Inhibition of Cyclic AMP-dependent Protein Kinase by Analogues of a Synthetic Peptide Substrate*

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Analogue s of the synthetic substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly in which the serine is replaced by other amino acids inhibited the activity of the catalytic subunit of cyclic AMP-dependent protein kinase from beef skeletal muscle (Peak I). All of the analogues were competitive with respect to peptide substrate but apparent Kᵢ values varied depending on the particular amino acid that was substituted for serine. Inhibition was also competitive with respect to mixed histone as determined in experiments utilizing one of the analogues. Acetylation of the terminal amino group of Leu-Arg-Arg-Ala-Ser-Leu-Gly in part followed by this substrate from 16 μM to 3 μM, but a similar modification of the inhibitory analogue Leu-Arg-Arg-Ala-Ala-Leu-Gly resulted in no major change in the Kᵢ value. An amount of inhibitory peptide sufficient to inhibit the cyclic AMP-dependent protein kinase by 90% caused less than 10% inhibition of several cyclic AMP-independent protein kinases indicating a high degree of specificity of inhibition by the peptide analogues. The experiments show that synthetic peptide analogues could be useful in identifying phosphorylation reactions catalyzed by cyclic AMP-dependent protein kinase as distinguished from other protein kinase reactions.

The primary structure of protein substrates for the cyclic AMP-dependent protein kinase (E.C. 2.7.1.37, ATP: protein phosphotransferase) was first shown to be an important specificity feature in studies in which denatured lysozyme or small protein fragments were used as substrates for the reaction (1–3). Since then, synthetic peptides corresponding to phosphorylated sites in protein substrates have been used in studies of the sequence specificity of this enzyme (4–7) and that of phospholipase kinase (8).

The synthesis of a peptide inhibitor of the cyclic AMP-dependent protein kinase was first reported by Kemp et al. (4). Replacement of serine by an alanine residue in a synthetic peptide substrate gave an analogue that inhibited the reaction competitively with protein or peptide substrates (4). The present report is concerned with the ability of a series of synthetic peptides to inhibit the cyclic AMP-dependent protein kinase. The peptides studied are analogues of the parent peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly, which corresponds closely to the phosphorylation site in pig liver pyruvate kinase (9). Inhibition constants for peptides containing different amino acid residues instead of serine and for chemically modified peptides show that the nature of the replacement is important in determining the inhibitory potency of the analogues.

MATERIALS AND METHODS

Protein Kinases—Homogeneous catalytic subunit of beef skeletal muscle cyclic AMP-dependent protein kinase (Peak I) (referred to as protein kinase unless stated otherwise) was prepared by Method I of Bechet et al. (10). Partially purified rabbit skeletal muscle "phosvitin" kinase was prepared by the method of Goldstein and Hasty (11). The preparation catalyzed the phosphorylation of casein and phosvitin, but not that of mixed histone. Homogeneous beef lung cyclic GMP-dependent protein kinase and a partially purified rabbit liver phosphorylase kinase were generously provided by Drs. David B. Glass and Ted D. Chrisman of this laboratory, respectively.

Synthetic Peptides—Solid phase peptide synthesis was carried out according to the methods of Gutte and Merrifield (12). The following amino acid derivatives were used: Boc'-Ala, Boc-Arg (NO₂), Boc-Asn, Boc-Asp (benzyl), Boc-Gly, Boc-His(tosyl), Boc-Leu, and Boc-Val (Peninsula Laboratories); and Boc-O-Ser (benzyl) (Bachem, Inc.). Trifluoroacetic acid (Pierce), 33% (v/v), and triethylamine (Gallard-Schlesinger Chemical Manufacturing Corp.), 10% (v/v), in methylene chloride were used for removal of Boc groups and subsequent neutralization, respectively. Coupling reactions were performed in methylene chloride (or dimethyl formamide when Boc-Arg (NO₂) was coupled) using dicyclohexylcarbodiimide as the coupling agent. Cleavage of the completed peptides from the 2% cross-linked polystyrene resin (Peninsula Laboratories) was accomplished with HBr in trifluoroacetic acid as described by Stewart and Young (13). The amino acid side chains of the cleaved peptides were depolymerized by catalytic hydrogenation. Crude deprotected peptides were purified on sulfopropyl (SP)-Sephadex and Sephadex G-25 columns as described previously (4). Peptide material in column eluents was monitored for arginine by the Sakaguchi test (13) and for amino groups with fluorescamine (Fluraml, Roche Diagnostics Corp.) (14). The amino groups of Leu-Arg-Arg-Ala-Ser-Leu-Gly and Leu-Arg-Arg-Ala-Ala-Leu-Gly were acetylated by treatment of the purified peptides with acetic anhydride (15). N-Octanoyl-Leu-Arg-Arg-Ala-Ser-Leu-Gly was prepared by the solid phase method with octanoic acid and dicyclohexylcarbodiimide. The dipeptide Arg-Arg was purchased from Vega-Fox Biochemicals.

Before use, all peptides were shown to be pure as determined by high voltage electrophoresis at pH 1.9 by use of ninhydrin or Sakaguchi staining. Concentrations of stock solutions were determined by amino acid analysis using the Durrum D-500 analyzer after hydrolysis in 5.7 N HCl for 24 h at 110°C. The determined amino acid compositions were as expected (data not shown).

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The abbreviations used are: Boc, t-butyloxycarbonyl; Mes, 2-(N-morpholino)ethanesulfonic acid.

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Phosphorylation Reactions—Reaction mixtures for the cyclic AMP-dependent protein kinase having a final volume of 0.08 ml ordinarily contained 62.5 mM Mes (pH 6.2), 12.5 mM magnesium acetate, 0.25 mM [γ-32P]ATP (50 to 200 cpm/pmol), 0.25 mM ethylene glycol bis(β-aminoethyl ether) N,N′-tetraacetic acid, 0.3 mg/ml of bovine serum albumin, 0.08 to 0.13 μg/ml of protein kinase, and histone (type II-A, Sigma Chemical Co.) or peptide substrate at concentrations indicated in Fig. 1, a and b. The concentrations of peptide inhibitor in the reaction mixtures are indicated in the legend to Fig. 1. Reactions were initiated by the addition of enzyme and carried out for 1 or 2 min at 30°C. Assays were terminated by the addition of sufficient glacial acetic acid to make the mixture 25% (v/v) in acetic acid. Substrate utilization was always less than 10% and product formation was linear with respect to both time and amount of enzyme. For the experiments of Table III in which the specificity of inhibition was studied, the conditions for protein phosphorylation of the enzyme were altered as explained under "Results." [γ-32P]ATP was prepared by the method of Glisson et al. (10) as modified by Reimann et al. (17). [32P]Phosphopeptide and phosphohistone were determined by the method of Glass et al. using phosphocellulose paper squares (18). [32P]Phosphocasein and phosphophosphorylase were quantified as described by Reimann et al. (17).

**Determination of Kinetic Constants**—Double reciprocal plots were constructed from initial rate measurements fitted to the Michaelis-Menten equation by the method of weighted least squares (19). Inhibition constants and their standard errors were determined by linear regression analysis of K_m/V_max (the slope obtained from plots of 1/v versus 1/s) versus inhibitor concentration plots.

**RESULTS**

**Inhibition of Cyclic AMP-dependent Protein Kinase by Analogues of Peptide Substrates**—Analogues of the synthetic peptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly in which the serine was replaced by other amino acids inhibited the activity of bovine skeletal muscle cyclic AMP-dependent protein kinase. All of the analogues were competitive with respect to the peptide substrate as illustrated for Leu-Arg-Arg-Ala-Ala-Leu-Gly in Fig. 1a. A secondary plot of slope against inhibitor concentration (Fig. 1a, inset) indicated linear inhibition with an apparent K_i value of 0.49 mM. The K_i value ranged from 0.3 to 0.5 mM for several determinations. A similar experiment was performed using mixed histone as the variable substrate (Fig. 1b). Again, linear competitive inhibition was observed; the apparent K_i value was 0.36 mM.

The results of kinetic analyses for all of the analogues tested are shown in Table I. The analogues that contained either an arginine, or valine, or in place of serine were the most potent inhibitors with K_i values of 0.49 and 0.81 mM, respectively. Peptides that contained either a positively charged (histidine) or negatively charged (aspartic acid) residue had similar K_i values (2.15 mM). Replacements with the neutral residues glycine and asparagine gave rise to analogues with similar K_i values (2.15 mM). The dipeptide Arg-Arg also competitively inhibited protein kinase (Table I) but not as effectively as the heptapeptide analogues. However, Arg-Arg was a more potent inhibitor than arginine itself, which has been reported to have an apparent K_i value of 21 mM (20).

The substitution of L-serine with D-serine resulted in a peptide analogue that produced only slight inhibition (≤10%) of protein kinase when used at concentrations up to 12 mM (not illustrated). A small fraction of this material (0.03%) was phosphorylatable and was most likely a minor contamination of the L-serine-containing peptide.

Acetyl-Leu-Arg-Arg-Ala-Leu-Gly had essentially the same inhibitory potency as the nonacylated peptide having a K_i value of 0.62 mM instead of 0.49 mM (Table I). It was of interest, however, that acetyl-Leu-Arg-Arg-Ala-Ser-Leu-Gly proved to be a better substrate than its nonacylated counterpart having a K_m of 3 ± 0.4 μM in contrast to a K_m of 16 ± 0.9 μM for the nonacylated peptide. The V_max values were similar (acylated, 19 ± 0.6; nonacylated, 20 ± 0.5 μmol/min/mg). The octanoylated peptide substrate had kinetic constants similar to those for the acetylated peptide (data not shown).

**Selectivity of Inhibition by Leu-Arg-Arg-Ala-Ala-Leu-Gly**—It was of interest to determine whether the inhibition of cyclic AMP-dependent protein kinase by analogues of peptide substrates was specific. Accordingly, the most potent inhibitor
Protein Kinase Specificity

The assays mixtures contained 62.5 mM Mes (pH 6.9), 3.13 mM magnesium acetate, 0.25 mM [γ-32P]ATP (50 to 150 cpm/pmol), 0.25 mg/ml of bovine serum albumin in addition to the indicated protein substrate and enzyme. Phosphorylase kinase and cyclic GMP-dependent protein kinase were assayed in the presence of 50 mM sodium fluoride or 1 μM cyclic GMP, respectively.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Leu-Arg-Ala-Ala-Leu-Gly</th>
<th>Protein substrate</th>
<th>Per cent activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclic AMP-dependent protein kinase</td>
<td>0</td>
<td>Histone (type II-A)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>II-A (0.23) mg/ml</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>mg/ml</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Phosphorylase kinase</td>
<td>0</td>
<td>Phosphorylase</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>b (2 mg/ml)</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td></td>
<td>92</td>
</tr>
<tr>
<td>Phosvitin kinase</td>
<td>0</td>
<td>Casein (0.04 mg/ml)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>mg/ml</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td></td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td></td>
<td>99</td>
</tr>
<tr>
<td>Cyclic GMP-dependent protein kinase</td>
<td>0</td>
<td>Histone H2B</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>(0.03 mg/ml)</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td></td>
<td>115</td>
</tr>
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<td></td>
<td>3.0</td>
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<td>115</td>
</tr>
</tbody>
</table>

* The data are expressed as per cent of activity in the absence of Leu-Arg-Ala-Ala-Leu-Gly.

of the various peptides examined, Leu-Arg-Ala-Ala-Leu-Gly, was tested as a potential inhibitor of other protein kinases. Each kinase was assayed at a substrate concentration close to or less than the $K_m$ value. For liver phosphorylase kinase, 2 mg/ml of rabbit skeletal muscle phosphorylase b was used ($K_m$ approximately 2 mg/ml). For cyclic GMP-dependent protein kinase, 0.03 mg/ml of histone H2B was employed ($K_m = 0.02$ mg/ml) (21). For "phosvitin" kinase, casein was used as the substrate at a concentration of 0.04 mg/ml ($K_m$ approximately 0.40 mg/ml). The results are shown in Table II. Concentrations of Leu-Arg-Ala-Ala-Leu-Gly sufficient to inhibit cyclic AMP-dependent protein kinase by 90% (the apparent $K_i$ value is slightly higher than 0.4 μM under these conditions) caused little or no inhibition of "phosvitin" kinase or cyclic GMP-dependent protein kinase and only an 8% inhibition of phosphorylase kinase. No explanation for the slight but reproducible apparent increase in the activity of cyclic GMP-dependent protein kinase is available.

**DISCUSSION**

In the present study, synthetic analogues of the peptide substrate Leu-Arg-Ala-Ala-Leu-Gly were found to inhibit the cyclic AMP-dependent protein kinase competitively. The "best" inhibitor was Leu-Arg-Ala-Ala-Leu-Gly, the analogue in which the serine residue was replaced by an alanine. This inhibitor had a $K_i$ value of 0.4 μM (Table I), approximately an order of magnitude lower than was found for the inhibitory peptide, Arg-Gly-Tyr-Ala-Leu-Gly, examined in a previous study. The large difference is probably due to the presence of the second basic residue in the NH2-terminal portion of the more potent inhibitor (5, 6). The $K_m$ values for the serine-containing substrate peptides used as models for the two inhibitors are different as well: 0.016 μM (5) and 4.2 μM (4), respectively.

The cyclic GMP-dependent protein kinase and phosvitin kinase were not inhibited by Leu-Arg-Ala-Ala-Leu-Gly at concentrations that inhibited the cyclic AMP-dependent protein kinase by 90%. Phosphorylase kinase was slightly inhibited (8%) (Table II). These data show selective inhibition of cyclic AMP-dependent protein kinase by an inhibitory peptide and are in consonance with the finding that the substrate specificity of various protein kinases is due, at least in part, to the linear sequence of residues near the phosphorylatable site in the respective substrates (5, 7, 9, 21, 22). In this regard, it is of interest to note that Gill et al. (23) have found that the naturally occurring heat-stable cyclic AMP-dependent protein kinase inhibitor protein does not inhibit the activity of the cyclic GMP-dependent protein kinase. The specificity of inhibition produced by the peptide Leu-Arg-Ala-Ala-Leu-Gly (Table II), as well as the finding, indicates that there are considerable differences in the abilities of the cyclic AMP- and cyclic GMP-dependent protein kinases to bind these peptides. It should be pointed out, however, that the results presented in Table II were obtained with the use of different substrates for cyclic AMP- and cyclic GMP-dependent protein kinases. Also, it has been reported that the two enzymes have similar substrate specificities (24). Clearly, more kinetic studies are required to further explain the similarities and differences in the specificities of these two enzymes.

The discrepancy between the $K_m$ of the parent peptide (0.16 μM) and the $K_i$ for the alanine-substituted analogue (0.4 μM) is not yet fully understood. One possible explanation is that the hydroxyl group present in the serine residue of the substrate peptide and absent in the alanine residue is critical for binding. However, recent studies in this laboratory have shown that the peptide acetyl-Leu-Arg-Ala-Ala-Leu-Gly binds to the catalytic subunit of protein kinase with an apparent dissociation constant ($K_d$) of approximately 0.25 μM in the presence or absence of Mg2+ and adenylyl-5'-yl imidodiphosphate, but the peptide has (as shown in this report) an apparent $K_m$ of 0.003 μM. These data rule out the possibility that the hydroxyl group in serine is responsible for the low $K_m$ value through binding effects. The aforementioned difference between the observed $K_m$ and $K_i$ values could be explained by certain types of kinetic mechanisms (e.g. Theorell-Chance (25) or Ordered Bi-Bi (26)) but not by others (e.g. rapid equilibrium random mechanism where the binding of one substrate either does not change or increase the affinity of the enzyme for the other substrate (i.e. $a = 1$) (19)).

Steady state kinetic measurements made in this laboratory with synthetic peptides suggest a kinetic mechanism of protein kinase that is compatible with the observations of a low $K_m$ (i.e. 0.003 μM) but a high $K_i$ (i.e. 0.25 μM). These results will be presented elsewhere.

Demaille et al. (20) have recently reported that the naturally occurring heat-stable protein kinase inhibitor (first described by Walsh et al. (27)) acts as a competitive inhibitor of protein kinase with protein substrate. An earlier report described the partially purified inhibitor as a noncompetitive inhibitor (28). This inhibitor was found to have a $K_i$ of approximately 2 × 10−3 μM (20), much lower than the value for the best synthetic peptide substrate analogue. Demaille et al. (20) have also shown that most of the inhibitory activity is present in a small peptide fragment of the inhibitor molecule, and that arginine and hydrophobic residues are important components of the inhibitor molecule. These results imply that the inhibitor molecule acts as a substrate analogue for protein kinase. Another report has suggested that the heat-stable inhibitor interacts with both the protein- and nucleotide-binding sites on the catalytic subunit (29). The interaction

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2 T. D. Chrisman, unpublished results.

3 J. R. Feramisco and E. G. Krebs, manuscript in preparation.
of one inhibitor molecule with two different binding sites may
give rise to the high affinity binding observed for the heat-
stable inhibitor.

Recent experiments in this laboratory with Xenopus oocytes
have shown that the parent peptide used in this study is
phosphorylated in the intact cell (30). This finding implies
that the synthetic inhibitor Leu-Arg-Arg-Ala-Ala-Leu-Gly
could be used to block the action of cyclic AMP-dependent
protein kinase in the intact cell. It should be useful in both
biochemical and pharmacological studies of the enzyme.

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