.003* |
| PKI(5-24)amide | 111 | 0.002* |
| PKI(10-24)amide | 42 | 4.2 |
| PKI(14-24)amide | 62 | 0.37* |
| PKI(14-24)amide | 52 | 36* |
| (Ala32)H2B(29-35) | 800* | 550* |
| (Ala)Kemptide | 376* |

* Data from Cheng et al. (25).
† Data from Cheng et al. (26).
‡ Data from Glass (9).
Protein kinases are probably ordered Bi-Bi reactions in which MgATP$^{2-}$ binds first (9, 40, 42, 43). Protein kinase activity was assayed with (Ala$^{3'}$)H$_2$B(29-35) as variable substrate as described under "Experimental Procedures." From the secondary replot of the slopes of the lines as a function of inhibitor peptide concentrations, the apparent $K_i$ value was determined to be 26.0 $\mu$M and 13.1 $\mu$mol/min/mg, respectively. Protein kinase activity was assayed with (Ala$^{5-22}$)amide and 188 $\mu$M. From these, the calculated values were: $K_a$ (mixed-type) inhibition constants in accord with Cleland (46). The $K_a$ (competitive) inhibition constants were determined similarly from replots versus $1/V_{max}$ from which were calculated the $K_a$, (uncompetitive) inhibition constants (Fig. 2).

FIG. 2. Inhibition of cGMP-dependent protein kinase activity by inhibitor peptides. A, competitive inhibition versus peptide substrate by inhibitor peptide PKI(14-24)amide. Inhibitor peptide concentrations ($\mu$M) were 0 (C), 40 (B), 80 (C), and 160 (D). Protein kinase activity was assayed with (Ala$^{14}$)H$_2$B(29-35) as variable substrate as described under "Experimental Procedures." From the secondary replot of the slopes of the lines as a function of inhibitor peptide concentration (inset), the $K_i$ value was determined to be 15 $\mu$M. The apparent $K_a$ and $V_{max}$ values of the enzyme for (Ala$^{14}$)H$_2$B(29-35) in the absence of inhibitor peptide were 25.8 $\mu$M and 13.9 $\mu$mol/min/mg, respectively. The apparent $K_a$ and $V_{max}$ values of the enzyme for (Ala$^{5-22}$)H$_2$B(29-35) in the absence of inhibitor peptide were 26.0 $\mu$M and 13.1 $\mu$mol/min/mg, respectively.

The kinetic mechanisms of the cyclic nucleotide-dependent protein kinases are probably ordered Bi-Bi reactions in which MgATP$^{2-}$ binds first (9, 40, 42, 43). It has also been shown that the interaction of the heat-stable inhibitor protein with the catalytic subunit of cAMP-dependent protein kinase is enhanced by preincubation with ATP (21, 22). Because of this, we examined whether the order of addition of reagents might affect the potency of inhibition of the cGMP-dependent protein kinase by the inhibitory peptides. The enzyme was preincubated at 30 °C with PKI(7-22)amide, MgATP$^{2-}$, and all the standard reaction components except phosphoryl-accepting substrate, and the reaction was then initiated by the addition of peptide substrate. Under these conditions, exactly the same inhibitory potency was obtained as when the reaction was initiated by the addition of cGMP-dependent protein kinase (data not shown). In addition to these experiments, PKI(5-22)amide, PKI(10-24)amide, and PKI(14-24)amide were 0 (C), 47 (B), 94 (C), and 188 (D). Protein kinase activity was assayed with [y$^{32}$P]ATP as variable substrate as described under "Experimental Procedures."
Recognition of Inhibitor Peptides by cGMP-dependent Kinase

24)amide were directly tested as possible substrates of the cGMP-dependent protein kinase. Reaction conditions were as described under "Experimental Procedures." None of the inhibitory peptides was phosphorylated after 120 min of incubation at substrate concentrations of 50 μM and a final concentration of cGMP-dependent protein kinase that was 50-fold greater than that used in the inhibition experiments. Under these same conditions, (Ala3')H2B(29-35) and Kemp tide were stoichiometrically phosphorylated in less than 15 min (data not shown).

Substrate-dependent Inhibition of the cGMP-dependent Protein Kinase—With previous studies, it has been shown that inhibition of the cGMP-dependent protein kinase by inhibitory peptides binding at the catalytic site was apparently peptide/protein substrate-dependent (9). Thus, (Ala3')H2B(29-35) and (Ala)Kemptide were found to be effective inhibitors when a range of peptides were used as the phosphoryl-acceptor substrate, but not when one of several histones were substrate. Similar differences were not observed for inhibition of the cAMP-dependent protein kinase by either of these two peptides (9). Because of these observations, the protein/peptide substrate dependency of inhibition of cGMP-dependent protein kinase by the PKI(5-24) derivatives was tested. For this study (Table II), three different substrates of the enzyme were used at or near their Kₘ concentrations. Of these, (Ala3')H2B(29-35), the substrate used in the experiments of Figs. 1–3, and histone H2B are kinetically excellent substrates for the cGMP-dependent protein kinase (Kₘ values = 28 μM and 1.5 μM, respectively; Refs. 28 and 3). Kemp tide, which has been used extensively as a model peptide substrate for the cAMP-dependent protein kinase, is readily phosphorylated by the cGMP-dependent enzyme, although its Kₘ value is much higher for the latter than for the former enzyme (Kₘ values = 231 μM and 5 μM, respectively; Refs. 40 and 42). In Table II are presented the results of testing the inhibition of cGMP-dependent protein kinase by (Ala3')H2B(29-35) and four of the inhibitor protein peptides using these three different protein/peptide substrates. As shown previously (9), (Ala3')H2B(29-35) is an effective inhibitor when either (Ala3')H2B(29-35) or Kemptide was used as substrate, but did not block the phosphorylation of histone H2B. The same result was observed for the four inhibitor protein peptides tested. Each of these peptides inhibited the phosphorylation of (Ala4')H2B(29-35) and Kemptide with similar potencies but none, over the concentrations tested, caused significant inhibition of histone H2B phosphorylation. The same results were also obtained independent of the order of addition of reaction components (not shown). The reasons for this apparent substrate-dependent action of the inhibitor peptides are not yet understood. It is not a consequence of the differences in Kₘ values for the peptides and histone, because each was examined at or near its Kₘ concentration. For (Ala3')H2B(29-35), the inability to inhibit substrate phosphorylation appears unique to histones since, whereas it does not inhibit the phosphorylation of various histones, it does inhibit cGMP-dependent protein kinase autophosphorylation (40) and the phosphorylation by the cGMP-dependent protein kinase of other proteins such as phosphorylase kinase, tropomin, and the Type I regulatory subunit of the cAMP-dependent protein kinase. Possibly, these effects are related to the "poly(L-arginine) binding site" of the cGMP-dependent kinases. As we have recently reported (25,26), the PKI(5-24)amide peptides inhibited the cGMP-dependent protein kinase, we have re-evaluated whether very high concentrations of inhibitor protein might be inhibitory. The data on this are presented in Fig. 4; only one peptide, (Ala3')H2B(29-35), was tested as substrate because of a shortage of available inhibitor protein at 100 μM (i.e. ~10⁻⁷ × Kₘ for the cAMP-dependent protein kinase), the inhibitor protein did not inhibit the cGMP-dependent protein kinase; this is a concentration where the peptides derived from it were markedly inhibitory.

TABLE II

| Inhibitor peptide | Concentration (μM) | Protein kinase activity | Kemptide Histone H2B |
|------------------|------------------|------------------------|----------------------|
| (Ala3')H2B(29-35) | 1                | 91.5                   | 112.4                |
|                  | 10               | 70.2                   | 86.4                 |
|                  | 100              | 22.2                   | 23.8                 |
| PKI(14-24)amide | 1                | 91.3                   | 100.0                |
|                  | 10               | 85.4                   | 87.0                 |
|                  | 100              | 38.7                   | 39.4                 |
| PKI(7-22)amide  | 1                | 91.7                   | 108.7                |
|                  | 10               | 82.8                   | 91.5                 |
|                  | 100              | 41.4                   | 44.3                 |
| PKI(5-24)amide  | 5.5              | 84.0                   | 103.9                |
|                  | 35               | 72.5                   | 77.0                 |
|                  | 350              | 21.9                   | 22.2                 |
| (Ala4')H2B(29-35)| 3                | 94.2                   | 98.2                 |
|                  | 30               | 65.5                   | 60.1                 |
|                  | 300              | 19.3                   | 17.5                 |

D. B. Glass, unpublished observations.

Discussion

The data presented in this report provide a comparison between the protein/peptide binding domains in the catalytic sites of the cGMP-dependent and cAMP-dependent protein kinases. As we have recently reported (25,26), the PKI(5-24) peptide derived from the inhibitor protein, and presumably the inhibitor protein itself, contain at least two critical regions of amino acids that are essential for the high affinity interaction at the catalytic site of the cAMP-dependent protein kinase. These two domains are the arginine cluster of Arg₁₅, Arg₂₉, and some of the first 7 NH₂-terminal residues (residues 5–11). For the cAMP-dependent protein kinase, deletion or substitution of residues in either of these two domains markedly modifies inhibitory peptide binding. In contrast to this, it is clear from the data provided in this report that the cGMP-dependent protein kinase contains in common with the cAMP-dependent protein kinase the recognition of the arginine cluster, but does not recognize the NH₂-terminal.
domain amino acid sequence. It is well documented that basic amino acid residues serve as determinants of peptide substrate specificity for the cGMP-dependent protein kinase (2-4, 28, 34). Thus, an ionic interaction with the guanidine groups of (Ala<sup>6</sup>)H<sub>2</sub>B(29-35) and the indicated concentrations of inhibitor protein (●) or PKI(14-24)amide (○) in a 20-μl reaction volume. Data are the average of duplicate determinations.

![Fig. 4. Comparison of the effects of inhibitor protein and PKI(14-24)amide on activity of the cGMP-dependent protein kinase. The reaction was performed as described under "Experimental Procedures" using substrate concentrations of 50 μM ATP and 30 μM (Ala<sup>6</sup>)H<sub>2</sub>B(29-35) and the indicated concentrations of inhibitor protein (●) or PKI(14-24)amide (○) in a 20-μl reaction volume. Data are the average of duplicate determinations.](image)

derived from it (Fig. 4), and may not bind at all. It would appear that the inhibitor protein has evolved not only specific recognition sequences for the substrate binding site of the cAMP-dependent protein kinase, but also additional features that have diminished its interaction with other protein kinases such as the cGMP-dependent enzyme.

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