The cAMP-Dependent Protein Kinase Discriminates between Prochiral Hydroxyl Groups

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Genetic changes, which result in the formation of unregulated, and therefore constitutively active protein kinases, are frequently responsible for carcinogenesis.[1] As a corollary, factors which are able to turn off these oncogenic protein kinases block uncontrolled cell growth. Consequently, protein kinase inhibitors may not only prove useful in defining the molecular events leading to cellular transformation, but, in addition, such inhibitors may also constitute a valuable new family of anticancer drugs. We have recently undertaken a detailed analysis of the active site substrate specificity of protein kinases.[2] Such an analysis serves as a critical prerequisite for the design of inhibitors containing elaborate functionality that can be readily accommodated within the enzyme active site. We now report on an additional facet of protein kinase substrate specificity, namely the ability to specifically phosphorylate only one out of a pair of prochiral hydroxyl moieties.

The cAMP-dependent protein kinase phosphorylates the aliphatic alcohols of serine and threonine residues in a variety of protein and peptide substrates.[3] Recently, we have shown that peptides bearing C-terminal alcohols will also serve as substrates, and we have employed these species to assess the active site subtrate specificity of the enzyme.[2] We found that the protein kinase has an absolute requirement for the proper stereochemistry at the α-position of the residue which undergoes phosphorylation. For example, while compound **1** is an excellent substrate (K_m = 35 ± 7 μM and V_{max} = 9.8 ± 0.9 μmol/min·mg), compound **2** fails to undergo enzyme-catalyzed phosphorylation (Figure 1). These results imply that the protein kinase may be able to discriminate between the prochiral hydroxyl moieties of **3** as well. We have now confirmed this prediction with the experiments described below.

The peptide Gly-Arg-Thr-Gly-Arg-Arg-Asn((dimethoxydiphenyl)methyl) was synthesized on Kaiser's oxime resin employing a previously described protocol (Scheme I).[4-6] Displacement of the heptapeptide from the solid support with *O*-benzylserinol, followed by deprotection with HF, provided species **3** (Figure 1). Under standard assay conditions,[2] the latter proved to be an excellent cAMP-dependent protein kinase substrate, exhibiting a K_m of 23.8 ± 3.6 μM and a V_{max} of 13.7 ± 0.8 μmol/min·mg. For comparison, the standard peptide substrate for this enzyme is Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), which exhibits a K_m of 16 μM and a V_{max} of 20 μmol/min·mg.[7] We subsequently phosphorylated **3** to near completion via incubation with excess ATP in the presence of the cAMP-dependent protein kinase at 30 °C for 6 h. The phosphopeptide was isolated, and fast atom bombardment mass spectral analysis revealed that the peptide is monophosphorylated [$(M + 1)^+$ = 969.5].

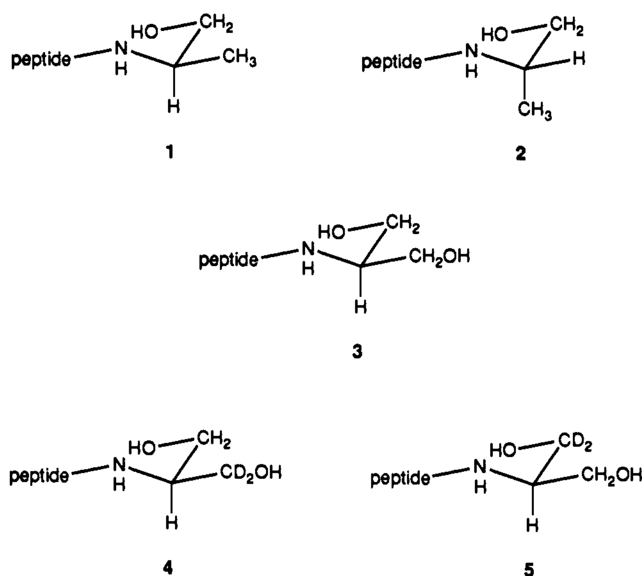
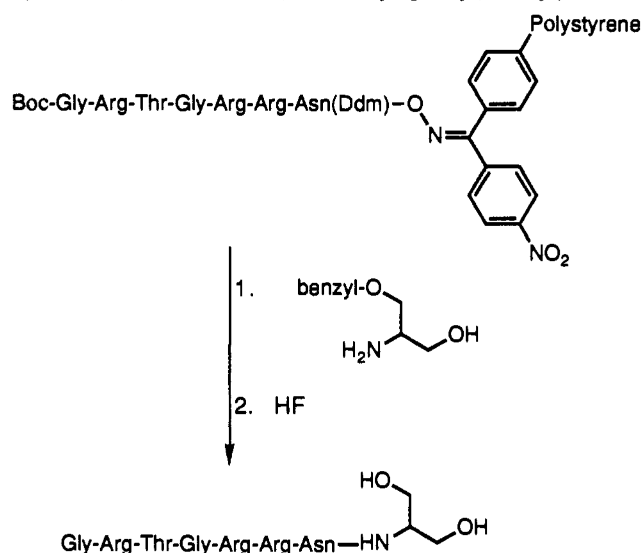


Figure 1. Peptides **1–5**, where "peptide" = Gly-Arg-Thr-Gly-Arg-Arg-Asn-. The deuterated serinol derivative **4** was prepared by the $LiAlD_4$ reduction of *O*-benzylated L-serine followed by the reactions outlined in Scheme I. Compound **5** was prepared in an analogous fashion via the reduction of *O*-benzylated D-serine.

Scheme I. Preparation of Peptides 3–5 Starting with Kaiser's Oxime Resin as a Support for Solid-Phase Peptide Synthesis (where Ddm = (dimethoxydiphenyl)methyl)



In order to elucidate the site of phosphorylation (i.e., *pro-R* versus *pro-S*), we synthesized the deuterated serinol-containing peptides **4** and **5** (Figure 1). The serinol derivative in the former was prepared via the $LiAlD_4$ reduction of L-*O*-benzylserine, and that in the latter in an identical fashion, but with the D-isomer of *O*-benzylserine.[8] In the case of **4**, the methylene group that does not contain the deuterium label corresponds to the *pro-R* group of serinol, whereas in **5**, it is the labeled methylene moiety which occupies the *pro-R* site. Both **4** and **5** were phosphorylated to near completion, purified, and then characterized by ^{31}P NMR

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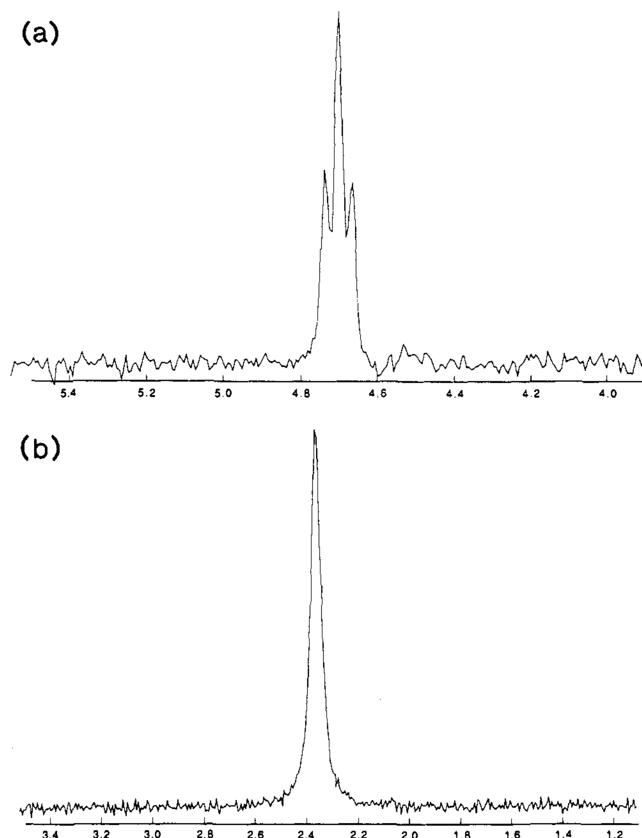

Figure 2. (a) ^1H-coupled ^{31}P NMR spectrum of phosphorylated **4**. Chemical shift = 4.68 ppm downfield from H_3PO_4 reference with a coupling constant of 5.3 Hz. (b) ^1H-coupled ^{31}P NMR spectrum of phosphorylated **5**. Chemical shift = 2.35 ppm. ^{31}P NMR experiments were performed at 161.903 MHz on a Varian-400S spectrometer. The samples contained approximately 6 mM phosphorylated peptide and 10 mM EDTA in D_2O. The following parameters were employed: spectral width 32 000 Hz; acquisition time 0.800 s; relaxation delay 0.500 s; pulse width 10.0 μs; ambient temperature. Since the samples were unbuffered, the difference in chemical shifts in spectra a and b is likely due to minor differences in pH between the two NMR samples. The phosphopeptides were prepared under the following reaction conditions: a 5 mM concentration of peptide was initially treated with 2 μM enzyme (150 mM KCl, 10 mM ATP, 12.5 mM $MgCl_2$, and 0.125 mg/mL of bovine serum albumin) for 90 min at 30 °C. An additional 2 μM enzyme was added at 90 min and then at 3 h to give a final enzyme concentration of 6 μM. The reaction mixture was added directly to CM-25 and eluted with a pH 3.5 50 mM NaOAc buffer (0.4 M to 1.0 M KCl gradient). The phosphopeptides eluted at approximately 0.55 M KCl and were subsequently lyophilized and desalted (G-10 with a 5% acetic acid/95% deionized water solvent system).

spectroscopy. The phosphorylated form of peptide **4** furnished a triplet with a ^1H–^{31}P coupling constant of 5.3 Hz; this indicates that the phosphate moiety is positioned adjacent to the -CH_2OH group (Figure 2a).[9] This also demonstrates that it is the *pro-R* position of serinol which serves as the site of phosphorylation. As noted above, the -CD_2OH group in **5** corresponds to the *pro-R* site. Since deuterium coupling constants are very small, split NMR signals of nuclei located near deuterium are not typically observed. In agreement with this expectation, the phosphopeptide of **5** provided a singlet (Figure 2b). Consequently, the results with both peptides unambiguously demonstrate that the *pro-R* hydroxyl group in serinol is the site that undergoes protein kinase-catalyzed phosphorylation.

The ability of enzymes to distinguish between prochiral groups or faces is generally attributed to the inherently asymmetric nature of enzyme active sites. Recently, the 3-dimensional structure of the catalytic subunit of the cAMP-dependent protein kinase containing bound ATP (or an ATP analog) and a truncated form of a naturally occurring protein inhibitor (i.e., "PKI") of the enzyme has been reported.[10–12] The region of the active site that accommodates the residue to be phosphorylated (i.e., typically serine or threonine) lies just above Thr201. Assuming that peptide **3** binds to the enzyme in a fashion that is analogous to that of PKI, the α-hydrogen is directed toward the Thr201 side chain. This appears to explain how the enzyme is able to distinguish between the prochiral hydroxyl groups of **3**. For example, in order for the enzyme to phosphorylate the -CH_2OH group of **4**, a conformation similar to that described for **3** above must be presented to the enzyme, a sterically allowed binding pattern. In contrast, if the enzyme attempted to phosphorylate the -CD_2OH group of **4** instead, then the -CH_2OH moiety would be directed toward Thr201, a potentially sterically unacceptable binding pattern. Although this analysis remains to be confirmed, it does suggest that replacement of Thr201 with a smaller residue, via site-directed mutagenesis, should furnish an enzyme that has an impaired ability to distinguish between prochiral hydroxyl moieties.

In summary, we have shown that the cAMP-dependent protein kinase is able to differentiate between prochiral alcohol groups on an active site directed peptide substrate. These observations mark the first time that any member of the protein kinase family has been shown to exhibit this form of substrate specificity.

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