

Phenol Kinase Activity of the Serine/Threonine-Specific cAMP-Dependent Protein Kinase: Steric and Electronic Effects[†]

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ABSTRACT: We have found that the cAMP-dependent protein kinase catalyzes the phosphorylation of a wide variety of peptide-based aromatic alcohols, thereby greatly amplifying the range of compounds recognized as substrates by this enzyme. This newly discovered enzyme-catalyzed reaction is sensitive to both steric and electronic effects. Substituents on the aromatic ring that are positioned *para* to the hydroxyl moiety lower the observed K_m , presumably via a favorable interaction with an adjacent hydrophobic pocket. In contrast, electron-withdrawing substituents have a slight adverse effect on the kinetics of phosphoryl transfer, an observation which is consistent with the notion that the rate of substrate turnover is dependent upon the nucleophilicity of the phosphorylatable hydroxyl moiety. As a corollary, electron-donating groups on the aromatic nucleus promote the rate of phosphoryl transfer to such an extent that the observed V_{max} values approach those exhibited by aliphatic alcohols. This suggests that analogously appended electron-donating groups on tyrosine moieties could dramatically improve the modest V_{max} values that are typical for tyrosine kinase-catalyzed reactions.

Compounds that selectively interfere with oncogene-mediated signal transduction pathways may ultimately serve as novel cancer chemotherapeutic agents. Protein kinases are particularly appealing targets in this regard since they are common participants in these signaling pathways. Indeed, with the recent acquisition of the three-dimensional structure of the cAMP-dependent protein kinase (PKA)¹ (Bossemeyer et al., 1993; Zheng et al., 1993a,b; Knighton et al., 1991a,b) as well as that of the cyclin-dependent kinase 2 (DeBondt et al., 1993), the opportunity now exists for rational drug design based on kinase active site structure. Nevertheless, there are several potential difficulties that could doom this approach to failure. First, if the active site structures of all serine/threonine-specific (or tyrosine-specific) protein kinases are absolutely identical, there may be little opportunity to create active site-targeted inhibitors that will specifically interfere with the activity of only one (or a few) kinase family member(s). In addition, these enzymes may be exquisitely selective in terms of the types of residues that can be readily accommodated within the active site. In short, if protein kinases exhibit a strict active site specificity, then the creation of inhibitors containing elaborate functionality may prove to be problematical. Fortunately, it is now likely that a desired protein kinase can be targeted for inhibition since these enzymes (especially those which are serine/threonine-specific) recognize substrates with a characteristic sequence of amino acids (Kemp & Pearson, 1990). Consequently, nonspecific inhibitory components can be covalently attached to enzyme-specific peptides. In addition, we have recently found that the *active site* substrate specificities of PKA and protein kinase C (PKC), which are limited in both cases to serine and threonine residues in protein substrates, exhibit a dramatic

divergence with respect to non-amino acid residues (Kwon et al., 1994). Finally, since the substrate specificity of both PKA and PKC is not restricted to amino acids present in eukaryotic proteins, but includes a wide variety of amino acid and non-amino acid residues, it is evident that there exists considerable flexibility in terms of the range of functionality that can be incorporated into the active site of these enzymes (Lee et al., 1993; Kwon et al., 1993a,b,c; Prorok et al., 1989). Therefore the latitude associated with *active site* specificity, coupled with the characteristic substrate *sequence* specificities of protein kinases, provides considerable optimism with respect to inhibitor design, particularly in terms of targeting specific protein kinases with compounds such as transition-state analogs and mechanism-based inhibitors. We now report a dramatic amplification of the range of compounds phosphorylated by PKA. Recently, we identified the structural motif responsible for the absence of tyrosine kinase activity in the serine/threonine-specific cAMP-dependent protein kinase (Lee et al., 1993). During this investigation, we discovered that PKA will phosphorylate an aromatic alcohol ("tyrosine kinase-like behavior") in an appropriately designed peptide. This observation represents the first example of PKA-catalyzed phosphoryl transfer to an acceptor moiety other than an aliphatic alcohol. We describe herein the scope of this new activity, including the effect of steric and electronic factors on the kinetics of phosphoryl transfer.

MATERIALS AND METHODS

All chemicals were obtained from Aldrich, except [γ -³²P]-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 purchased from Whatman. ¹H NMR and ¹³C NMR experiments were performed at 400 MHz (Varian VXR-400S) and 22.5 MHz (Varian Gemini-300), respectively. Chemical shifts are reported with respect to tetramethylsilane. Fast atom bombardment (peptides) and electron impact (aromatic

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¹ Abbreviations: Boc, *tert*-butoxycarbonyl; Ddm, dimethoxydiphenylmethyl; kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; SPPS, solid-phase peptide synthesis.

alcohols) mass spectral analyses were conducted with a VG-70SE mass spectrometer. A Perkin-Elmer Lambda 2 spectrophotometer was employed for the UV-visible assays.

cAMP-Dependent Protein Kinase Preparation. The catalytic subunit was purified to homogeneity using a previously described procedure (Prorok & Lawrence, 1989). 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 (Armstrong & Kaiser, 1978) and the known primary structure of the catalytic subunit (Shoji et al., 1983).

Aminophenol Synthesis. The following aminophenol derivatives were purchased from Aldrich: 2-amino-5-methylphenol, 2-amino-4-methylphenol, 2-amino-4-chlorophenol, 2-amino-4-(ethylsulfonyl)phenol, 2-amino-4-nitrophenol, 4-amino-2-nitrophenol, and 2-amino-4-*tert*-butylphenol. The remainder were synthesized as described below. In all cases, the aminophenols listed below proved to be prone to air oxidation and, consequently, were employed without extensive characterization. However, once attached to the peptide, the aromatic ring system was stable when exposed to air at room temperature.

2-Amino-3-methylphenol from the Reduction of 3-Methyl-2-nitrophenol. $\text{Na}_2\text{S}_2\text{O}_4$ (excess), 6.8 g, was directly added to a basic aqueous solution (0.82 g of NaOH in 40 mL water) containing 1.52 g of 3-methyl-2-nitrophenol. The reaction mixture was stirred in an ice bath. The white precipitate that formed was collected by filtration, washed with a solution of 0.5 g of $\text{Na}_2\text{S}_2\text{O}_4$ in 30 mL of water, dried, and immediately reacted with Boc-Gly-Arg-Thr-Gly-Arg-Arg-Asn[dimethoxydiphenylmethyl (Ddm)]-resin (see Peptide Synthesis section below).

2-Amino-6-methylphenol from the Nitration and Subsequent Reduction of *o*-Cresol. A 25-mL round-bottomed flask containing 4 mL of *o*-cresol was cooled in an ice bath. Nitric acid (30%, 7 mL) was added dropwise with stirring. The dark viscous solution was subsequently steam-distilled to provide a yellow solid, which was recrystallized from acetone/water (0.54 g, 9% yield). ^1H NMR (DMSO- d_6): 7.83 (d, 1H, H-3, $J = 7.6$ Hz), 7.54 (d, 1H, H-5, $J = 8.0$ Hz), 6.93 (t, 1H, H-4, $J = 8.0$), 2.34 (s, 3H, CH_3). ^{13}C NMR (DMSO- d_6): 151.7, 137.5, 135.4, 129.3, 122.7, 119.5. CI-MS m/e calcd for $\text{C}_7\text{H}_7\text{NO}_3$: 153.1. Found: 153.2 (M^+). The 6-methyl-2-nitrophenol was subsequently reduced to the desired amine via the reaction conditions described above for 2-amino-3-methylphenol.

Precursor to Compound 15 from the Acylation (Benzoyl Chloride) and Subsequent Reduction of 4-Amino-2-nitrophenol. A solution of 4-amino-2-nitrophenol (3.0 g, 0.02 mol) and benzoyl chloride (2.8 g, 0.02 mol) in 30 mL of pyridine was stirred for 12 h at room temperature. The pyridine was removed *in vacuo*, and the black residue was dissolved in aqueous base and filtered. The filtrate was acidified, and a yellow precipitate was obtained. The solid was dissolved in acetone and decolorized with activated carbon. Evaporation of the solvent *in vacuo* provided a solid, which was recrystallized from acetone/water (1.5 g, 30% yield). ^1H NMR (DMSO- d_6): 10.78 (s, 1H, phenol OH), 10.40 (s, 1H, amide NH), 8.46 (d, 1H, H-3 on phenol ring, $J = 2.8$ Hz), 7.95 (m, 2H, H-2 and H-6 on benzoyl ring), 7.89 (q, 1H, H-5 on phenol ring, $J = 2.8, 9.6$ Hz), 7.51–7.60 (m, 3H, H-3, H-4, and H-5 on benzoyl ring), 7.14 (d, 1H, H-6 on phenol ring, $J = 8.8$ Hz). ^{13}C NMR (DMSO- d_6): 165.9, 149.0, 135.9, 134.8, 132.0, 131.4, 128.7, 127.9, 128.5, 119.6, 116.4. CI-MS m/e

calcd for $\text{C}_{13}\text{H}_{10}\text{N}_2\text{O}_4$: 258.2. Found: 258.2. Reduction of 4-(benzoylamino)-2-nitrophenol was performed as described for 2-amino-3-methylphenol.

Precursor to Compound 16 from the Acylation (Phenylacetyl Chloride) and Subsequent Reduction of 4-Amino-2-nitrophenol. The acylation (30% yield) with phenylacetyl chloride was carried out as described above. ^1H NMR (DMSO- d_6): 10.71 (broad s, 1H, phenol OH), 10.32 (broad s, 1H, amide NH), 8.30 (d, 1H, H-3 on phenol ring, $J = 2.8$ Hz), 7.63 (dd, 1H, H-5 on phenol ring, $J = 2.8, 8.8$ Hz), 7.31 (d, 4H, H-2, H-3, H-5, and H-6 on phenyl ring, $J = 4.8$ Hz), 7.31 (m, 1H, H-4 on phenyl ring), 7.08 (d, 1H, H-6 on phenol ring, $J = 8.8$ Hz), 3.61 (s, 2H, CH_2). ^{13}C NMR (DMSO- d_6): 169.6, 148.6, 136.1, 135.9, 131.4, 129.4, 128.6, 127.2, 126.9, 119.8, 115.1, 43.1. CI-MS m/e calcd for $\text{C}_{14}\text{H}_{12}\text{N}_2\text{O}_4$: 272.3. Found: 272.2 (M^+). Reduction of 4-[(phenylacetyl)amino]-2-nitrophenol was performed as described for 2-amino-3-methylphenol.

Precursor to Compound 17 from the Acylation (Phenylpropionyl Chloride) and Subsequent Reduction of 4-Amino-2-nitrophenol. The acylation (30% yield) with phenylpropionyl chloride was carried out as described above. ^1H NMR (DMSO- d_6): 10.68 (broad s, 1H, phenol OH), 10.06 (s, 1H, amide NH), 8.29 (d, 1H, H-3 on phenol ring, $J = 2.0$ Hz), 7.59 (dd, 1H, H-5 on phenol ring, $J = 2.8, 8.8$ Hz), 7.22–7.29 (m, 4H, H-2, H-3, H-5, and H-6 on phenyl ring), 7.17 (t, 1H, H-4 on phenyl ring, $J = 7.2$ Hz), 7.07 (d, 1H, H-6 on phenol ring, $J = 8.8$ Hz), 2.89 (t, 2H, CH_2 adjacent to carbonyl, $J = 7.6$ Hz), 2.60 (t, 2H, CH_2 adjacent to phenyl ring, $J = 8.0$ Hz). ^{13}C NMR (DMSO- d_6): 170.9, 148.5, 141.4, 135.9, 131.4, 128.6, 128.5, 127.2, 126.3, 119.8, 115.0, 37.8, 30.7. CI-MS m/e calcd for $\text{C}_{15}\text{H}_{14}\text{N}_2\text{O}_4$: 286.3. Found: 286.3 (M^+). Reduction of 4-[(phenylpropionyl)amino]-2-nitrophenol was performed as described for 2-amino-3-methylphenol.

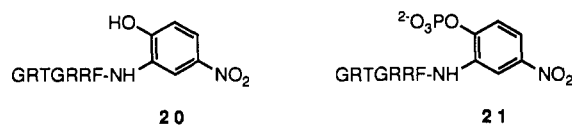
Precursor to Compound 19 from the Condensation of 4-Hydroxy-3-nitrophenylacetic Acid with Benzylamine and Subsequent Reduction. The condensation reaction (63% yield) was conducted according to a previously described procedure (DeMarinis et al., 1985). ^1H NMR (DMSO- d_6): 8.50 (t, 1H, H-3 on phenol ring, $J = 5.4$ Hz), 7.75 (s, 1H, H-5 on phenol ring), 7.22–7.34 (m, 5H, hydrogens on phenyl ring), 6.82 (d, 1H, H-6 on phenol ring, $J = 8.0$ Hz), 4.24 (d, 2H, CH_2 adjacent to amide nitrogen, $J = 5.6$ Hz), 3.79 (s, 2H, CH_2 adjacent to carbonyl). ^{13}C NMR (DMSO- d_6): 170.7, 159.8, 139.8, 136.3, 136.0, 128.5, 127.5, 127.0, 125.7, 122.1, 42.2, 40.9. FAB-MS m/e calcd for $\text{C}_{15}\text{H}_{14}\text{N}_2\text{O}_4$: 286.3. Found: 287.1 (M^+). The subsequent reduction was performed as described for 2-amino-3-methylphenol.

Peptide Synthesis. Boc-Gly-Arg-Thr-Gly-Arg-Arg-Asn[dimethoxydiphenylmethyl]-resin was prepared on Kaiser's oxime resin [substitution level = 0.43 mmol/(g of resin)] (Kaiser, 1989; DeGrado & Kaiser, 1980, 1982) with *tert*-butyloxycarbonyl (Boc) amino acids and subsequently displaced from the resin with *o*-aminophenol derivatives 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 (2.5×10 cm) connected in series [gradient (solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile), from 0 to 3 min (100% A); linear gradient from 3 min (100% A) to 25 min (75% A

and 25% B); from 25 to 30 min (25% B to 70% B); from 30 to 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 from 0.4 to 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.

The peptide predominantly employed in this study is Gly-Arg-Thr-Gly-Arg-Arg-Asn, which corresponds to a portion of a heat-stable protein kinase inhibitor protein that binds tightly to the cAMP-dependent protein kinase (Glass et al., 1989). We initially employed the doubly protected BocAsn-(xanthyl), which was attached to the oxime resin under standard conditions. Both Boc and xanthyl groups were removed with 25% trifluoroacetic acid in CH_2Cl_2 , and peptide synthesis proceeded according to the protocol described above. Upon liberation of the peptide from the oxime resin, the desired species was obtained in less than 0.1% yield. We attribute this low yield to the presence of the Asn-oximate ester linkage, which we suspect is unusually labile in the absence of a side-chain protecting group. Indeed, others have demonstrated that Asn-resin linkages are potentially unstable during SPPS due to an intramolecular cyclization reaction (Kisfaludy et al., 1975; Meyers et al., 1969; Zahn & Fölsche, 1969). In order to circumvent Asn-initiated peptide release from the oxime resin, the more robust dimethoxydiphenylmethyl (Ddm) protecting group for the asparagine side chain was employed (Konig & Geiger, 1970). Upon exposure of the Gly-Arg-Thr-Gly-Arg-Arg-Asn(Ddm)-oxime resin to 4-nitro-2-aminophenol, followed by treatment with 100% trifluoroacetic acid, the desired peptide species **14** [10 mg/(g of resin)] was obtained. In general, this yield of the asparagine-containing peptides was sufficient for our needs. However, in one particular series of experiments (*vide infra*), we required relatively large quantities of substrate and, consequently, we replaced the asparagine residue with an amino acid not prone to intramolecular cyclization. The phenylalanine-containing species Gly-Arg-Thr-Gly-Arg-Arg-Phe-oxime resin, upon treatment with 4-nitro-2-aminophenol, provided 35 mg of purified product per gram of resin.

Preparation and Isolation of Phosphopeptide 21. Peptide **20** (1 mM) was incubated with 4 μM catalytic subunit of the



cAMP-dependent protein kinase and 15 mM ATP at 30 °C (where pH = 7.1 and the buffer and salt concentrations employed are identical to those described below in the Kinase Assay section). After 3 h, the reaction mixture was introduced onto a C-25 ion-exchange column. The desired phosphopeptide was then isolated using the conditions described above in Peptide Synthesis.

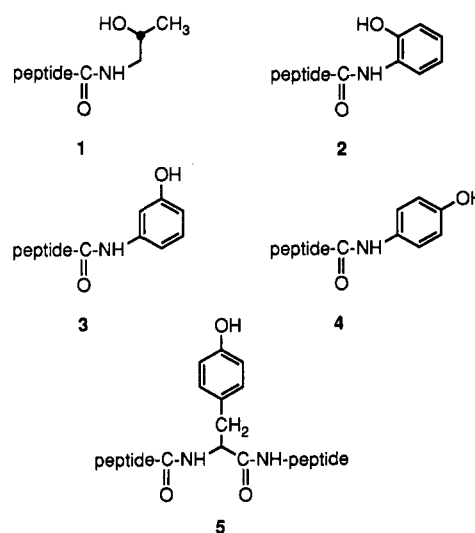
Kinase Assay. Assays were performed in triplicate at pH 7.1 and thermostated in a water bath maintained at 30 °C. Final assay volume totaled 50 μL and contained 100 mM 4-morpholinepropanesulfonic acid, 150 mM KCl, 12.5 mM MgCl_2 , 0.125 mg/mL bovine serum albumin, and 50 nM catalytic subunit of the cAMP-dependent protein kinase. For the determination of kinetic constants the following concentrations were employed: 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 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 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 4 volumes of 0.5% H_3PO_4 and 1 volume of water, followed by a final acetone rinse. The disks were air dried and placed in plastic scintillation vials containing 6 mL of Liquescent prior to scintillation counting for radioactivity. The conversion of phosphopeptide **21** to the unphosphorylated species **20** was monitored via UV-visible spectroscopy at 415 nm ($\epsilon = 11\,383\text{ M}^{-1}\text{ cm}^{-1}$). The conditions and reagent concentrations were identical to those described for the radioactivity assay except that the final assay volume was 1 mL and $[\text{ADP}] = 1\text{ mM}$. For K_m and V_{max} determination, the concentration of **21** was varied around its K_m .

Determination of Kinetic Constants. The apparent K_m ($\pm\text{SD}$) and V_{max} ($\pm\text{SD}$) values were determined from initial rate experiments. The data from these experiments were plotted using the Lineweaver–Burke procedure, and the corresponding plots proved to be linear.





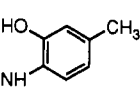
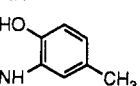

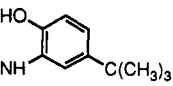
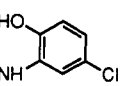
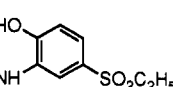
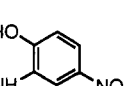
RESULTS AND DISCUSSION

The cAMP-dependent protein kinase is a serine/threonine-specific kinase that fails to phosphorylate tyrosine in an active site-directed peptide (Mendelow et al., 1993). We recently investigated the molecular basis for this substrate specificity (Lee et al., 1993). In order for phosphoryl transferase activity to transpire, the enzyme requires that the hydroxyl group on the substrate molecule be appropriately aligned in the active site (cf. compounds **1–5**). This notion is consistent with the



recent observations of Taylor, Sowadski, Gill, and their colleagues (Knighton et al., 1993). These investigators concluded that the phenolic oxygen of a tyrosine moiety (i.e., **5**) would not be correctly positioned for in-line attack on the γ -phosphate of ATP. In an analogous fashion, the alcohol functionalities in **3** and **4** are clearly unable to achieve the requisite geometrical relationship (cf. with respect to the neighboring amide bond) available to the hydroxyl moiety in **1**. As expected, **3** and **4** fail to undergo enzyme-catalyzed phosphorylation. In contrast, the aromatic alcohol in **2** can sweep a range of orientations that are similar to those possible in **1**. Indeed, **2** serves as a substrate exhibiting a K_m ($793 \pm$

Table 1: K_m and V_{max} Values for the Aromatic Alcohol-Containing Peptides 1, 2, and 6–14^a

	GRTGRRN-amino alcohol	K_m (μM)	V_{max} (nmol min ⁻¹ mg ⁻¹)
1		607 ± 83	1240 ± 100
2		793 ± 39	68 ± 4
6		114 ± 16	8290 ± 270
7		1691 ± 151	70 ± 5
8		1672 ± 78	57 ± 2
9		375 ± 42	90 ± 4
10		788 ± 32	59 ± 2
11		146 ± 8	36 ± 2
12		376 ± 28	58 ± 2
13		466 ± 43	55 ± 2
14		594 ± 15	37 ± 1

^a Kinetic constants were determined as described under Materials and Methods. The values are given as the average ± SD.

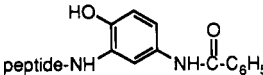
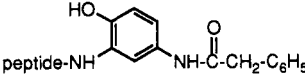
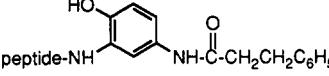
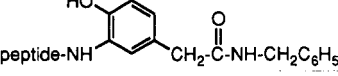
39 μM) that is reasonable for secondary alcohols (cf. 1; Table 1). In contrast, the V_{max} (67.9 ± 3.9 nmol min⁻¹ mg⁻¹) is substantially lower than that previously observed for the PKA-catalyzed phosphorylation of aliphatic alcohols (cf. 1; Table 1). However, this value is analogous to those typically obtained for tyrosine kinase-catalyzed reactions (Herrera et al., 1988; Kadowski et al., 1985; Pike et al., 1984). We now report on the scope of this newly discovered activity of PKA.

PKA Exhibits a Marked Preference for Phenols Containing Substituents That Are *para* to the Alcohol Functional Group. We have previously demonstrated that secondary alcohols serve as poorer PKA substrates than primary alcohols (Kwon et al., 1993a). For example, compound 1 exhibits a 7-fold lower V_{max} and a more than 5-fold larger K_m than its unsubstituted counterpart 6. Furthermore, when the *serine* in kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) is replaced with a threonine, a 40-fold increase in K_m is observed along with a 4-fold decrease in V_{max} (Kemp et al., 1977). These results demonstrate that the enzyme is sensitive to the presence of substituents on the alcohol-bearing carbon. This steric sensitivity may be a consequence of an active site functionality which partially interferes with the ability of the secondary alcohol to achieve the conformation required for phosphoryl transfer. Indeed, from the recently solved crystal structure

of PKA, we have identified one candidate in this regard, namely, Phe187 (Kwon et al., 1994). Consequently, the K_m obtained for the PKA-catalyzed phosphorylation of 2 not only is consistent with the values previously noted for other substituted alcohols (e.g., 1) but appears to reflect the structure of the active site as well. We have synthesized the methyl-substituted derivatives 7–10 in order to further evaluate the steric requirements of the active site. Both compounds 7 and 8 exhibit an approximately 2-fold increase in K_m relative to the unsubstituted parent compound 2. The position of the methyl groups on the aromatic ring (relative to the hydroxyl moiety) likely requires that these substituents be directed into the active site during catalysis. If one assumes that the K_m is a reflection of the affinity of the peptide for the enzyme, then unfavorable steric interactions may be responsible for the somewhat elevated K_m values associated with 7 and 8. In contrast, the V_{max} values for 2 and 7–10 are nearly identical. This result is particularly noteworthy for compound 7, since this suggests that a methyl substituent, adjacent to the phosphoryl-accepting aromatic alcohol, does not appear to significantly interfere with phosphoryl transfer to the hydroxyl moiety. In contrast to peptides 7 and 8, the *para*-substituted derivative 9 exhibits a 2-fold lower K_m than the parent phenol. This may be due to the presence of a hydrophobic pocket, which is located immediately adjacent to the active site region. One possible explanation for the relatively favorable K_m associated with the phosphorylation of 9 is that the methyl substituent on the aromatic ring may be ideally positioned to occupy the nearby lipophilic pocket. Indeed, the *tert*-butyl-substituted aromatic phenol 11 displays a K_m that is even lower than that of 9 (Table 1). Finally, since the kinetic constants associated with the phosphorylation of the methylated derivative 10 are nearly identical to those obtained for 2, it is apparent that the methyl substituent on this substrate has little impact on the manner in which the aromatic nucleus interacts with the enzyme. Although the aromatic hydroxyl moiety must clearly be oriented into the active site and toward ATP, the methyl group is likely directed out into solution during catalysis. Consequently one might predict that a substituent positioned adjacent to the aromatic amine (as in 10) would have a negligible effect on reaction kinetics.

Electron-Withdrawing Substituents at the *para* Position Produce a Mildly Deleterious Effect on the Maximal Velocity for Phosphoryl Transfer. Graves and his colleagues have proposed that the maximal velocities associated with the enzyme-catalyzed phosphorylation of serine/threonine and tyrosine residues may be controlled, in part, by the relative nucleophilicities of the attacking alkoxides (Martin et al., 1990). The aliphatic alkoxides of serine/threonine are expected to be more nucleophilic than the aromatic alkoxide of tyrosine. Consequently, these investigators have argued that this behavior may be responsible for the relatively low maximal velocities typically found for tyrosine kinase-catalyzed reactions (Herrera et al., 1988; Kadowski et al., 1985; Pike et al., 1984). Certainly, this suggestion is also consistent with the comparatively low V_{max} values of 2 and 7–11 versus those of 1 and 6. We have explored this possibility more fully by preparing peptides 12–14, each of which contains an electron-withdrawing group at the *para* position of the aromatic nucleus. From a comparison of the kinetic values associated with the phosphorylation of 9 and 12–14, it is evident that the stronger the electron-withdrawing group, the greater the K_m and the smaller the V_{max} . However, the overall effect is rather modest. This implies that, in terms of reaction rate, the aromatic group is primarily responsible for the observed kinetics of these substrates. In addition, any

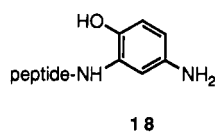
Table 2: K_m and V_{max} Values for the Aromatic Alcohol-Containing Peptides 15–17 and 19^a

	GRTGRRN-amino alcohol	K_m (μM)	V_{max} (nmol min ⁻¹ mg ⁻¹)
15		153 \pm 14	659 \pm 31
16		81.2 \pm 2.9	919 \pm 7
17		56.7 \pm 1.9	892 \pm 16
19		153 \pm 4	214 \pm 4

^a Kinetic constants were determined as described under Materials and Methods. The values are given as the average \pm SD.

further reduction in the nucleophilicity of the alcohol moiety by electron-withdrawing substituents has a relatively minor influence on substrate efficacy.

Electron-Donating Substituents at the para Position Markedly Stimulate the Maximal Velocity for Phosphoryl Transfer. Although the electron-withdrawing substituents in 12–14 exert a comparatively unremarkable effect on the ability of these compounds to serve as substrates for PKA, the observed trend in V_{max} does indicate that electronic effects can influence catalytic behavior in a predictable fashion. Nevertheless, it is evident that the inherent electron-withdrawing power of the aromatic nucleus is the predominant governing element. Consequently, we sought to reverse the properties of the aromatic moiety by preparing derivatives containing powerful electron-donating substituents on the ring system. Compounds 15–17 all exhibit a pronounced enhancement in maximal velocity relative to the other phenols described in this study (Table 2). Indeed, the V_{max} values for 15–17 approach that exhibited by the aliphatic alcohol 1. In addition, the favorable K_m values associated with these compounds are likely due to the insertion of the hydrophobic tail of the amide substituents into the lipophilic pocket located near the active site (Bossemeyer et al., 1993; Zheng et al., 1993a,b; Knighton et al., 1991a,b). However, it is also possible that the carbonyl moiety of the amide functionality may be responsible for the enhanced kinetic constants exhibited by peptides 15–17 by serving as a hydrogen bond acceptor. Such an interaction between enzyme and peptide not only may enhance the relative affinity of these two components for one another but, simultaneously, could increase the reaction rate by helping to correctly position the aromatic hydroxyl group in the active site. We attempted to prepare 18 in order to



address what contribution, if any, the presence of the carbonyl moiety makes to the improved kinetics associated with the phosphorylation of 15–17. Unfortunately, this compound proved to be unstable. However, we were able to synthesize an isomeric counterpart of 17, namely, 19. In the latter compound, the relative position of the carbonyl is similar to that in 15–17. Nevertheless, since the amide nitrogen in 19 is not directly attached to the aromatic ring system, the

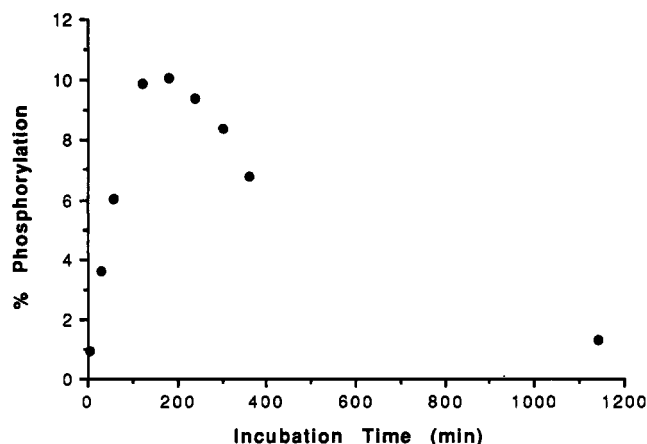


FIGURE 1: Percent phosphorylation of 2 versus time (min). The amount of peptide substrate that underwent phosphorylation (10% of total ATP present) peaked at 3 h. The assay was performed at pH 7.1 (100 mM 4-morpholinepropanesulfonic acid) and 30 °C in a mixture containing 150 mM KCl, 12.5 mM MgCl₂, 100 μM [γ -³²P]-ATP, 0.125 mg/mL bovine serum albumin, 1.0 μM PKA, and 500 μM peptide 2. Twenty-five-microliter aliquots were removed at selected time intervals, and the reaction was terminated upon spotting onto phosphocellulose disks. The disks were processed as described in the Materials and Methods section (under Kinase Assay) and subsequently counted for radioactivity.

electronic properties associated with this species are more analogous to those of 9. Interestingly, 19 does exhibit a 2-fold enhancement in maximal velocity relative to 9. However, this is a small increase compared to what is observed with 17. Consequently, we conclude that the special substrate efficacy associated with 15–17 is most likely due to the electron-donating ability of the amide substituents. These results are notable for two reasons. First, they validate the suggestion of Graves and his co-workers that electronic effects can control the rate of protein kinase-catalyzed phosphoryl transfer. Second, they imply that active site-directed peptides containing appropriately substituted tyrosine residues may serve as unusually efficient tyrosine kinase substrates.

PKA Catalyzes the Conversion of Phosphorylated Phenol to the Unphosphorylated Aromatic Alcohol. In our studies on the active site substrate specificity of PKA we typically isolate at least one phosphorylated peptide in order to characterize a representative product by ³¹P NMR and mass spectrometry (Lee et al., 1993; Kwon et al., 1993b,c). Generally, the peptide is incubated with ATP for several hours in the presence of PKA, which is added in aliquots during the course of the reaction. The phosphopeptide is then purified by chromatography. While these experiments reliably provide large quantities of phosphorylated aliphatic alcohols, we initially encountered some difficulty with the acquisition of the phosphorylated form of 2. We subsequently monitored the course of the reaction with [γ -³²P]ATP and found, much to our surprise, that the amount of phosphopeptide generated peaked at approximately 3 h, but thereafter plummeted in concentration (Figure 1). Ultimately we were able to isolate the desired phosphopeptide by terminating the incubation after 3 h. Nevertheless, the eventual loss of phosphorylated peptide during long incubation times is intriguing. There are two mechanisms which could account for this phenomenon. PKA may simply serve as a phosphatase by catalyzing the hydrolysis of the phosphorylated phenol. Alternatively, the enzyme may catalyze the "back reaction" in the direction of ADP phosphorylation (Qamar et al., 1992). However, while the latter explanation is consistent with a leveling off of phosphopeptide concentration, it does not fully explain the observed loss of phosphorylated product. We, as well as others, have noted

that PKA serves as a weak ATPase (Mendelow et al., 1993; Salerno et al., 1990; Yoon & Cook, 1987; Armstrong et al., 1979; Sugden et al., 1976; Moll & Kaiser, 1976). Consequently, long incubation times in the presence of the relatively high enzyme concentrations employed in these experiments would ultimately convert much of the starting ATP to ADP. Subsequently, the phosphopeptide generated during the initial stages of the incubation could serve as a phosphoryl donor for the conversion of ADP to ATP, a process that is catalyzed by PKA. Under these circumstances, the back reaction would eventually predominate, leading to the depletion of the phosphopeptide product. In order to distinguish between the PKA-catalyzed back reaction and simple phosphatase activity, we employed a spectrophotometric assay, an assay which requires relatively large quantities of phosphorylated peptide. Consequently, we prepared the peptide Gly-Arg-Thr-Gly-Arg-Arg-Phe- on the oxime resin. As noted in the Materials and Methods section, a C-terminal phenylalanine generates a higher yield of the desired peptide than the corresponding asparagine. The peptide was subsequently displaced from the oxime resin with 4-nitro-2-aminophenol. The PKA-catalyzed phosphorylation of the resulting peptide (**20**) exhibits a V_{\max} of 20 ± 1 nmol min⁻¹ mg⁻¹ and a K_m of 259 ± 12 μ M. We subsequently isolated the corresponding phosphopeptide **21**. Release of the phosphoryl group from this species can be readily followed by UV-vis spectrophotometry at 415 nm. In the presence of 1 μ M PKA, but in the absence of ADP, **21** was not appreciably converted to **20**. Consequently, we conclude that PKA does not serve as a phosphatase. However, upon addition of 1 mM ADP, we did observe a significant increase in the formation of **20**. The PKA-catalyzed phosphorylation of ADP, in the presence of the phosphoryl donor **21**, displays a V_{\max} of 98 nmol min⁻¹ mg⁻¹ and a K_m of 1.72 mM. In short, the PKA-catalyzed back reaction proceeds at a rate that is nearly 5-times greater than that of the forward reaction. However, the phosphorylated aromatic alcohol exhibits a K_m that is 6-fold larger than that of its unphosphorylated counterpart. We conclude that the loss of phosphopeptide during long incubation times with PKA (Figure 1) is a consequence of the back reaction, most likely driven by the PKA-catalyzed hydrolysis of ATP to ADP.

In summary, we have found that a serine/threonine-specific protein kinase catalyzes the phosphorylation of peptide-linked phenols. This reaction is subject to both steric and electronic effects. In the case of the former, phenols which contain substituents *para* to the aromatic alcohol serve as the most efficient substrates, whereas for the latter, powerful electron-donating groups strongly promote the kinase-catalyzed reaction. These observations mark a dramatic amplification in the range of compounds that serve as substrates for PKA and could ultimately prove useful in the design of novel inhibitors for protein kinases.

REFERENCES

- Armstrong, R. N., & Kaiser, E. T. (1978) *Biochemistry* 17, 2840–2845.
- Armstrong, R. N., Kondo, H., & Kaiser, E. T. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 722–725.
- Bossemeyer, D., Engh, R. A., Kinzel, V., Postingl, H., & Huber, R. (1993) *EMBO J.* 12, 849–859.
- DeBondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O., & Kim, S.-H. (1993) *Nature* 363, 595–602.
- DeGrado, W. F., & Kaiser, E. T. (1980) *J. Org. Chem.* 45, 1295–1300.
- DeGrado, W. F., & Kaiser, E. T. (1982) *J. Org. Chem.* 47, 3258–3261.
- DeMarinis, R. M., Lavanchy, P., Hieble, J. P., Jim, K. F., & Matthews, W. D. (1985) *J. Med. Chem.* 28, 245–248.
- Herrera, R., Lebwohl, D., de Herreros, A. G., Kallen, R. G., & Rosen, O. M. (1988) *J. Biol. Chem.* 263, 5560–5568.
- Kadowski, T., Fujita, Y., Yamaguichi, E. N., Takaku, F., Akiyama, T., Kathuria, S., Akanuma, Y., & Kasuga, M. (1985) *J. Biol. Chem.* 260, 4016–4020.
- Kaiser, E. T. (1989) *Acc. Chem. Res.* 22, 47–54.
- Kemp, B. E., & Pearson, R. B. (1990) *Trends Biochem. Sci.* 15, 342–346.
- Kemp, B. E., Graves, D. J., Benjamini, E., & Krebs, E. G. (1977) *J. Biol. Chem.* 252, 4888–4893.
- Kisfaludy, L., Shön, I., Rényi, M., & Görög, S. (1975) *J. Am. Chem. Soc.* 97, 5588–5589.
- Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N.-H., Taylor, S. S., & Sowadski, J. M. (1991a) *Science* 253, 407–414.
- Knighton, D. R., Zheng, J., Ten Eyck, L. F., Xuong, N.-H., Taylor, S. S., & Sowadski, J. M. (1991b) *Science* 253, 414–420.
- Knighton, D. R., Cadena, D. L., Zheng, J., Ten Eyck, L. F., Taylor, S. S., Sowadski, J. M., & Gill, G. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90, 5001–5005.
- König, W., & Geiger, R. (1970) *Chem. Ber.* 103, 2041–2051.
- Kwon, Y.-G., Srinivasan, J., Mendelow, M., Lee, T. R., Pluskey, S., Salerno, A., & Lawrence, D. S. (1993a) *J. Biol. Chem.* 268, 10713–10716.
- Kwon, Y.-G., Srinivasan, J., Mendelow, M., Lee, T. R., Pluskey, S., & Lawrence, D. S. (1993b) *J. Biol. Chem.* 268, 16725–16729.
- Kwon, Y.-G., Srinivasan, J., Mendelow, M., Pluskey, S., & Lawrence, D. S. (1993c) *J. Am. Chem. Soc.* 115, 7527–7528.
- Kwon, Y.-G., Mendelow, M., & Lawrence, D. S. (1994) *J. Biol. Chem.* 269, 4839–4844.
- Lee, T. R., Mendelow, M., Srinivasan, J., Kwon, Y.-G., & Lawrence, D. S. (1993) *J. Am. Chem. Soc.* 115, 9888–9891.
- Martin, B. L., Wu, D., Jakes, S., & Graves, D. J. (1990) *J. Biol. Chem.* 265, 7108–7111.
- Mendelow, M., Prorok, M., Salerno, A., & Lawrence, D. S. (1993) *J. Biol. Chem.* 268, 12289–12296.
- Meyers, C. A., Havran, R. T., Schwartz, I. L., & Walters, R. (1969) *Chem. Ind. (London)*, 136–137.
- Moll, G. W., Jr., & Kaiser, E. T. (1976) *J. Biol. Chem.* 251, 3993–4000.
- Pike, L., Kuenzel, E. A., Casnellie, J. E., & Krebs, E. G. (1984) *J. Biol. Chem.* 259, 9913–9921.
- Prorok, M., & Lawrence, D. S. (1989) *J. Biochem. Biophys. Methods* 18, 167–176.
- Prorok, M., Sukumaran, D. K., & Lawrence, D. S. (1989) *J. Biol. Chem.* 264, 17727–17733.
- Qamar, R., Yoon, M.-Y., & Cook, P. F. (1992) *Biochemistry* 31, 9986–9992.
- Salerno, A., Mendelow, M., Prorok, M., & Lawrence, D. S. (1990) *J. Biol. Chem.* 265, 18079–18082.
- Shoji, S., Ericsson, L. H., Walsh, K. A., Fischer, E. H., & Titani, K. (1983) *Biochemistry* 22, 3702–3709.
- Sugden, P. H., Holladay, L. A., Reimann, E. M., & Corbin, J. D. (1976) *Biochem. J.* 159, 409–422.
- Yoon, M.-Y., & Cook, P. F. (1987) *Biochemistry* 26, 4118–4125.
- Zahn, H., & Fölsche, E. T. (1969) *Chem. Ber.* 102, 2158–2159.
- Zheng, J., Trafney, E. A., Knighton, D. R., Xuong, N.-H., Taylor, S. S., Ten Eyck, L. F., & Sowadski, J. M. (1993a) *Acta Crystallogr. D* 49, 362–365.
- Zheng, J., Knighton, D. R., Ten Eyck, L. F., Karlsson, R., Xuong, N.-H., Taylor, S. S., & Sowadski, J. M. (1993b) *Biochemistry* 32, 2154–2161.