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INTRASUBSTRATE STERIC INTERACTIONS IN THE ACTIVE SITE CONTROL THE SPECIFICITY OF THE cAMP-DEPENDENT PROTEIN KINASE

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The cAMP-dependent protein kinase catalytic subunit phosphorylates serine residues more efficiently than threonine residues in synthetic peptides. In marked contrast, both amino acids are phosphorylated at similar rates when contained within the appropriate intact protein substrate. The structural basis for the discriminatory behavior observed in small peptides has been investigated and found to be a result of intrapeptide steric interactions in the vicinity of the threonine alcohol moiety. Leu-Arg-Arg-Gly-Thr-Leu-Gly, which is nearly free of these interactions, is phosphorylated at a rate that is almost comparable to its serine-containing counterpart.

The bovine cardiac muscle cAMP-dependent protein kinase (EC 2.7.1.37) phosphorylates serine residues in a wide variety of proteins (1). In addition, many low molecular weight peptides, such as Leu-Arg-Arg-Ala-Ser-Leu-Gly, serve as efficient A-kinase substrates (2). Synthetic peptides containing threonine residues are also phosphorylated, albeit at drastically reduced rates compared to their serine counterparts (2-6). For example, Leu-Arg-Arg-Ala-**Thr**-Leu-Gly has a much lower k_{cat} (5.5-fold) and a vastly higher K_M (21-fold) than kemptide [(2) and *vide infra*]. It is tempting to ascribe the poor substrate efficacy of threonine-containing peptides to some steric barrier associated with the enzyme active site. However, the A-kinase phosphorylates a threonine residue in phosphatase inhibitor-1 at a rate that is nearly comparable to that observed for the phosphorylation of the serine-kemptide sequence in pyruvate kinase (3). The local sequence surrounding the threonine residue in phosphatase inhibitor-1 is Arg-Arg-Pro-Thr-Pro-Ala. Nevertheless, synthetic peptides corresponding to this sequence have also been found to be poor substrates (3, 5). It is clear that either (a) the intact phosphatase inhibitor-1 protein induces a conformational change in the A-kinase that renders the active site more suitable for threonine or (b) the intact protein constrains the peptide chain in the vicinity of the threonine

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Abbreviations: cAMP, cyclic 3',5'-adenosine monophosphate; kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; A-kinase, cAMP-dependent protein kinase; ATP, adenosine triphosphate; MOPS, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin; DTT, dithiothreitol.

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residue to lie in a conformation that is highly prone to phosphorylation. The latter point is particularly intriguing in light of the conformation occupied by kemptide in the enzyme active site (7-9). A notable feature of this conformation is that both the side chain of alanine and the amide hydrogen of leucine are oriented toward the phosphorylation site (10). Using CPK models, we have found that either of these groups could interact with the more sterically demanding threonine side chain and thereby disrupt the conformation that is required for binding and/or phosphorylation. The experimental results described in this paper confirm this structural analysis.

Materials And Methods

All chemicals were obtained from Aldrich, except for [γ-^{32}P] ATP (New England Nuclear), cAMP (Fluka), protected amino acid derivatives and Merrifield's 1% cross-linked resin (U.S. Biochemical), and Universol scintillation cocktail (ICN Radiochemical). 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.

Enzyme isolation and purity: The catalytic subunit was purified to homogeneity using a previously described procedure (11). Purity was assessed *via* SDS polyacrylamide gel electrophoresis, which displayed a single band at a molecular weight of 41,000 daltons. 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 (12) and the known primary structure of the catalytic subunit (13).

Peptide synthesis: Peptides were prepared on a 1% cross-linked chloromethylated polystyrene resin using t-BOC amino acids according to Merrifield's solid phase methodology (14). Peptides were cleaved from the resin with 90% HF/10% anisole (SUNY at Buffalo Microlabs), and subsequently extracted into 10% acetic acid and lyophilized. All peptides were gel-filtered on Sephadex G-10 (1% acetic acid), chromatographed on SP-Sephadex C-25 (0.2-0.7 M NaCl gradient in a 50 mM sodium acetate pH 3.5 buffer), and again gel-filtered on Sephadex G-10. All peptides gave satisfactory amino acid analyses and displayed a greater than 95% purity by HPLC.

Enzyme assay: Assays were performed in triplicate at pH 7.1 in a water bath thermostatted at 30 °C. Final assay volume totalled 100 µL and contained 100 mM MOPS, 150 mM KCl, 12.5 mM $MgCl_2$, 50 µM [γ-^{32}P] ATP (80-200 cpm/pmole), .125 mg/mL BSA, 1.5-6.0 nM catalytic subunit, and the peptide substrate. 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 MOPS, 150 mM KCl, 1 mM DTT, and 0.125 mg/mL BSA at pH 7.1]. Aliquots of 25 µL were withdrawn at 1.5, 2, or 3 minute intervals and quickly spotted onto 2.1 cm diameter phosphocellulose paper disks. After 10 seconds the disks were immersed in 10% glacial acetic acid and allowed to soak with occasional stirring for at least 1 hour. The acetic acid was decanted and the disks were collectively washed with six volumes of distilled water followed by a final acetone rinse. The disks were dried with a heat gun and placed in plastic scintillation vials containing 6 mL of a toluene-based fluor prior to scintillation counting for radioactivity. k_{cat} and K_M values were obtained from Lineweaver-Burk plots.

Results And Discussion

The k_{cat} and k_{cat}/K_M values for the A-kinase-catalyzed phosphorylation of Leu-Arg-Arg-Ala-**Ser**-Leu-Gly, Leu-Arg-Arg-Ala-**Thr**-Leu-Gly and Leu-Arg-Arg-**Gly-Ser**-Leu-Gly are provided in table I. Substitution of the serine in kemptide with a threonine residue results in a

TABLE I. k_{cat} and k_{cat}/K_M values for serine- and threonine-containing A-kinase substrates

Peptide	k_{cat} (min^{-1})	k_{cat}/K_M ($\mu M^{-1}min^{-1}$)
Leu-Arg-Arg-**Ala-Ser**-Leu-Gly	734 ± 8	40.3 ± .02
Leu-Arg-Arg-**Ala-Thr**-Leu-Gly	132 ± 6	0.34 ± .12
Leu-Arg-Arg-**Gly-Thr**-Leu-Gly	576 ± 28	0.62 ± .12

5.5-fold decrease in k_{cat} and a nearly 120-fold decrease in k_{cat}/K_M. Several other investigators have noted that threonine-containing peptides are notoriously poor A-kinase substrates (2-6). We have analyzed the structure of Leu-Arg-Arg-Ala-**Thr**-Leu-Gly based on the known active site conformation of kemptide (10). CPK models reveal that unfavorable steric interactions between the threonine side chain and the alanine side chain and/or the leucine amide hydrogen can occur if the peptide is forced to occupy the requisite active site substrate conformation. These interactions may preclude proper active site positioning of the hydroxyl moiety. Alternatively, the severe spatial crowding in the vicinity of the hydroxyl group may interfere with efficient phosphoryl transfer from ATP to the peptide substrate. These potentially disruptive intrapeptide steric interactions are depicted in two (of several possible) conformations in figure I.

The peptide Leu-Arg-Arg-**Gly**-Thr-Leu-Gly contains a **glycine** residue at the former alanine site. It is important to note that the corresponding serine peptides, Leu-Arg-Arg-**Ala**-Ser-Leu-Gly and Leu-Arg-Arg-**Gly**-Ser-Leu-Gly, have identical k_{cat} values and nearly identical K_M values (9). Therefore, any changes in the catalytic parameters that occur with the glycine-for-alanine substitution in the threonine containing-peptide should be due to an alleviation of intrapeptide steric interactions between the alanine and threonine side chains.

Leu-Arg-Arg-**Gly-Thr**-Leu-Gly displays a more than 4-fold increase in k_{cat} relative to its Ala-Thr-counterpart. Indeed, the Gly-Thr-peptide is phosphorylated .78 times as fast as kemptide. This figure is strikingly close to the value obtained (.75) when the rates of phosphorylation of the threonine-containing phosphatase inhibitor-1 and the serine-containing pyruvate kinase are compared (3).

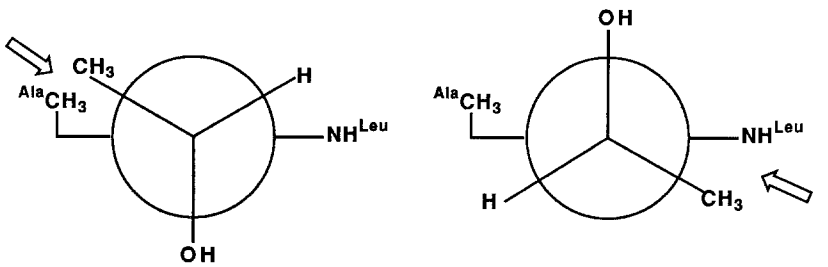

FIGURE I. Two conformations of Leu-Arg-Arg-**Ala-Thr**-Leu-Gly that possess disruptive steric interactions.

TABLE II. Rates of A-kinase-catalyzed phosphorylation of threonine-containing peptides

Peptide	k_{cat} (min^{-1})	Reference
Leu-Arg-Arg-Gly-Thr-Leu-Gly	576	this study
Leu-Arg-Arg-Ala-Thr-Leu-Gly	132	this study
Arg-Arg-Arg-Arg-Pro-Thr-Pro-Ala-NH_2	9.33	5
Arg-Arg-Pro-Thr-Pro-Ala-NH_2	4.04	5
Arg-Arg-Pro-Thr-Val-Ala	3.06	3*
Arg-Arg-Ala-Thr-Val-Ala	2.02	3*
Arg-Arg-Arg-Thr-Val-Ala-OEt	2.0	4*
Arg-Pro-Thr-Pro-Ala-NH_2	0.24	5
Arg-Arg-Arg-Arg-Pro-Thr-Pro-Ala	0.15	3*
Arg-Arg-Pro-Thr-Pro-Ala	0.14	3*
Arg-Arg-Ala-Thr-Pro-Ala	0.047	3*

**based on 10 μg/mL of holoenzyme*

To our knowledge, the k_{cat} for Leu-Arg-Arg-Gly-Thr-Leu-Gly is the largest ever reported for a threonine-containing peptide substrate (table II). However, the impressive k_{cat} is accompanied by a relatively poor K_M (930 ± 106 μM) compared to Leu-Arg-Arg-**Ala**-Thr-Leu-Gly (383 ± 45 μM). It is curious that the glycine-for-alanine substitution should lead to such a dramatic rise in K_M. One possibility is that the high K_M reflects a decreased substrate affinity for the enzyme active site. However, the glycine-for-alanine alteration on the corresponding serine-containing peptides leads to only a barely perceptible change in K_M (9). If the glycine substitution is responsible for a loss in active site affinity for the threonine-bearing peptide, it is difficult to imagine why the same substitution would not produce a similar effect on the analogous serine-containing peptide.

Whitehouse et. al. have proposed that the A-kinase catalyzes the phosphorylation of kemptide *via* a steady-state ordered Bi-Bi kinetic mechanism [scheme I where E = enzyme, P = peptide substrate, and P* = product; with $K_M = (k_4 + k_5)/k_3$] (15-16). CPK models, and the results described in this paper, indicate that the hydroxyl moiety of the threonine residue may be forced to reside in a sterically crowded region when the peptide occupies the enzyme active site. It is therefore possible that the rate determining step (k_{cat}) is phosphoryl transfer (k_5) from ATP to the obstructed alcohol functional group. As a result, the rise in K_M would reflect the observed increase in k_{cat}. Even if this mechanistic interpretation is correct, the salient feature is that the K_M values for both threonine-containing peptides are still high with respect to kemptide (18.2 ± 0.3 μM). Work is currently in progress to address the structural basis for this discrepancy.

In summary, peptides that contain threonine residues are typically poor A-kinase substrates. In at least one case, this appears to be a consequence of unfavorable steric factors in the vicinity

$$E + ATP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}ATP + P \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{-}ATP\text{-}P \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E\text{-}ADP\text{-}P^* \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E\text{-}ADP + P^* \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E + ADP$$

SCHEME 1.

of the hydroxyl moiety of the substrate molecule. A peptide has now been described in which these detrimental steric restrictions are at least partially alleviated and a corresponding increase in the phosphorylation rate is observed. It is possible that the sequence surrounding the threonine residue in phosphatase inhibitor-1 is forced to lie in a conformation that is consistent with the active site constraints yet provides sufficient steric freedom to ensure rapid phosphoryl transfer from ATP to the substrate.

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