Distinguishing between Closely Related Protein Kinases: A Variation on the Bisubstrate Inhibitor Theme

Xiongwei Yan and David S. Lawrence*

Department of Biochemistry
The Albert Einstein College of Medicine of
Yeshiva University, 1300 Morris Park Avenue
Bronx, New York 10461-1602

Jackie D. Corbin and Sharron H. Francis

Department of Molecular Physiology and Biophysics
Vanderbilt University School of Medicine
Nashville, Tennessee 37232

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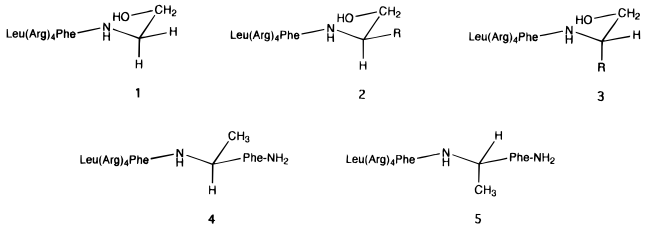
Protein kinases are key participants in a variety of signal transduction pathways, including those responsible for cell growth and differentiation.[1] These enzymes catalyze a relatively simple reaction, namely, phosphoryl transfer from ATP to serine, threonine, and/or tyrosine residues in proteins. Protein kinases may ultimately prove to be useful targets for chemotherapeutic intervention since the constitutive activation of specific members of the kinase family is known to result in cellular transformation.[2] However, it has been estimated that the human genome likely codes for more than 2000 protein kinases,[3] a figure which suggests that the design of kinase-specific inhibitors may prove problematic. One strategy employed to overcome this potential difficulty utilizes peptides whose primary sequences are based upon the characteristic substrate sequence specificities of individual protein kinases.[4] Unfortunately, it is now recognized that these enzymes often exhibit overlapping specificities *with respect to peptide substrates*. Nowhere is this difficulty more evident than with the cAMP- ("PKA") and cGMP-dependent ("PKG") protein kinases. The former is responsible for transducing the signals of the second messenger cAMP, whereas the latter plays a key role in NO-induced signaling.[5] These closely related enzymes exhibit a high degree of primary sequence homology, particularly within their catalytic cores. Consequently, it is not too surprising that every peptide that has been reported to serve as a substrate for PKG is also phosphorylated by PKA.[6] In addition, inhibitors related in structure to ATP also fail to significantly distinguish between these closely related enzymes.[7] We report herein the first example of a highly selective reversible inhibitor for PKG. Furthermore, the selectivity of this inhibitory species is based, in part, upon a novel variation on the "bisubstrate inhibitor" strategy.[8,9]

Although PKA and PKG both recognize and phosphorylate the naturally occurring L-amino acids, serine and threonine, we have previously demonstrated that these enzymes exhibit pronounced differences in their ability to phosphorylate unnatural residues.[10] For example, compound **1** is an excellent substrate for PKA and PKG. This species contains an unnatural amino alcohol fused to an active site-directed peptide. Furthermore, residues (**2**) containing α-substituents also serve as a substrates for both enzymes. In the latter instance, the α-configuration corresponds to that found in L-amino acids. In contrast, when the configuration at this stereocenter is analogous to that in D-amino acids, the resultant species (**3**) are no longer recognized as substrates for PKA but retain their ability to be phosphorylated by PKG. Is it possible to utilize this pronounced difference in the active site substrate specificity of these two closely related enzymes to construct a PKG-selective inhibitor? To address this question, we prepared peptides **4** and **5**. In the former case, a nonphosphorylatable L-alanine residue resides in the position normally reserved for serine. In the latter case, a D-alanine moiety is located at this site.



Peptide **4** is an effective inhibitor of both PKA and PKG (Table 1). These results are consistent with the notion that L-amino acids, and related analogs (cf. peptide **2**), are readily accommodated within the active sites of both enzymes. In addition, the nearly identical K_i values exhibited by peptide **4** for both PKA and PKG highlight the previously encountered difficulties in distinguishing between these closely related enzymes with conventional peptides. In contrast, although peptide **5** is also an excellent inhibitor of PKG, its inhibitory potency toward PKA is markedly diminished. These results are in agreement with the substrate specificities displayed by these enzymes for alcohol-bearing residues analogous to D-amino acids (cf. peptide **3**). In the presence of fixed [ATP] (100 μM) and variable [peptide substrate], **5** is a 50-fold more potent inhibitor of PKG than of PKA. Most importantly, however, this observed selectivity of **5** for PKG should be even greater under *in vivo* conditions (*vide infra*). In short, peptide **5** is the first example of an active site-directed species that exhibits a significant inhibitory preference for PKG over that of PKA.[11]

As expected, both peptides **4** and **5** are competitive inhibitors versus variable peptide substrate.[12] Surprisingly, however, **5** also serves as a competitive inhibitor versus variable ATP for the PKA-catalyzed reaction. On the basis of these results, it is clear that **5** competes directly with the ability of *both* ATP and peptide substrate to bind to their appropriate sites in PKA. Dual competitive inhibition patterns are often exhibited by transition state or bisubstrate analogs,[13] and consequently, the simplest interpretation of these results is that **5** simultaneously associates with at least a portion of both the ATP and peptide substrate binding sites. Alternatively, since ATP is known to induce changes in the conformation of PKA,[14] it is also possible that

* To whom correspondence should be addressed. Telephone (718) 430-2813; Fax (718) 430-8565.

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Table 1. The K_i Values and Inhibition Patterns of Peptides 4 and 5 with PKA and PKG[a]

	cAMP-dependent protein kinase		cGMP-dependent Protein Kinase	
inhibitor	K_i (kemptide)	K_i (ATP)	K_i (kemptide)	K_i (ATP)
4	4.9 ± 0.8 μM (C)	13.5 ± 0.9 μM (U)	10.0 ± 0.7 μM (C)	15.1 ± 1.8 μM (U)
5	380 ± 40 μM (C)	490 ± 40 μM (C)	7.5 ± 2.5 μM (C)	17.2 ± 1.5 μM (U)

[a] The peptide substrate employed in these studies was kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly). Assays were performed in triplicate at pH 7.1 and thermostated in a water boath at 30 °C. Final assay volume totaled 40 μL and contained 100 mM MOPS, 150 mM KCl, 12.5 mM $MgCl_2$, 0.125 mg/mL bovine serum albumin, and 0.5 nM PKA or 2 nM PKG dimer (with 20 μM cGMP). Inhibitor concentrations were varied from 5−8-fold about their K_i values. [ATP] was varied from 4 to 24 μM at fixed [kemptide] (50 μM for PKA and 100 μM for PKG) and [kemptide] was varied from 10 to 60 μM (PKA) or 30 to 200 μM (PKG) at fixed [ATP] (100 μM [γ-^{32}P]ATP (300−2000 cpm/pmol)). Phosphorylated reactions were initiated by addition of 10 μL solution of either PKA or PKG and terminated after 6 min (PKA) or 20 min (PKG) by spotting 25 μL aliquots onto 2.1 cm phosphocellulose paper disks. After 10 s the disks were immersed in 10% glacial acetic acid (30 mL per disk) and allowed to soak for at least 60 min. The acetic acid was decanted, and the disks were collectively washed with 4 volumes of 0.5% phosphoric acid, 1 volume of water, and a final acetone rinse (2 mL/disk). The disks were air-dried and placed in plastic scintillation vials containing 6 mL of Liquiscint prior to scintillation counting for radioactivity.

ATP and **5** bind to different, and mutually exclusive, conformational states. However, regardless of the actual mechanism, it is evident that **5** cannot associate with PKA when ATP is bound in the active site of this enzyme. Consequently, given the weak inhibitory potency of **5** at 100 μM ATP (i.e., $K_i = 380 \pm 40$ μM) and the average intracellular concentration of ATP in normal (2.5 mM) and tumor cells (3.1 mM),[15] it is evident that this species will be an exceedingly poor inhibitor of PKA under *in vivo* conditions. *The bisubstrate structural motif is typically regarded as an attractive feature since it should confer additional inhibitory potency relative to simple mono-substrate analogs. However, as exemplified by the poor inhibitory performance of* ***5*** *with PKA, an inhibitor that directly competes with more than one substrate can be spectacularly ineffective under appropriate conditions as well.*

Finally, it is important to note that **5** does not display the characteristics of a bisubstrate inhibitor with PKG. In short, since it is not a competitive inhibitor versus variable ATP, high *in vivo* concentrations of the nucleotide cannot prevent peptide **5** from associating with PKG. As a consequence, **5** is the first example of a peptide-based PKG reversible inhibitor that exhibits no significant activity toward PKA. Although the structural basis for this discriminatory behavior remains to be elucidated, it is evident that even closely related protein kinases can now be distinguished by their characteristic active site specificities.[10,16]

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(12) The competitive inhibition patterns from the Lineweaver−Burk plots intersect slightly off the *y*-axis (data not shown). Others (see: Feramisco, J. R.; Krebs, E. G. *J. Biol. Chem.* **1978**, *253*, 8968−8971) have observed this behavior with inhibitory peptides using the substrate kemptide (i.e., Leu-Arg-Arg-Ala-Ser-Leu-Gly), which was employed in this study as well. Indeed, kemptide appears to be responsible for this phenomenon, as both peptides **4** and **5** give competitive inhibition patterns that precisely intersect on the *y*-axis using the substrate Leu-Arg-Arg-Arg-Arg-Phe-Ser-$NHCH_2C_6H_5$ in the PKA-catalyzed reaction. Unfortunately, the latter arginine-rich peptide substrate cannot be employed with PKG since, in addition to serving as a substrate for this enzyme, it also appears to bind to a "polyarginine binding site" (Walton, G. M.; Gill, G. N. *J. Biol. Chem.* **1981**, *256*, 1681−1688), which significantly modulates PKG activity (Yan, X.; Lawrence, D. S. Unpublished results).

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