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MULTIPLE ARGININE RESIDUES CONTRIBUTE TO THE INCREASED EFFICACY OF PEPTIDE SUBSTRATES FOR THE cAMP-DEPENDENT PROTEIN KINASE

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Efficient cAMP-dependent protein kinase substrates typically contain an arginine dyad one amino acid removed from the residue which undergoes phosphorylation (ie. Arg-Arg-X-Ser). However, several naturally occurring protein kinase inhibitors and substrates possess additional basic residues that are proximal to the arginine dyad, implying the presence of either an extended or an additional acidic subsite on the enzyme. In this study, we investigated the substrate efficacy of several multiple arginine-bearing peptides. The most efficient substrate studied, Arg-Arg-Leu-Arg-Arg-Ala-Ser-Leu-Gly, exhibits a nearly eleven-fold increase in k_{cat}/K_m relative to Leu-Arg-Arg-Ala-Ser-Leu-Gly. The enhanced k_{cat}/K_m is primarily a consequence of a reduced K_m. These results suggest that a double arginine dyad, separated by a single amino acid, represents the optimal sequence for basic residues on cAMP-dependent protein kinase substrates.

The cAMP-dependent protein kinase ("A-kinase") phosphorylates peptides and proteins that possess a basic residue or cluster of residues near the site of phosphorylation (1,2). Krebs and his coworkers have specifically demonstrated that the most effective substrates are those which contain an arginine dyad one amino acid removed from the phosphorylatable residue (3,4). The heptapeptide, Leu-**Arg**-**Arg**-Ala-**Ser**-Leu-Gly, which contains this critical structural feature, serves as a potent substrate for the A-kinase (3). However, several investigators have reported that additional arginine residues proximal to the basic dyad enhance the kinetic efficiency of certain protein kinase peptide substrates. For example, replacement of the **alanine** residue highlighted in the peptide Val-Leu-Glu-**Ala**-Arg-Arg-Gly-Ser-Ser-Ile-Pro-Glu with an arginine results in a 5-fold increase in V_{max}/K_m (5). Substitution of the N-terminal **alanine** in **Ala**-Lys-Arg-Ser-Arg-Lys-Glu with arginine results in an even more dramatic increase in V_{max}/K_m (57-fold) (6). A similar enhancement in V_{max}/K_m (56-fold) is observed when two arginines are added to the N-terminus of Arg-Arg-Pro-Thr-Pro-Ala (7). These, and other results (8,9), strongly suggest that basic residues near the arginine dyad augment substrate efficacy. However, definitive statements regarding their optimal locale and number remains an open question, in part due to the sizable incongruities in peptide sequence existing among the aforementioned substrates. The present study was undertaken to address this issue. Leu-Arg-Arg-Ala-Ser-Leu-Gly was selected as the substrate archetype on

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Abbreviations: A-kinase, cAMP-dependent protein kinase; ATP, adenosine triphosphate; cAMP, cyclic 3',5'-adenosine monophosphate; PKI, protein kinase inhibitor.

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which the multiple arginine-containing peptides were based. The results described below confirm that those peptides containing adjunct arginines display kinetic constants superior to those associated with the parent peptide.

Materials and Methods

All chemicals were obtained from Aldrich, except for [γ-^{32}P]ATP (New England Nuclear), cAMP (Sigma), protected amino acid derivatives (U.S. Biochemical) and Universol scintillation cocktail (ICN Radiochemical). Dialysis tubing was purchased from Fisher Scientific. Affi-gel Blue resin was acquired from Bio-Rad. Phosphocellulose P81 paper disks were obtained from Whatman. Fresh beef hearts were purchased from Klinck Bros. Inc. (Buffalo, NY).

Enzyme isolation and purity: The catalytic subunit was purified to homogeneity and cryogenically maintained according to a previously described protocol (10).

Peptide Synthesis and Purification: Peptides were synthesized on a 1% cross-linked chloromethylated polystyrene resin using *tert*-butyloxycarbonyl amino acids according to Merrifield's solid phase methodology (11). Peptides were cleaved from the resin and deprotected with 90% HF/10% anisole (SUNY at Buffalo MicroLabs). Purification of Leu-Arg-Arg-Ala-Ser-Leu-Gly was carried out as outlined previously (12). All other peptides were extracted from the resin using 10% acetic acid and chromatographed on SP-sephadex C-25 using a 0.45-0.85 M NaCl gradient in 50 mM sodium acetate buffer, pH 3.5. Desalting was performed on Sephadex G-15. Peptides were at least 95% pure as determined by HPLC. The concentration of the peptide stock solutions were determined by the quantitative ninhydrin reaction (11). Amino acid analysis after acid hydrolysis in 6 N HCl (22 hrs) for the peptides listed in Table 1 is as follows:

Peptide **1**: Ala (1.0), Arg (2.0), Gly (1.0), Leu (1.9), Ser (0.9)
Peptide **2**: Ala (1.0), Arg (2.9), Gly (0.9), Leu (2.0), Ser (1.0)
Peptide **3**: Ala (1.0), Arg (4.2), Gly (1.0), Leu (1.8), Ser (0.9)
Peptide **4**: Ala (1.0), Arg (2.9), Gly (0.9), Leu (1.0), Ser (1.0)
Peptide **5**: Ala (1.0), Arg (3.8), Gly (1.0), Leu (1.0), Ser (1.0)

Enzyme assay: All assays were performed in triplicate on the same day to minimize errors due to pipetting and minor daily changes in enzyme activity. Final assay volume totalled 100 µL and contained 100 mM 4-morpholinepropanesulfonic acid, 150 mM KCl, 12.5 mM $MgCl_2$, 100 µM [γ-^{32}P]ATP (500 cpm/pmol), .125 mg/mL bovine serum albumin, 16.2-60.9 ng/mL catalytic subunit and peptide substrates at concentrations spanning their K_m. 25 µL aliquots were spotted onto phosphocellulose paper disks and immersed in 10% acetic acid. Disks were washed with four volumes of 5 mM H_3PO_4 and rinsed with water followed by acetone. Disks were counted in 6 mL of scintillation cocktail. V_{max} and K_m values were obtained from Lineweaver Burk plots. Less than 10% of substrate was consumed for any given peptide concentration.

Results and Discussion

The kinetic parameters for the A-kinase-catalyzed phosphorylation of synthetic peptides employed in this study appear in Table 1. Peptides **2-5** all exhibit enhanced k_{cat}/K_m parameters versus the parent peptide **1**, mainly through the contribution of reduced K_m values. The quadruple-arginine-containing peptides **3** and **5** display markedly better K_m's than their triple arginine counterparts **2** and **4**. In fact, peptide **3** manifests one of the lowest K_m values known for a small peptide not bearing an amide-capped carboxyl terminus. Peptides **2** and **3**, which contain a leucine spacer between the arginine dyad and the terminal arginine(s), afford improved substrate efficacy versus peptides **4** and **5**, which possess the contiguous arginine sequence.

It is tempting to attribute the reduced K_m values of peptides **2-5** to an enhancement in binding affinity for the A-kinase catalytic subunit. This notion is particularly compelling in light of the

TABLE 1. Rates of A-kinase-catalyzed phosphorylation of multiple arginine-containing peptides

Peptide	Sequence	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	$\frac{k_{cat}}{K_m}$ (min^{-1} μM^{-1})
1	LRRASLG	14.7±0.7	13.5±0.4	37.3
2	RLRRASLG	3.1±0.3	15.2±0.5	199.0
3	RRLRRASLG	1.6±0.1	16.1±0.3	408.3
4	RRRASLG	5.2±0.2	13.1±0.3	102.2
5	RRRRASLG	2.1±0.1	14.9±0.3	287.9

results from studies involving segments of the heat-stable inhibitor protein (PKI) of A-kinase (Table 2). Deletion or replacement of arginine 15 in the PKI-related segments significantly reduces the inhibitory potency of the resultant peptides. Since arginines 18 and 19 comprise the same key recognition subsite commonly found in A-kinase substrates (15), arginine 15 would correspond to a site six residues separate from a phosphorylatable residue. The N-terminal arginine of peptide **3** occupies a site six amino acids removed from the target serine and displays the lowest K_m value of the investigated peptides. Given these subsite analogies, it seems tenable to ascribe the reduced K_m's of peptides **2-5** to an increase in binding capacity. However, a certain degree of caution must be exercised when drawing these parallels, since K_m cannot always be equated with K_d.

Only slight variances in V_{max} values occur among peptides **1-5** (Table 1). In the previously described studies, addition or insertion of an arginine effected an approximate doubling of V_{max} (5,7,8). In one instance, the increase was nearly 14-fold (6). Since V_{max} is a complex composite of individual microscopic rate constants, these discrepancies may signal the likelihood of different rate determining step(s) for the peptide substrates in these various studies (16-18).

In summation, addition of arginine residues to the peptide fragment Arg-Arg-Ala-Ser-Leu-Gly, augments the efficiency of the A-kinase reaction, primarily through a reduction in K_m. The optimal peptide substrate contains two arginine dyads separated by a single amino acid (i.e. Arg-Arg-X-Arg-Arg-X-Ser). Basic residues, usually arginine, proximal to the double-arginine subsite also exist in a number of naturally occurring substrates and inhibitors (5-9,13,14). The local sequence of the autophosphorylated **serine**-99 in the regulatory subunit of bovine type I cAMP-dependent

TABLE 2. K_i values for truncated segments of PKI

Sequence	K_i (nM)	Reference
$R^{15}TGR^{18}R^{19}NAIHA\text{-}NH_2$	250	13
$TGR^{18}R^{19}NAIHA\text{-}NH_2$	1250	13
$YADFIASGR^{15}TGR^{18}R^{19}NAIHDILVSSA$	240	14
$YADFIASGG^{15}TGR^{18}R^{19}NAIHDILVSSA$	6600	14

protein kinase is Gly-**Arg**-**Arg**-**Arg**-**Arg**-Gly-Ala-Ile-**Ser** (19). For bovine type II kinase, the region proximal to the autophosphorylated **serine**-95 includes Gly-**Arg**-Phe-Asp-**Arg**-**Arg**-Val-**Ser** (20). The arginine degeneracy in these naturally-occurring substrates, in conjunction with the results described herein, appear to be diagnostic of either an extended or a second acidic subsite on the A-kinase.

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