Molecular Basis for the Substrate Specificity of a Serine/Threonine-Specific Protein Kinase

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Abstract: Protein kinases typically phosphorylate the aliphatic alcohols of serine/threonine residues or the aromatic alcohol of tyrosine residues *but not both*. We report herein the first example of aromatic alcohol phosphorylation by a serine/threonine-specific protein kinase. The cAMP-dependent protein kinase phosphorylates the C-terminal aromatic alcohol in the active site-directed peptide Gly-Arg-Thr-Gly-Arg-Arg-Asn-(*o*-aminophenol). In contrast, corresponding peptides containing *m*- and *p*-aminophenols failed to serve as protein kinase substrates. These results indicate that the orientation of the aromatic hydroxyl group relative to the adjacent peptide backbone bond is a critical structural motif employed in substrate recognition by the enzyme. This observation also provides an explanation for the demonstrated inability of the cAMP-dependent protein kinase to catalyze the phosphorylation of tyrosine residues in proteins. The observed K_m (793 ± 39 μM) for the *o*-aminophenol-containing substrate is consistent with values previously reported for other alcohol-bearing residues containing β-substituents. In contrast, the k_{cat} (2.77 ± 0.16 min^{-1}) is significantly lower than those generally observed for the cAMP-dependent protein-kinase-catalyzed phosphorylation of *aliphatic* alcohols. However, this k_{cat} does compare favorably with values reported for tyrosine-kinase catalyzed phosphorylations. The corresponding phosphopeptide of Gly-Arg-Thr-Gly-Arg-Arg-Asn-(*o*-aminophenol) was isolated and subsequently characterized by fast atom bombardment mass spectrometry and ^1H-coupled ^{31}P nuclear magnetic resonance spectroscopy. In the latter case, a singlet was observed at −1.87 ppm upfield from H_3PO_4, which is the expected coupling pattern and chemical shift for a phosphorylated aromatic alcohol. These observations indicate that a serine/threonine-specific protein kinase possesses the inherent ability to catalyze phosphoryl transfer to an acceptor moiety other than an aliphatic alcohol and consequently expands the realm of possibilities with respect to inhibitor design.

In the last few years, research in the general area of signal transduction has exploded with activity. From vision[1] to cell division,[2,3] protein kinases serve as key components along signal transduction pathways. In addition, there are compelling reasons to believe that specific protein kinase inhibitors could potentially serve as novel cancer chemotherapeutic agents. However, in contrast to our rapidly developing appreciation of signal transduction and the role of protein kinases along these pathways, the creation of specific protein inhibitors (other than the standard alanine-for-serine substitution in active site-directed peptides) has been painfully slow. This is, in large part, due to our incomplete understanding of the scope and limitations of the active site substrate specificity of protein kinases, namely the range of compounds that can be readily accommodated and processed (*i.e.*, phosphorylated) by the catalytic apparatus of this enzyme family. Perhaps no where is our dearth of knowledge concerning substrate specificity more evident than at the well-known partition dividing the protein kinase family into two separate subfamilies. Protein kinases typically exhibit a strict substrate specificity, either phosphorylating aliphatic (*i.e.*, serine and threonine) or aromatic (*i.e.*, tyrosine) hydroxyl groups in proteins.[4] For example, $P130^{gag\text{-}fps}$-catalyzed tyrosine autophosphorylation is blocked if the target tyrosine moiety is mutated to either a serine of threonine residue.[5] In addition, while Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly serves as a substrate for the insulin receptor/kinase, the corresponding serine- and threonine-containing peptides are not phosphorylated and merely act as competitive inhibitors.[6] In this context, we have demonstrated the corollary that the serine/threonine-specific cAMP-dependent protein kinase is unable to phosphorylate a tyrosine residue in an active site-directed peptide.[7] Indeed, until recently, all members of the protein kinase family were thought to exhibit the absolute substrate specificity illustrated in the previous examples. However, several kinases have now been identified which are able to phosphorylate all three hydroxyl-bearing amino acid residues in peptide or protein substrates.[8-18] In light of the latter observations, we became particularly intrigued with identifying those structural features on the substrate molecule that enable amino-acid-specific protein kinases to discriminate between aromatic and aliphatic alcohols. We have now addressed this question for the serine/threonine-specific cAMP-dependent protein kinase, and we have discovered that the substrate specificity

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(1) Smith, D. P.; Ranganathan, Hardy, R. W.; Marx, J.; Tsuchida, T.; Zuker, C. S. *Science* **1991**, *254*, 1478-1484.
(2) Lewin, B. *Cell* **1990**, *61*, 743-752.
(3) Pelech, S.; Sanghera, J. S. *Science* **1992**, *257*, 1355-1356.
(4) Hanks, S. K.; Quinn, A. M.; Hunter, T. *Science* **1988**, *241*, 42-52.
(5) Weinmaster, G.; Pawson, T. *J. Biol. Chem.* **1986**, *261*, 328-333.
(6) Walker, D. H.; Kuppuswamy, D.; Visvanathan, A.; Pike, L. J. *Biochemistry* **1987**, *26*, 1428-1433.
(7) Mendelow, M.; Prorok, M.; Salerno, A.; Lawrence, D. S. *J. Biol. Chem.*, **1993**, *268*, 12289-12296.
(8) Dailey, D.; Schieven, G. L.; Lim, M. L.; Marquardt, H.; Gilmore, T.; Thorner, J.; Martin, G. S. *Mol. Cell. Biol.* **1990**, *10*, 6244-6256.
(9) Featherstone, C.; Russell, P. *Nature* **1991**, *349*, 808-811.
(10) Seger, R.; Ahn, N. G.; Boulton, T. G.; Yancopoulos, G. D.; Panayotatos, N.; Radziejewska, E.; Ericsson, L.; Bratlien, R. L.; Cobb, M. H.; Krebs, E. G. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6142-6146.
(11) Stern, D. F.; Zheng, P.; Beidler, D. R.; Zerillo, C. *Mol. Cell. Biol.* **1991**, *11*, 987-1001.
(12) Ben-David, Y.; Letwin, K.; Tannock, L.; Bernstein, A.; Pawson, T. *EMBO J.* **1991**, *10*, 317-325.
(13) Crews, C. M.; Alessandrini, A. A.; Erikson, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8845-8849.
(14) Howell, B. W.; Afar, D. E. H.; Lew, J.; Douville, E. M. J.; Icely, P. L. E.; Gray, D. A.; Bell, J. C. *Mol. Cell. Biol.* **1991**, *11*, 568-572.
(15) Barrett, C. B.; Erikson, E.; Maller, J. L. *J. Biol. Chem.* **1992**, *267*, 4408-4415.
(16) Letwin, K.; Mizzen, L.; Motro, B.; Ben-David, Y.; Bernstein, A.; Pawson, T. *EMBO J.* **1992**, *11*, 3521-3531.
(17) Parker, L. L.; Atherton-Fessler, S.; Piwnica-Worms, H. *Proc. Natl. Acad. Sci U.S.A.* **1992**, *89*, 2917-2921.
(18) Lindberg, R. A.; Quinn, A. M.; Hunter, T. *Trends Biochem. Sci.* **1992**, *17*, 114-119.

0002-7863/93/1515-9888$04.00/0

of this enzyme is not limited to aliphatic alcohols but extends into the realm of aromatic alcohols as well.

Materials and Methods

All chemicals were obtained from Aldrich, except for [γ-^{32}P]ATP (New England Nuclear), cAMP (Fluka), protected amino acid derivatives (Advanced Chemtech and U.S. Biochemical), and Liquiscint (National Diagnostics). Dialysis tubing was purchased from Fisher Scientific, CM C-50 Sephadex and GS-100 Superfine Sephadex were obtained from Pharmacia, and Affi-gel Blue resin was acquired from BioRad. Phosphocellulose P 81 paper disks were purchases from Whatman.

cAMP-Dependent Protein Kinase Preparation. The catalytic subunit was purified to homogeneity using a previously described procedure.[19] Purity was assessed *via* SDS polyacrylamide gel electrophoresis, which displayed a single band at a molecular mass of 41 000 Da. Ellman's reagent titrated the cysteine residues to 2.05–2.10 sulfhydryls per molecule of enzyme, which is in excellent agreement with previously reported studies[20] and the known primary structure of the catalytic subunit.[21]

Peptide Synthesis. Boc-Gly-Arg-Thr-Gly-Arg-Arg-Asn[dimethoxydiphenylmethyl(Ddm)]-resin was prepared on Kaiser's oxime resin (substitution level = 0.43 mmol/g of resin)[22-24] with *tert*-butoxycarbonyl (Boc) amino acids and subsequently displaced from the resin with the *ortho*-, *meta*-, and *para*-derivatives of aminophenol by heating to reflux in chloroform for 4 h. The reaction mixture was subsequently filtered and the solvent removed *in vacuo*. Each peptide was then treated with 100% trifluoroacetic acid for 3 h at room temperature to remove the Ddm and Boc protecting groups. The trifluoroacetic acid was evaporated under reduced pressure, and the peptides were purified by preparative HPLC using three Waters radial compression modules (2.5 cm × 10 cm) connected in series [gradient (solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile): 0–3 min (100% A), a linear gradient from 3 min (100% A) to 25 min (75% A and 25% B), 25–30 min (25%–70% B), and 30–40 min (70% B)]. The peptides were then lyophilized and purified by ion exchange on CM-25 (50 mM NaOAc at pH 3.5 with a KCl gradient of 0.4–1.0 M). Finally, the peptides were rapidly gel-filtered *via* preparative HPLC using the HPLC solvent gradient described above. All of the collected peptides were then lyophilized and gave satisfactory fast atom bombardment mass spectral analyses.

Kinase Assay. Assays were performed in triplicate at pH 7.1 and thermostated in a water bath maintained at 30 °C. Final assay volume totaled 50 μL and contained 100 mM 4-morpholinepropanesulfonic acid, 150 mM KCl, 12.5 mM $MgCl_2$, 0.125 mg/mL of bovine serum albumin, and 50 nM catalytic subunit of the cAMP-dependent protein kinase. For the determination of kinetic constants, concentrations were employed as follows: 100 μM [γ-^{32}P] ATP (300–400 cpm/pmol) and a substrate concentration over a 6-fold range around the apparent K_m. Phosphorylation reactions were initiated by the addition of 10 μL of catalytic subunit diluted from a concentrated stock solution (1.5 mg/mL in 100 mM 4-morpholinepropanesulfonic acid, 150 mM KCl, 1 mM dithiothreitol, and 0.125 mg/mL of bovine serum albumin at pH 7.1). Reactions were terminated after 5.0 min by spotting 25-μL aliquots onto 2.1-cm diameter phosphocellulose paper disks. After 10 s, the disks were immersed in 10% glacial acetic acid and allowed to soak with occasional stirring for at least 1 h. The acetic acid was decanted, and the disks were collectively washed with four volumes of 0.5% H_3PO_4 and one volume of water followed by a final acetone rinse. The disks were air-dried and placed in plastic scintillation vials containing 6 mL of liquiscint prior to scintillation counting for radioactivity.

Determination of Kinetic Constants. The apparent K_m (±SD) and k_{cat} (±SD) values were determined from initial rate experiments. The data from these experiments were plotted using the Lineweaver–Burke procedure, and the corresponding plot proved to be linear.

Preparative Scale Phosphorylation of Peptide 2 and Isolation of the Phosphorylated Product. The *o*-aminophenol-containing peptide **2** was phosphorylated to near completion using the following reaction conditions: a 1 mM concentration of peptide was initially treated with 1 μM enzyme (150 mM KCl, 10 mM ATP, 12.5 mM $MgCl_2$, and 0.125 mg/mL of bovine serum albumin) for 60 min at 30°C. An additional 1 μM of enzyme was added at 60 min and then at 120 min to give a final enzyme concentration of 3 μM. The reaction mixture was gel-filtered (G-10 with 5% acetic acid/95% deioinized water as eluent) and then further purified using the HPLC gradient described above. The phosphopeptide eluted at 25.5 min and its unphosphorylated counterpart at 26.5 min.

^{31}P Nuclear Magnetic Resonance Experiments. ^{31}P NMR experiments were performed at 161.903 MHz on a Varian-400S spectrometer. The sample contained approximately 8 mM phosphorylated peptide in D_2O. Parameters were employed as follows: spectral width 32 000 Hz, acquisition time 0.800 s, relaxation delay 0.500 s, pulse width 10.0 μs, ambient temperature.

Results and Discussion

The cAMP-dependent protein kinase readily phosphorylates both serine (K_m = 16.0 μM; k_{cat} = 824 min^{-1}) and threonine (K_m = 590 μM; k_{cat} = 228 min^{-1}) residues at Xaa in the active site-directed peptide Leu-Arg-Arg-Ala-Xaa-Leu-Gly.[25-26] In contrast, the tyrosine-containing peptide Leu-Arg-Arg-Ala-Tyr-Leu-Gly fails to serve as a substrate for this enzyme.[7] These results are representative of the absolute substrate specificity that has been generally attributed to the vast majority of protein kinases. There are a number of explanations which may account for this phenomenon. For example, the relatively large hydrophobic tyrosine side chain could preclude ATP from binding to the active site or might prevent the requisite conformational change the enzyme undergoes prior to catalyzing phosphoryl transfer.[27-29] However, we have previously shown that Leu-Arg-Arg-Ala-Tyr-Leu-Gly is a noncompetitive inhibitor versus ATP, indicating that ATP and a peptide can simultaneously bind to the enzyme.[7] In addition, we found that the low-level ATPase activity associated with the cAMP-dependent protein kinase is unaffected by the tyrosine-containing peptide, implying that the enzyme retains its ability to catalyze phosphoryl transfer in the presence of the heptapeptide.[7] These results suggest that the ability of the protein kinase to discriminate between serine/threonine and tyrosine residues is not due to interference of the normal catalytic operation of the enzyme by the tyrosine moiety. Instead, it is evident that, as a consequence of some structural attribute unique to the tyrosine moiety, enzyme-catalyzed phosphorylation is simply not triggered. The structural characteristics of tyrosine that may account for this behavior include the following.

(i) The tyrosine side chain is more sterically demanding than the side chains of serine and threonine. Previously, we,[30] as well as others,[25,31] have noted that serine and its structural counterparts are more readily phosphorylated than β-substituted residues, such as threonine. One obvious interpretation is that the larger threonine moiety is not as readily accommodated in the enzyme active site as serine. Consequently, the even bulkier aromatic alcohol side chain of tyrosine may be totally excluded from the active site.

(ii) The hydroxyl functionality of the tyrosine side chain occupies a comparatively rigid geometry by virtue of being attached to a planar aromatic ring. This feature might preclude the requisite side-chain conformation needed for interaction with the catalytic apparatus of the enzyme.

(iii) The alkoxides of aromatic alcohols are generally less nucleophilic than their corresponding aliphatic analogues. This suggests that the former might not be reactive enough to serve as a phosphoryl acceptor in reactions catalyzed by serine/threonine kinases.

(iv) The orientation of the tyrosine hydroxyl moiety relative to the adjacent peptide bond is clearly different than that for the alcohol functionalities in serine and threonine. This could preclude proper alignment of the tyrosine hydroxyl moiety with respect to the active site functional groups engaged in catalysis.

(19) Prorok, M.; Lawrence, D. S. *J. Biochem. Biophys. Methods* **1989**, *18*, 167–176.
(20) Armstrong, R. N.; Kaiser, E. T. *Biochemistry*, **1978**, *17*, 2840–2845.
(21) Shoji, S.; Ericsson, L. H.; Walsh, K. A.; Fischer, E. H.; Titani, K. *Biochemistry* **1983**, *22*, 3702–3709.
(22) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1980**, *45*, 1295–1300.
(23) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1982**, *47*, 3258–3261.
(24) Kaiser, E. T. *Acc. Chem. Res.* **1989**, *22*, 47–54.
(25) Kemp, B. E.; Graves, D. J.; Benjamini, E.; Krebs, E. G. *J. Biol. Chem.* **1977**, *252*, 4888–4893.
(26) The cAMP-dependent protein kinase has recently been reported to phosphorylate a Tyr-Glu copolymer to a very limited extent.[11] These results are somewhat surprising in light of the known substrate specificity of the enzyme, which is limited to positively charged substrates.[25]
(27) Buechler, J. A.; Vedvick, T. A.; Taylor, S. S. *Biochemistry* **1989**, *28*, 3018–3024.
(28) Buechler, J. A.; Taylor, S. S. *Biochemistry* **1990**, *29*, 1937–1943.
(29) Zheng, J.; Knighton, D. R.; Parello, J.; Taylor, S. S.; Sowadski, J. M. *Methods Enzymol.* **1991**, *200*, 508–521.
(30) Kwon, Y.-G.; Mendelow, M.; Srinivasan, J.; Lee, T. R.; Pluskey, S.; Salerno, A.; Lawrence, D. S. *J. Biol. Chem.* **1993**, *268*, 10713–10716.
(31) Glass, D. B.; El-Maghrabi, M. R.; Pilkis, S. J. *J. Biol. Chem.* **1986**, *261*, 2987–2993.

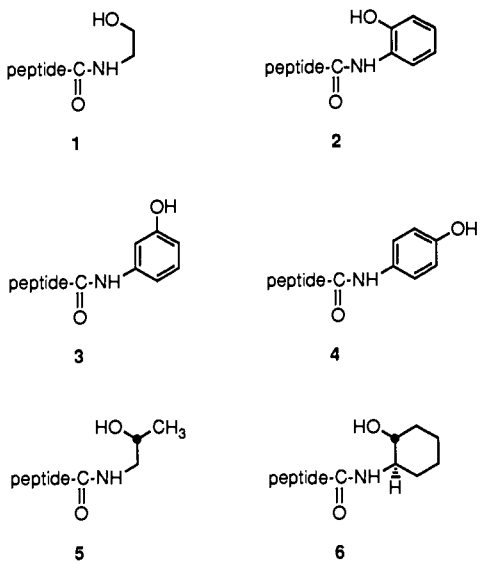

Figure 1. The structure of peptides **1-6**. Peptide = Gly-Arg-Thr-Gly-Arg-Arg-Asn.

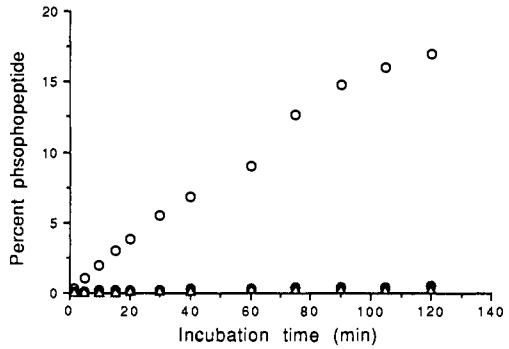

Figure 2. Percent phosphorylation of peptides **2-4** as a function of reaction time. The conditions employed are those described in the Kinase Assay section of the Materials and Methods section with the following exceptions: [peptide] = 500 μM, [enzyme] = 500 nM at 0 min and then an additional 500 nM of protein kinase was added at 60 min and, [ATP] = 1.5 mM. Aliquots of the reaction mixture were removed at selected time points and spotted onto phosphocellulose paper disks, which were subsequently washed and counted for radioactivity. Both peptides **3** and **4** were phosphorylated to less than 0.5% (○) peptide 2, (●) peptide 3, and (△) peptide 4.

In order to address these possibilities, a series of peptides were prepared on the basis of our recent observation that peptides containing C-terminal alcohols are readily phosphorylated by the cAMP-dependent protein kinase.[30] For example, compound **1** exhibits a K_m of 114 μM and a k_{cat} of 338 min^{-1}. The target hydroxyl group in this compound is positioned in a manner similar to that present in serine, namely two carbon atoms removed from the adjacent backbone peptide bond. In a somewhat analogus fashion, the aromatic alcohol in peptide **2** can sweep a range of orientations, relative to the adjacent peptide bond, that are similar to those possible in **1**. In contrast, the geometries between the hyroxyl moiety and the backbone peptide bond in **3** and **4** are quite distinct from those in **1** and **2** (Figure 1). Furthermore, the substitution pattern of the aromatic system in **4** is actually reminiscent of that present on the side chain of tyrosine.

Peptides **2-4** were synthesized employing a previously described methodology[30] and were then incubated with the catalytic subunit of the cAMP-dependent protein kinase. A plot of percent peptide phosphorylation versus reaction time is provided in Figure 2. It is evident from this graph that only the *ortho*-substituted derivative **2** is phosphorylated to any significant extent. These results demonstrate that a properly aligned aromatic hydroxyl moiety on an active site-directed peptide will serve as a substrate for the cAMP-dependent protein kinase, an enzyme which was previously thought to be only capable of catalyzing the phosphorylation of aliphatic alcohols. Moreover, the data from Figure 2 explicitly eliminate the other explanations for serine/threonine specificity mentioned above. All three compounds (**2-4**) contain aromatic residues of analogous size to tyrosine, all three possess alcohol functional groups that are attached directly to a planar ring system (as in tyrosine), and all three alcohol moieties should be as nucleophilic as the tyrosine hydroxyl functionality; yet only peptide **2** is phosphorylated.

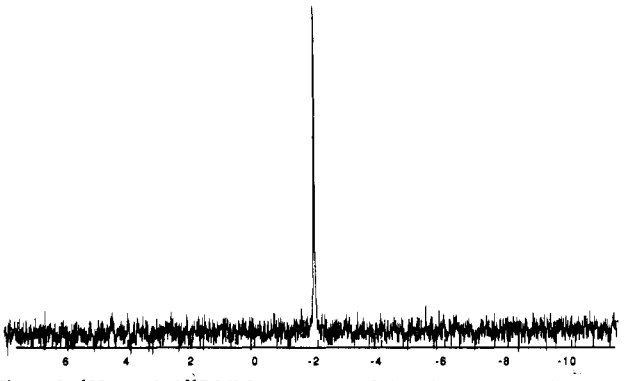

Figure 3. ^{1}H-coupled ^{31}P NMR spectrum of phosphorylated **2**. Chemical shift = −1.87 ppm upfield from external H_3PO_4. See the Materials and Methods section for experimental details.

Table I. K_m and k_{cat} Values for the *o*-Aminophenol-Containing Peptide **2** and the Primary and Secondary Alcohol-Bearing Peptides **1**, **5**, and **6**[a]

GRTGRRN-amino alcohol	K_m (μM)	k_{cat} (min^{-1})
(1)	114 ± 6	338 ± 12
(2)	793 ± 39	2.77 ± 0.16
(5)	607 ± 83	49.0 ± 4.1
(6)	617 ± 37	77.5 ± 1.6

[a] Kinetic constants were determined as described under Materials and Methods. Those for peptides **1**, **5**, and **6** have been previously described elsewhere.[30] The values are given as the average ± SD.

In order to demonstrate that phosphorylation had indeed taken place at the aromatic hydroxyl moiety in **2**, we incubated this peptide with the cAMP-dependent protein kinase and excess ATP for 3 h as described under Materials and Methods. The phosphopeptide was subsequently purified and then characterized by fast atom bombardment mass spectrometry as well as by ^{31}P NMR spectroscopy. Mass spectral analysis revealed a $(M + 1)^+$ peak of 987.5, the expected value for the monophosphorylated form of **2**. The ^{1}H-coupled ^{31}P NMR spectrum for the phosphopeptide is provided in Figure 3. The singlet observed at −1.87 ppm (referenced to external H_3PO_4) is the expected coupling pattern and chemical shift for a phosphorylated aromatic hydroxyl moiety. For example, phosphotyrosine exhibits a singlet at −2.1 ppm (spectrum not shown). In contrast, under the conditions employed in this study, phosphorylated aliphatic alcohols generally furnish chemical shifts in the 2–6 ppm range.[32-33]

We subsequently determined the K_m and k_{cat} values for **2** from initial rate experiments. These values, along with those previously reported on related peptides,[30] are provided in Table I. The K_m for **2** is approximately 7-fold larger than that acquired with the ethanolamine peptide **1**. However, the former is a β-substituted alcohol, and we have previously noted that these types of residues display somewhat elevated Michaelis constants.[30] Indeed, compounds **2** and **5**, both of which are substituted at the β-position of the alcohol moiety, exhibit similar K_m values, suggesting that the K_m obtained from the phosphorylation of **2** is neither unusual nor unexpected. In contrast, the k_{cat} obtained for **2** is substantially lower than those previously reported for aliphatic alcohols in general and peptides **1** and **5** in particular.[30] Interestingly, Graves and his co-workers have noted that the velocities typically observed with protein-kinase-catalyzed

(32) Ho, C.; Magnuson, J. A.; Wilson, J. B.; Magnuson, N. S.; Kurland, R. J. *Biochemistry* **1969**, *8*, 2074–2082.
(33) Kwon, Y.-G.; Srinivasan, J.; Mendelow, M.; Pluskey, S.; Lawrence, D. S. *J. Biol. Chem.* **1993**, *268*, 7527–7528.

serine phosphorylations are substantially greater than those obtained from protein-kinase catalyzed tyrosine phosphorylations.[34] Our results are remarkably consistent with their suggestion that this general behavior may be a consequence of the nucleophilicity associated with the attacking alkoxide, which is expected to be greater for aliphatic alcohols.[35] However, in this specific case, alternative explanations are also possible. Namely, the depressed turnover number obtained with **2** may be a consequence of the relatively large size of the six-membered ring. Alternatively, the low k_{cat} may be due to specific structural attributes associated with the aminophenol ring system, such as the fixed O–C–C–N dihedral angle. We have previously shown that compound **6**, which contains a cyclohexyl ring system, exhibits a reasonably efficient k_{cat} of 77.5 min^{-1}.[30,36] This result appears to rule out ring size as the structural feature responsible for the depressed maximal velocity associated with **2**. Since the k_{cat} values exhibited by **5** and **6** are nearly identical, it is also clear that the conformational constraints imposed by the cyclohexyl ring system do not deleteriously affect the velocity at which **6** undergoes phosphorylation. These results imply that the low k_{cat} associated with the phosphorylation of **2** is likely due to either the reduced nucleophilicity of aromatic alkoxides or the planar geometry associated with this system. Whichever explanation ultimately proves valid, it is noteworthy that the k_{cat} exhibited by **2** does compare favorably with k_{cat} values reported for tyrosine-kinase catalyzed reactions in general. For example, substrates for the insulin receptor kinase exhibit values from 0.8 to 6.8 min^{-1}.[37,38]

Recently, "dual specificity" protein kinases have been identified.[8-18] These enzymes are apparently able to indiscriminately phosphorylate serine, threonine, and tyrosine residues. Although the cAMP-dependent protein kinase is unable to phosphorylate a tyrosine moiety in an active site-directed peptide,[7] we have now shown that this serine/threonine-specific protein kinase is able to catalyze phosphoryl transfer to an aromatic alcohol. The results described herein indicate that the relative orientation and distance of the alcohol moiety from the peptide backbone are the key structural features responsible for the active site substrate specificity of the cAMP-dependent protein kinase. The nondiscriminatory behavior associated with dual specificity protein kinases may be due to a relaxation of constraints that otherwise control the positioning of the hydroxyl moiety with respect to critical active site functional groups.[18] Linberg, Quinn, and Hunter have proposed that Thr201 in the cAMP-dependent protein kinase may serve as this control point, since the 201 position tends to indicate whether the protein kinase is serine/threonine-specific (position 201 occupied by a serine or threonine residue) or tyrosine-specific (position 201 occupied by a proline residue).[18] In the 3-dimensional structure of the cAMP-dependent protein kinase, Thr201 lies just below the phosphoryl acceptor residue of the substrate.[39-43] In addition, we have recently suggested that Thr201 may also play a key role in precluding phosphoryl transfer to residues in which the stereochemistry at the α-position is inverted relative to that of L-serine.[44]

In summary, we have found that the cAMP-dependent protein kinase catalyzes the phosphorylation of an aromatic alcohol in an active site-directed peptide. This indicates that a serine/threonine-specific protein kinase possesses the inherent ability to catalyze phosphoryl transfer to an acceptor moiety other than an aliphatic alcohol and therefore expands the realm of possibilities with respect to inhibitor design. Furthermore, we have shown that an aromatic alcohol will not serve as a site of phosphorylation if it is misaligned relative to the amide backbone of the peptide substrate, thereby suggesting that serine/threonine-specific protein kinases discriminate against the tyrosine phenolic hydroxyl moiety in a similar fashion.

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(34) Martin, B. L.; Wu, D.; Jakes, S.; Graves, D. J. *J. Biol. Chem.* **1990**, *265*, 7108–7111.

(35) Yuan, C.-Y.; Jakes, S.; Elliot, S.; Graves, D. J. *J. Biol. Chem.* **1990**, *265*, 16205–16209.

(36) Peptide **6** is actually a mixture of two compounds, the 1*R*,2*R* isomer shown in Table I and the corresponding 1*S*,2*S* isomer. On the basis of previously demonstrated substrate specificity requirements for the cAMP-dependent protein kinase,[30] the latter will not serve as a substrate but should act as a competitive inhibitor versus its active diastereomeric counterpart. Under these circumstances, V_{max} is unaffected by the presence of the 1*S*,2*S* isomer.

(37) Kadowski, T.; Fujita, Y.; Yamaguchi, E. N.; Takaku, F.; Akiyama, T.; Kathuria, S.; Akanuma, Y.; Kasuga, M. *J. Biol. Chem.* **1985**, *260*, 4016–4020.

(38) Pike, L.; Kuenzel, E. A.; Casnellie, J. E.; Krebs, E. G. *J. Biol. Chem.* **1984**, *259*, 9913–9921.

(39) Knighton, D. R.; Zheng, J.; Eyck, L. F. T.; Ashford, V. A.; Xuong, N.-H.; Taylor, S. S.; Sowadski, J. M. *Science* **1991**, *253*, 407–414.

(40) Knighton, D. R.; Zheng, J.; Eyck, L. F. T.; Xuong, N.-H.; Taylor, S. S.; Sowadski, J. M. *Science* **1991**, *253*, 414–420.

(41) Zheng, J.; Trafny, E. A.; Knighton, D. R.; Xuong, N.-H.; Taylor, S. S.; Ten Eyck, L. F.; Sowadski, J. M. *Acta Crystallogr.* **1993**, *D49*, 363–365.

(42) Zheng, J.; Knighton, D. R.; Eyck, L. F. T.; Karlsson, R.; Xuong, N.-H.; Taylor, S. S.; Sowadski, J. M. *Biochemistry* **1993**, *32*, 2154–2161.

(43) Bossemeyer, D.; Engh, R. A.; Kinzel, V.; Postingl, H.; Huber, R. *EMBO J.* **1993**, *12*, 849–859.

(44) Kwon, Y.-G.; Srinivasan, J.; Mendelow, M.; Pluskey, S.; Lawrence, D. S. *J. Am. Chem. Soc.* **1993**, *115*, 7527–7528.