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Synthetic hexapeptide substrates and inhibitors of 3′:5′-cyclic AMP-dependent protein kinase

(peptide phosphorylation/amino-acid sequence)

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ABSTRACT **The substrate specificity of the catalytic subunit of rabbit skeletal muscle 3′:5′-cyclic AMP-dependent protein kinase (EC 2.7.1.37; ATP:protein phosphotransferase) has been studied using the synthetic peptide Arg-Gly-Tyr-Ser-Leu-Gly corresponding to the sequence around serine 24, a phosphorylation site in reduced, carboxymethylated, maleylated (RCMM) chicken egg white lysozyme. This peptide served as a substrate for the enzyme and exhibited a 6-fold higher V_{max} and a 100-fold higher K_m than RCMM-lysozyme. Replacement of the arginine with glycine, histidine, or lysine resulted in a dramatic reduction in the V_{max}. These results support the concept that arginine is an important residue in determining the substrate specificity of the protein kinase, predominantly influencing the V_{max} of the phosphorylation reaction. Two synthetic peptides in which serine was replaced by an alanine acted as competitive inhibitors of phosphorylation of the synthetic peptide substrate and RCMM-lysozyme.**

Recent studies in this (1, 2) and other (3, 4) laboratories have provided support for the concept that the local primary structure of the substrate includes important specificity determinants for the 3′:5′-cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase; EC 2.7.1.37). Daile and Carnegie (3) demonstrated that the protein kinase could phosphorylate a seventeen residue peptic peptide derived from myelin basic protein. This finding and the observation of Bylund and Krebs (2) that the protein kinase phosphorylates specific sites in chemically denatured chicken lysozyme are most easily interpreted in terms of a local primary structure specificity requirement. Similar conclusions have recently been drawn by Humble *et al.* (4) from studies on the phosphorylation of a cyanogen bromide peptide derived from liver pyruvate kinase. Further support for this concept has been obtained by Kemp *et al.* (1) from studies on the phosphorylation of genetic variants of β-casein. The variant most readily phosphorylated was β-casein-B. It is significant that phosphorylation of this variant occurred at a single site, serine 124, near the arginine substitution at position 122. Appropriate comparisons with the other variants indicated that this arginine was responsible for the enhanced phosphorylation of β-casein-B. Moreover it was noted that arginine occurs on the NH_2-terminal side of the phosphoserine in almost all of the cyclic AMP-dependent protein kinase phosphorylation sites that have been sequenced (summarized in refs. 1 and 2). The proximity of basic residues to the phosphorylation site has been noted by other authors (2, 4–8).

In order to investigate further the role of arginine in protein kinase specificity a study of the phosphorylation of a variety of synthetic peptides was made. In this communication we report the effect of substituting other amino-acid residues for arginine in the hexapeptide, Arg-Gly-Tyr-Ser-Leu-Gly, corresponding to the amino acid sequence around serine 24 in chicken lysozyme. It is also reported that synthetic peptide analogs in which the serine has been replaced by an alanine act as competitive inhibitors of both peptide and protein phosphorylation.

Abbreviation: RCMM-lysozyme, reduced, carboxymethylated, maleylated lysozyme.

MATERIALS AND METHODS

Cyclic AMP-Dependent Protein Kinase. Homogeneous catalytic subunit of rabbit skeletal muscle cyclic AMP-dependent protein kinase (Peak I) was prepared by the method of Beavo *et al.* (9). The catalytic subunit will be referred to simply as protein kinase.

Solid Phase Synthesis of Peptides. The hexapeptide substrates were custom synthesized by Peninsula Laboratories and supplied in crude form following HF cleavage from the resin. The hexapeptide Arg-Gly-Ile-Ala-Leu-Gly was synthesized in this laboratory by the solid phase techniques as described by Gutte and Merrifield (10). The completed peptide was cleaved (HBr) from the resin and deprotected (catalytic hydrogenation) according to the procedures described by Stewart and Young (11). The following amino-acid derivatives [protected on the α-amino position with the *t*-butoxycarbonyl (Boc) group] were purchased from Peninsula Laboratories: Boc-Ala, Boc-Arg(NO_2), Boc-Gly, Boc-Leu, Boc-Ile, Boc-Ser(Bzl).

Purification of Synthetic Peptides. The deprotected synthetic hexapeptides were purified by ion exchange chromatography on a SP-Sephadex (1.5 × 100 cm) column eluted with a concave gradient (0.2–2.0 M) of pyridine–acetate buffer, (pH 3.1–3.65) at 50°. The peptide material present in the column effluent was detected with fluorescamine (Fluram, Roche Diagnostics Corp.) (12) and by absorbance, A_{280}, for peptides containing aromatic residues. The peptide peak was concentrated by rotary evaporation and chromatographed on a Sephadex G-10 (100 × 0.9 cm) column eluted with 30% (vol/vol) acetic acid. The resultant peptide was concentrated by rotary evaporation and lyophilized repeatedly from H_2O to give a fluffy white powder. Peptide purity was assessed by high voltage electrophoresis on paper at pH 1.9 and pH 6.5. The amount of purified peptide was determined from amino-acid analysis of acid hydrolysates using a Durrum D-500 amino-acid analyzer.

Phosphorylation of Synthetic Peptides and Reduced, Carboxymethylated, Maleylated (RCMM)-Lysozyme by the Protein Kinase. The reaction mixture, with a final volume of 0.08 ml, contained 1 mM [γ-^{32}P]ATP (10–200 cpm/pmol), 62.5 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) (pH 6.9), 12.5 mM magnesium acetate, 0.25 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), protein kinase, and the peptide or protein substrate at concentrations as indicated in the appropriate figure legends. Enzyme dilutions were made in buffer containing 0.5

mg/ml of bovine serum albumin as recommended by Beavo *et al.* (9). Additional bovine serum albumin was added to reaction mixtures containing synthetic peptide as substrate to ensure that the final protein concentration was 0.125 mg/ml. For experiments in which RCMM-lysozyme was used as a substrate the reaction mixture differed from above in that it contained only 25 mM 2-(*N*-morpholino)ethanesulfonic acid at pH 6.9 and 4 mM magnesium acetate (2). After incubation at 30° for 2 min reactions were terminated with 0.5 ml of 30% acetic acid.

Separation of the Phosphorylated Product from [γ-^{32}P]ATP. Either phosphorylated RCMM-lysozyme or the phosphopeptides were separated from [γ-^{32}P]ATP by ion exchange chromatography. For routine assays the terminated reaction mixture was applied to polypropylene columns (Bio-Rad) containing 2 ml of anion exchange resin (AG 1 × 8) equilibrated with 30% acetic acid. The phosphopeptide was eluted with 30% acetic acid directly into a liquid scintillation vial, whereas the [γ-^{32}P]ATP remained bound to the resin. Recovery of the phosphorylated peptides from the anion exchange column was checked by repeated passage through the column and found to be quantitative. Liquid scintillation counting (Cerenkov radiation) was used to measure the extent of peptide phosphorylation.

Esterification and Acetylation. The methyl ester of the synthetic peptide Arg-Gly-Try-Ser-Leu-Gly was prepared according to the procedure described by Wilcox (13), using 0.1 M HCl in methanol. Acetic anhydride (50-fold excess) was used to acetylate peptide amino groups according to the method of Riordan and Vallee (14). Acetylation of peptides containing tyrosine gave rise to *O*-acyltyrosine. In these cases neutral hydroxylamine (pH 7.5, 0.833 M) was used to regenerate the tyrosyl hydroxyl group (14).

RESULTS

Phosphorylation of the synthetic peptide Arg-Gly-Tyr-Ser-Leu-Gly

At the commencement of this study it was not known whether the protein kinase would phosphorylate relatively small peptides. For this reason the sequence around a known phosphorylation site in chicken egg white lysozyme, serine 24, was chosen as a model. Since the sequence of this protein was known, it provided an opportunity for systematically increasing the length of the synthetic peptide had the small peptides failed to be phosphorylated. Accordingly, the synthetic hexapeptide Arg-Gly-Tyr-Ser-Leu-Gly, corresponding to the sequence of residues from 21 through 26 in lysozyme, was tested as a substrate for the protein kinase. This peptide was found to be readily phosphorylated by the protein kinase. A time course of the phosphorylation of the synthetic hexapeptide demonstrated that it was phosphorylated stoichiometrically (Fig. 1*A*), thus excluding the possibility that a minor contaminant in the preparation was serving as the phosphate acceptor.

In addition to the investigation of the stoichiometry of the reaction (see above), additional evidence that the hexapeptide itself was being phosphorylated was obtained by characterization of the product. The amino-acid composition of the isolated phosphorylated peptide agreed with the composition of the parent peptide (see Table 1). During the complete acid hydrolysis of the peptide all the radioactivity was released as $^{32}P_i$. After partial acid hydrolysis (6 M HCl, 110°, 2 hr in an evacuated tube) 21% and 33% of the radioactivity migrated with the phosphoserine and inorganic phosphate markers, respectively, when examined by high

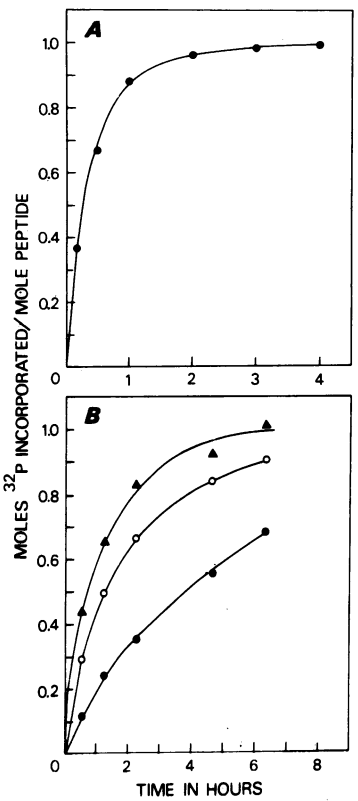

FIG. 1. Synthetic peptide phosphorylation. (*A*) Time course of Arg-Gly-Tyr-Ser-Leu-Gly phosphorylation. Peptide (0.71 mM) was incubated with protein kinase (18 μg/ml) in a reaction mixture (800 μl) having the composition described in *Materials and Methods*. Aliquots (5 μl) were removed at intervals, and the incorporation of ^{32}P was measured as described. (*B*) Time course of phosphorylation of Gly-Gly-Tyr-Ser-Leu-Gly (●), His-Gly-Tyr-Ser-Leu-Gly (▲), and Lys-Gly-Tyr-Ser-Leu-Gly (○). Experimental conditions were as described in (*A*) except that the protein kinase concentration was 200 μg/ml, the reaction volume was 80 μl, and 2 μl aliquots were removed at intervals. The concentration of peptide was 2.41 mM, 3.45 mM, and 3.6 mM, respectively for the three peptides.

voltage electrophoresis at pH 1.9. The remaining 45% of the phosphorylated product was incompletely hydrolyzed and migrated towards the cathode. High voltage electrophoresis on paper (at pH 1.9 and pH 6.5) was also used to assess the purity of the phosphorylated product. The radioactivity corresponding with the ninhydrin spot accounted for 88% of the radioactivity applied to the electrophoretogram. Approximately 6% of the applied radioactivity was associated with an unidentified minor contaminant (see Fig. 2A). Under the conditions employed to study reaction kinetics (see below) only the major radioactivity peak was observed. Phosphorylation of the contaminant was seen only under conditions of high enzyme concentration and long reaction times.

The effect of increasing concentration of synthetic peptide on reaction velocity was studied. The apparent K_m and V_{max} values measured at pH 6.9 were approximately 4.4 ± 0.2 mM and 12.2 ± 0.3 μmol/min per mg, respectively (Table 2). Both the K_m and V_{max} values for the hexapeptide differed significantly from those for the intact RCMM-lysozyme, which were 0.04 mM and 2.4 μmol/min per mg, respectively. Thus the K_m for the hexapeptide was approximately 100-fold higher than that for the intact RCMM-lysozyme, whereas the V_{max} for the hexapeptide was approximately 5-fold greater.

Table 1. Amino-acid analysis of synthetic peptides

Peptide	Residues									
	Ala	Arg	Gly	His	Ile	Leu	Lys	Ser*	Tyr†	$^{32}P_i$‡
Arg-Gly-Tyr-Ser-Leu-Gly	—	1.01	1.97	—	—	1.02	—	0.95	1.05	—
Arg-Gly-Tyr-Ser(P)-Leu-Gly	—	0.94	2.10	—	—	1.01	—	0.88	1.07	1.01
Gly-Gly-Tyr-Ser-Leu-Gly	—	—	2.98	—	—	1.06	—	0.96	1.01	—
His-Gly-Tyr-Ser-Leu-Gly	—	—	1.99	0.99	—	1.04	—	0.96	1.04	—
Lys-Gly-Tyr-Ser-Leu-Gly	—	—	1.99	—	—	1.05	0.99	0.97	0.99	—
Arg-Gly-Tyr-Ala-Leu-Gly	1.00	0.97	1.98	—	—	1.08	—	—	0.99	—
Arg-Gly-Ile-Ala-Leu-Gly	1.00	0.99	1.97	—	1.01	1.04	—	—	—	—

Values given are mol of amino acid per mol of peptide.
* Corrected by 10% for destruction during hydrolysis.
† Corrected by 5% for destruction during hydrolysis.
‡ Determined by liquid scintillation counting.

Phosphorylation of peptides without arginine

The results of studies on the phosphorylation of genetic variants of β-casein-B (1) indicated that it might be essential to have an arginine near the serine in order to ensure its phosphorylation by the protein kinase, and inspection of the published phosphorylation site sequences (1) indicated that this arginine occurred between two and five residues on the NH_2-terminal side of the serine. To obtain further information on this point synthetic hexapeptides containing histidine, lysine, and glycine instead of the arginine were tested as substrated for the protein kinase. Surprisingly, it was found that these peptides could all be phosphorylated at measurable rates, although it was immediately apparent that much higher enzyme concentrations and longer reaction times were required than with the arginine-containing peptide. Time courses for these reactions are shown in Fig. 1*B*. With the histidine-substituted peptide approximately 1 mol of phosphate per mol of peptide was incorporated in 6 hr; with the lysine-substituted peptide, 0.9 mol; and with the glycine-substituted peptide, 0.7 mol. Again high voltage electrophoresis was used to assess the purity of the phosphorylated peptides containing the glycine, histidine, and lysine substitutions. In each case a single radioactive peak, accounting for 93% or more of the radioactivity applied to the electrophoretogram, was observed; this corresponded with the position of the ninhydrin spot and thus confirmed that the substituted peptides and not minor contaminants were being phosphorylated (see Fig. 2B, C, and D).

The apparent K_m and V_{max} values were determined for the substituted peptides. As can be seen (Table 2), the K_m for each of the peptides was higher than for the arginine-containing peptide as compared at pH 6. In the case of the histidine-containing peptide, the apparent K_m showed a strong pH dependence in that the K_m at pH 6.9 was much higher than at 6.0. This suggested that the peptide with the protonated imidazole group was the preferred substrate.

The ratio V_{max}/K_m was calculated for the synthetic pep-

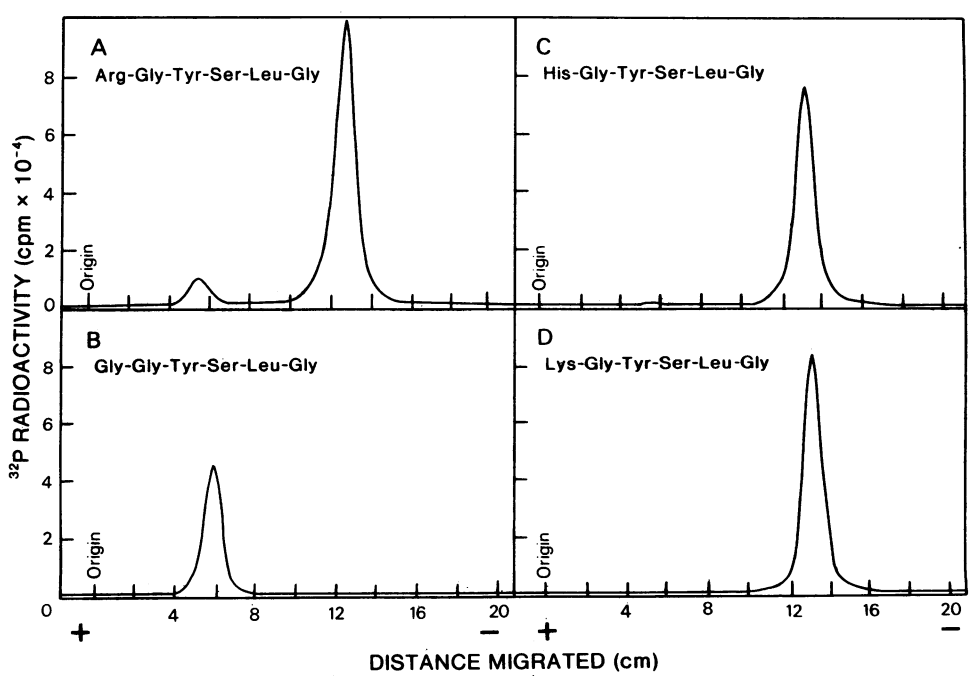

FIG. 2. High voltage electrophoresis of the phosphorylated peptides. The phosphorylated peptides obtained from the time course study (see Fig. 1) were electrophoresed at pH 1.9 as described previously (2). The electrophoretograms were stained with ninhydrin and scanned with a Packard radiochromatogram scanner. In order to gain greater precision the areas under the peaks were cut out and counted by liquid scintillation counting. The appropriate peptide sequences are given in each of the figure frames (A through D). For further details refer to the *text*.

Table 2. Kinetic constants for synthetic peptide substrates

Peptide	pH	$K_m^{Apparent}$ (mM)	V_{max} (μmol/min per mg)	Ratio $\frac{V_{max}}{K_m}$
Arg-Gly-Tyr-Ser-Leu-Gly	6	4.2 ± 0.2	16.4 ± 0.3	3.87
	6.9	4.4 ± 0.2	12.2 ± 0.3	2.77
Gly-Gly-Tyr-Ser-Leu-Gly	6	12.4 ± 1.3	0.41 ± 0.02	0.03
His-Gly-Tyr-Ser-Leu-Gly	6	13.4 ± 0.5	0.95 ± 0.02	0.07
	6.9	53.6 ± 6.9	1.8 ± 0.2	0.03
Lys-Gly-Tyr-Ser-Leu-Gly	6	14.7 ± 0.4	0.86 ± 0.01	0.06
RCMM-Lysozyme	6.9	0.040 ± 0.002	2.41 ± 0.05	60.30

Peptide phosphorylation was measured as described in *Materials and Methods* with protein kinase concentration of 2 μg/ml. Kinetic constants (± SEM) were estimated by fitting the data to the Michaelis–Menten equation using the method of least squares.

tide substrates (Table 2) to provide an arbitrary yardstick for quantitating the "specificity" of the protein kinase toward these peptides in a manner analogous to that used in measuring the specificity of proteases towards synthetic substrates (16). The arginine-containing peptide had approximately a 50-fold higher V_{max}/K_m ratio than the histidine-containing peptide, which in turn was greater than the ratio for either the lysine- or glycine-containing peptides. All the synthetic peptides had a much lower ratio than was found for RCMM-lysozyme.

The effect of modifying the synthetic peptide terminal residues

One feature of the synthetic peptide substrate Arg-Gly-Tyr-Ser-Leu-Gly which distinguishes it from RCMM-lysozyme and which might account for the large difference in K_m values between these two substrates is the proximity of the terminal primary amino group and the COOH-terminal carboxyl group to the serine. Accordingly, the methyl ester of the synthetic peptide was prepared and tested as a substrate; however, it was found that this modification caused only a minor (25%) reduction in the apparent K_m. Acetylation of the terminal primary amino group doubled the apparent K_m (Table 3).

Synthetic peptide substrate analog inhibitors

Phosphorylation of the synthetic peptide Arg-Gly-Tyr-Ser-Leu-Gly by the protein kinase suggested that it might be feasible to prepare synthetic substrate analogs that could be used to inhibit protein kinase activity. Two synthetic peptide analogs in which alanine was substituted for the serine, Arg-Gly-Tyr-Ala-Leu-Gly and Arg-Gly-Ile-Ala-Leu-Gly, were tested. Each peptide inhibited the phosphorylation of either the synthetic peptide substrate or RCMM-lysozyme competitively (Figs. 3 and 4). An estimate of the K_i for the peptide inhibitor Arg-Gly-Tyr-Ala-Leu-Gly, made from secondary plots of the apparent K_m or the ratio K_m/V_{max} against inhibitor concentration, gave a value of approximately 5 mM with either synthetic peptide or RCMM-lysozyme as the substrate. The K_i for the other inhibitor peptide was approximately 7 mM. Thus the K_i values for these peptides are comparable to the apparent K_m values for the synthetic peptide substrates.

Table 3. The effect of chemical modification on synthetic peptide

Substrate	Apparent K_m (mM)
RCMM-Lysozyme	0.040 ± 0.002
H_2N-Arg-Gly-Tyr-Ser-Leu-Gly-OH	4.53 ± 0.33
H_2N-Arg-Gly-Tyr-Ser-Leu-Gly-OCH_3	2.97 ± 0.30
CH_3-CO-NH-Arg-Gly-Try-Ser-Leu-Gly-OH	9.97 ± 0.89

Peptide phosphorylation at pH 6.9 was measured as described in *Materials and Methods* with a protein kinase concentration of 2 μg/ml. Kinetic constants (± SEM) were estimated by fitting the data to the Michaelis–Menten equation using the method of least squares.

DISCUSSION

The results reported here demonstrate that the cyclic AMP-dependent protein kinase catalytic subunit can phosphorylate the synthetic hexapeptide, Arg-Gly-Tyr-Ser-Leu-Gly, which corresponds to residues 21–26 in chicken egg white lysozyme. This finding provides further support for the idea that the protein kinase recognizes components of the local primary sequence around the phosphorylation site.

The kinetic constants for the phosphorylation of the synthetic peptide differ from those for the phosphorylation of RCMM-lysozyme in that the K_m for the synthetic peptide is approximately 100-fold higher and the V_{max} 5-fold higher than that for the protein substrate. The reason for the substantial difference in the K_m between the peptide and the protein substrate is not yet understood, but it is possible that

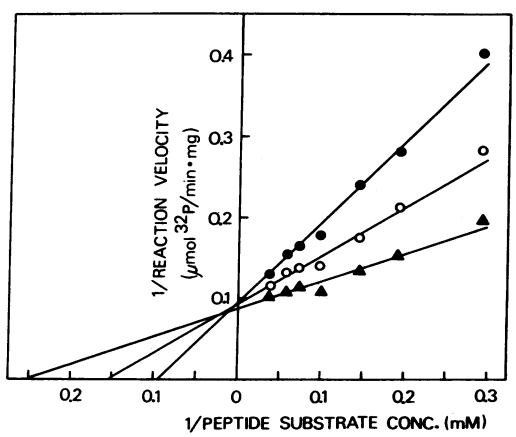

FIG. 3. Competitive inhibition of peptide phosphorylation by the analog peptide Arg-Gly-Tyr-Ala-Leu-Gly. A double reciprocal plot of the rate of peptide Arg-Gly-Tyr-Ser-Leu-Gly phosphorylation as a function of substrate concentration is given. The inhibitor concentrations were (mM): 0 (▲); 6.7 (○); 12.5 (●). Initial rates were determined under the conditions described in *Materials and Methods* at pH 6.0.

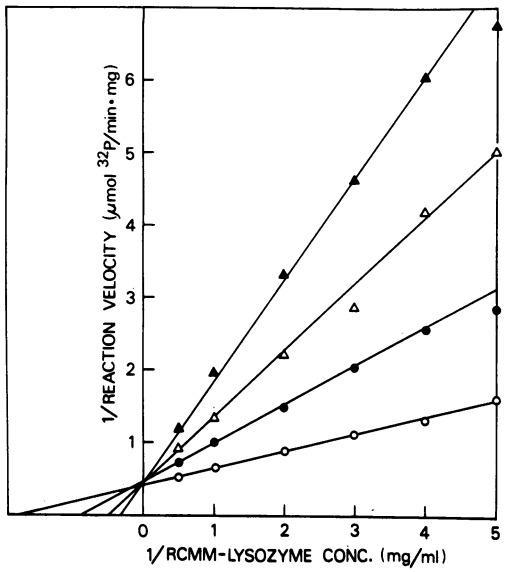

FIG. 4. Competitive inhibition of RCMM-lysozyme by the analog peptide Arg-Gly-Tyr-Ala-Leu-Gly. A double reciprocal plot of the rate of RCMM-lysozyme phosphorylation as a function of substrate concentration is given. The inhibitor concentrations were (mM): 0 (○); 8.4 (●); 16.7 (△); 25.9 (▲). Initial rates were determined under the conditions described in *Materials and Methods* at pH 6.9.

there may be a chain length requirement for optimal productive binding of the substrate to the enzyme. In this respect it is interesting that the K_m of proline hydroxylase for synthetic polypeptides of the type $(Pro\text{-}Gly\text{-}Pro)_n$ has a strong dependence on chain length (15). The presence of amino- and carboxyl-terminal groups near the phosphorylation site did not appear to account for the difference in the K_m between the peptide and RCMM-lysozyme in that modification by acetylation of the peptide NH_2-terminus or esterification of the COOH-terminus had a minimal effect on the apparent K_m.

Replacement of the arginine with glycine, histidine, or lysine in the hexapeptide results in a dramatic reduction in the V_{max} and a lesser change in the apparent K_m. This further substantiates the conclusion concerning the importance of an arginine residue made previously from the study of the specificity of the protein kinase towards genetic variants of β-casein (1). The predominant effect of arginine on the V_{max} of the synthetic peptide substrate is not surprising in that the catalytic efficiency of other enzymes that act on oligomeric substrates is known to be influenced by secondary enzyme–substrate interactions (16).

The present paper reports a synthetic inhibitor of the cyclic AMP-dependent protein kinase. In fact the only other inhibitor that has been described for this enzyme is the naturally occurring heat-stable inhibitor found in many tissues (17). The latter acts as a noncompetitive inhibitor with respect to the protein substrate, whereas the present inhibitor, which is an analog of a phosphorylation site, serves as a competitive inhibitor. The availability of a specific low-molecular-weight inhibitor of the cyclic AMP-dependent protein kinase would be extremely useful in many types of studies, e.g., in probing the question of whether a given function of cyclic AMP is mediated by protein phosphorylation. The greatest demand would be for a specific inhibitor that can be used for studies on intact cells. Obviously, the design of such an intracellular inhibitor will need to overcome any difficulties associated with gaining access to the cell interior and susceptibility of the inhibitor to intracellular proteolysis. In a few specialized cases, such as the *Xenopus* oocyte, it is possible to circumvent the permeability problem by microinjection. Although the peptide inhibitors described in this report could be used in the latter system and for cell-free studies, the development of more potent inhibitors with lower K_i values would be an advantage.

During the preparation of this manuscript Dr. P. Carnegie kindly communicated the results of a study on the specificity of the cyclic AMP-dependent protein kinase towards a synthetic octapeptide (Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg) corresponding to the sequence around serine-110 in myelin basic protein. The importance of an arginine residue in specificity was demonstrated by proteolytic cleavage of the octapeptide. This work has since been published (18).

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