Phosphorylation of Acyl and Dansyl Derivatives of the Peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly by the cAMP-dependent Protein Kinase

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A series of acyl derivatives and a dansyl derivative of the synthetic peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly have been tested as substrates for the catalytic subunit of the cAMP-dependent protein kinase (ED 2.7.1.37; ATP: phosphotransferase). The purpose of this study was to test whether substitution of the NH2-terminal leucine with acyl substituents of varying chain length and hydrophobicity would influence the kinetics of phosphorylation of these peptides. Increasing the acyl substituent from acetyl to hexanoyl had no marked effect on the kinetics of phosphorylation. The trifluoroacetyl derivative was also phosphorylated with kinetic parameters comparable to the parent peptide, namely an apparent $K_m$ of 2.5 $\mu$M and a $V_{max}$ of 30 $\mu$mol min$^{-1}$ mg$^{-1}$. Substitution of the pentapeptide Arg-Ala-Ser-Leu-Gly with acyl substituents also had little effect on the kinetics of phosphorylation. The $\omega$-N dansyl derivative of Leu-Arg-Arg-Ala-Ser-Leu-Gly-COOH was phosphorylated by the protein kinase with an apparent $K_m$ of 3 $\mu$M and a $V_{max}$ of 27 $\mu$mol min$^{-1}$ mg$^{-1}$. The dansyl peptide appeared to act as a specific substrate for the cyclic AMP-dependent protein kinase as indicated by DE-52 ion exchange chromatography of crude extracts of rat muscle. Since the phosphorylated form of the dansyl peptide was readily separated from the dephospho form by high pressure liquid chromatography on a reverse phase octadecasilane column the protein kinase could be assayed spectrophotometrically using this technique.

Protein phosphorylation is not recognized as an important regulatory mechanism in the control of a wide variety of physiological processes (1). A number of protein phosphotransferases have been identified and, in the case of the most thoroughly studied examples, cAMP-dependent protein kinase and phosphorylase $b$ kinase, it is clear that these enzymes have restricted specificity, phosphorylating only a limited number of proteins in vivo (2). The molecular basis of this substrate specificity has been investigated for these and several other protein kinases and it has been found that they are capable of phosphorylating synthetic peptides corresponding to the local phosphorylation site sequences in their natural substrates (2). It is believed that particular residues in these fragments act as specificity determinants. In the case of the cAMP-dependent protein kinase, the synthetic peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly corresponding to the local phosphorylation site sequence in porcine pyruvate kinase was found to act as an excellent substrate (3, 4). The adjacent arginine residues in this peptide were found to have a strong influence on the kinetics of phosphorylation (3, 4). Recent studies with synthetic peptides corresponding to the phosphorylation site sequences in other natural substrates of the cAMP-dependent protein kinase have demonstrated that the presence of multiple arginines per se is insufficient to ensure optimal kinetics of phosphorylation (5). These findings together with theoretical proposals (6, 7) have suggested that other structural features besides arginine residues may be important in specificity.

Studies in other systems in which enzymes utilize macromolecular substrates have suggested that there may be multiple interactions between the enzyme and substrate. In the case of peptic digestion, Fruton and his colleagues have studied the influence of a number of substituent groups on model peptide substrates for this enzyme (8). The purpose of the present study has been to investigate the effect of substituting the $\omega$-amino group of the NH2-terminal leucine in the heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly and the pentapeptide Arg-Ala-Ser-Leu-Gly with a variety of substituents.

RESULTS AND DISCUSSION

Data concerned with the characterization of the synthetic peptides including amino acid analysis, chromatographic and electrophoretic properties are presented in the adjacent miniprint. In the case of the dansyl$^2$ derivative additional data including fluorescence excitation and emission spectra, uv absorption spectra, stoichiometry of phosphorylation, and gel chromatography of the phosphorylated product are also given.

Phosphorylation of Acyl Derivatives—A series of acyl derivatives of the pyruvate kinase analog peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly-amide were synthesized and tested as substrates for the catalytic subunit of the cyclic AMP-dependent protein kinase. The $V_{max}$ and apparent $K_m$ values obtained for the derivative acetyl through to hexanoyl were essentially the same as those of the parent peptide ($K_m$, 2.5 $\mu$M; and $V_{max}$, 30 $\mu$mol min$^{-1}$ mg$^{-1}$) (Table IV). In addition the trifluoroacethyl

1 Portions of this paper (including Figs. 1 to 10 and Tables I to VI) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full-size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-943, cite author(s), and include a check or money order for $2.25 per set of photocopies.

2 The abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl-; Mes, 2-(N-morpholino)ethanesulfonic acid; EDTA, ethylene glycol bis(β-aminoethylether) $N,N,N',N'$-tetraacetic acid; HPLC, high pressure liquid chromatography.
and dansyl derivatives had kinetic parameters comparable to the parent peptide (Tables IV and V). These results demonstrate that modification of the NH₂ terminus of the peptide with these substituents had little effect on the capacity of the peptide to act as a substrate for the cAMP-dependent protein kinase. In other systems, however, acylation of synthetic peptides has been found to have a pronounced influence on their specific interactions with proteins. In particular, Benjamin et al. (19) found that acylation of synthetic peptides representing residues 109 to 112 of tobacco mosaic virus protein significantly improved their interaction with antibodies to the parent protein. More recently, Fruton et al. (8) have shown that the presence of substituent groups on peptide substrates for pepsin influenced the kinetics of peptide bond cleavage catalyzed by this enzyme. In the case of elastase trifluoroacetyl peptides have been found to act as potent inhibitors (21) however this modification does not alter the kinetics of phosphorylation of the protein kinase peptide substrate.

Since the diarganine-containing pyruvate kinase analog peptide is a particularly efficient substrate for the cAMP-dependent protein kinase small effects of the acyl substituents on the kinetic parameters may have been masked. This possibility is suggested by the Kₐ values of synthetic substrates for measuring the CAMP-dependent protein kinase (Table IV). In the case of elastase trifluoroacetyl peptides have been found to act as potent inhibitors (21) however this modification does not alter the kinetics of phosphorylation of the synthetic elastase substrate.

The HPLC procedure employed here is perhaps the only procedure for routine use is the inability to process large numbers of samples simultaneously as is readily achieved with the radioisotope procedures for both protein (22) and synthetic substrates (18).

Specificity of Synthetic Peptide as Substrate—The practical value of synthetic substrates for measuring the cAMP-dependent protein kinase in crude systems depends on these substrates being specific and not susceptible to phosphorylation by unrelated protein kinases. In the present study, the specificity of the dansyl peptide and heptapeptide amide were examined using DEAE-cellulose-fractionated muscle extracts (Fig. 10). The peptide phosphotransferase activity peaks corresponded with the three major histone kinase activity peaks observed by others. In order of elution the protein kinase activities correspond to the free catalytic subunit of the cAMP-dependent protein kinase and the isoenzymes I and II of the holoenzyme, respectively. All three peaks were inhibited by the aet-able inhibitor (results not shown). Detergent (Triton X-100) was included in the extraction buffer and column elution buffers to facilitate the extraction of any organellar or membrane-bound phosphotransferase activity capable of phosphorylating the dansyl peptide. These results indicate that the pyruvate kinase analog peptide (Leu-Arg-Ala-Ser-Leu-Gly) as well as its dansyl derivative act as specific substrates for the cAMP-dependent protein kinase in extracts of rat muscle.

Acknowledgments—The technical assistance of Jenny Williams and Glenda Vaughton is gratefully acknowledged. Amino acid analyses were done by Meryl Heitman. I am indebted to Mr. Ian Daniels for glass blowing and modifications to the manual peptide synthesis apparatus.

REFERENCES


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Protein Kinase Synthetic Substrates

30: Phosphorylation of Cys- and Cys-Derivatives of the Peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly by the Cyclic AMP-Dependent Protein Kinase. Bruce E. Kemp

**Experimental Procedures**

**MATERIALS AND METHODS**

The catalytic subunit of the cyclic AMP-dependent protein kinase was isolated from beef heart muscle (Fig II) as described previously (9, 10). Peptide Synthesis and Purification. The synthetic peptides were synthesized by the Merrifield solid phase peptide synthesis (11) using a manual 2916 residue on the C-terminal amide of the acetyl derivative for which acetic anhydride was used. The completion of coupling of the experimental procedures was determined by quantitative amino acid analysis of hydrolysates using a. 

**TABLE III**

The catalytic subunit of the cyclic AMP-dependent protein kinase was isolated from beef heart muscle (Fig I) as described previously (9, 10). Peptide Synthesis and Purification. The synthetic peptides were synthesized by the Merrifield solid phase peptide synthesis (11) using a manual method (3). The purity of the synthetic peptide was assessed by amino acid analysis (Table I). High voltage paper electrophoresis at pH 2.4 and 3.4 (Table II) and thin layer chromatography on cellulose plates (Table I).

**TABLE I**

Acidic acid analysis of synthetic peptides following acid hydrolysis. Values given are mol % of arginine per mol peptide. Synthesized peptide was hydrolyzed at 110 °C for 24 h at 105. All derivations were linked through the α-N terminal amino acid.

<table>
<thead>
<tr>
<th>Peptide Derivative</th>
<th>Arg</th>
<th>Gly</th>
<th>Residue</th>
<th>Ala</th>
<th>Leu</th>
<th>Arg</th>
</tr>
</thead>
</table>

**TABLE II**

High voltage electrophoresis of synthetic peptides. The synthetic peptides were recrystallized in 0.5 M acetic acid, 60-280 (v/v) for 45 min, 250°C and at pH 6.4 (pyridine, acetic acid, H₂O, 7:0:3) for 60 min, 250°C. The elution chromatography was performed with both butylphosphonate and phenylphosphonate (v:v) mixtures as described previously. After the completion of deproteinization in perchloric acid. All derivations were linked through the α-N terminal amino acid.

**Electrophoretic Mobility**

<table>
<thead>
<tr>
<th>Peptide Derivative</th>
<th>pH 3.4</th>
<th>pH 6.4</th>
</tr>
</thead>
</table>

**TABLE IV**

Thin layer chromatography of synthetic peptides. Synthetic peptide samples were applied to cellulose thin layer plates. The plates were developed in the following solvent (v:v) systems: 1. Acetic acid, 30% ethanol and water (A). 2. Acetic acid, 30% ethanol and water (B). 3. Acetic acid, 30% ethanol and water (C). All derivations were linked through the α-N terminal amino acid.

**Peptide Derivative**

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
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**Phosphorylation of Cys- and Cys-Derivatives of the Peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly by the Cyclic AMP-Dependent Protein Kinase.** Bruce E. Kemp

In the case of the Cys-derivative, the same was further assessed by measurement of the phosphorylation of 125I-phosphatase. Approximately 0.50 ml of 125I-phosphatase was incorporated per ml of peptide (Fig. 1).

**Figure 1**

Time course of Cys-dimethylphosphate formation. The Cys-dimethylphosphate (1,008) was incubated with protein kinase I (3) in a reaction mixture (200 μl) containing Cys (4). The phosphorylated peptide was recovered from the reaction mixture by thin-layer chromatography on Silica gel 60 (2.5% acetic acid, 90% ethanol, 250°C, 10 min, 0.65) in the presence of 1% acetic acid. The peptides were visualized by staining with ninhydrin and phenanthrenequinone (1). The peptides were linked through the α-N terminal amino acid. The purity of the synthetic peptide was assessed by amino acid analysis (Table I). High voltage paper electrophoresis at pH 2.4 and 3.4 (Table II) and thin layer chromatography on cellulose plates (Table I).
Readings were made manually.

Higher pH values from the column matrix. The solvent composition chosen for routine isocratic separation of the mobile phase increased the retention of the dephospho-form of the peptide (Fig. 5). Increasing the mobile phase methanol concentration increased the retention of the dephospho-form of the peptide. By using two detectors to detect the small peak of product chromatographing near the large peak of reactant dansyl-phosphorylated dansyl-peptide it was possible to obtain the percent conversion of the phosphorylated dansyl-peptide. The percent conversion was calculated from the peak heights which effectively provided an internal standard. The elution parameter k' was calculated as described previously [28].

% CONVERSION

Table IV

<table>
<thead>
<tr>
<th>Peptide Derivative</th>
<th>Max umol/mg*min</th>
<th>K'^2</th>
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</thead>
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<td>Acetyl</td>
<td>3.7 x 10^-3</td>
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</tr>
<tr>
<td>Propanoyl</td>
<td>3.7 x 10^-3</td>
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<td>Butanoyl</td>
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</tr>
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<td>Phenylacetyl</td>
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<tr>
<td>Phenylglycine</td>
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<tr>
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<td>0.0</td>
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*Values reported previously for parent peptide [28].

Table V

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Table VI

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References

The references cited in this supplement are listed in the main text.
Figure 9. Panel A: Dose-response plot of daptomycin-peptide phosphorylation as a function of peptide concentration. Daptomycin-peptide phosphorylation was measured in a kinetic assay lasting 10 min in a total volume of 0.2 ml and a composition as described under Experimental Methods. Phosphate incorporation was quantitated in a phosphoimager. Each reaction was conducted in triplicate. The reaction was terminated after 1 min by adding 0.5 ml ice-cold methanol. Daptomycin-peptide phosphorylation was detected by HPLC following injection of 0.2 ml aliquots of the terminated reaction mixtures as described under Fig. 4.

Panel B: Time course of daptomycin-peptide phosphorylation measured by [32P] phosphate incorporation and HPLC. Daptomycin-peptide phosphorylation was measured as described under Experimental Methods except the [32P] adenosine 5' triphosphate (ATP) concentration was 75 ng per ml. Aliquots (500 µl) were withdrawn at the times indicated and processed as described under Experimental Methods. [32P] phosphate transferred (x), phosphorylation of daptomycin-peptide measured by HPLC (y) as described under Fig. 8.

Figure 10. Fractionation of rat muscular protein kinase activities on DEAE-cellulose. The extract was prepared by homogenizing 1 g of rat hind leg muscle in 50 ml of buffer A (50 mM Tris-HCl, 1 mM DTT, 25 pmol p[NHE], 0.2 units p[NHE] inhibitor, 15 % glycerol). The extract was prepared by using an ultrasonic and Branson glass homogenizers, centrifuged for 15 min at 26,000 g. The supernatant was filtered through glass wool and a 20 ml sample (200 mg protein) applied to a DEAE-cellulose column preincubated with the extract for 2 hr. The column was eluted with a linear gradient of A buffer and the fractions were monitored for protein (A). Protein kinase activity was measured using 32P ATP as described under Experimental Methods and 17 assays included 2 mM ATP. Panel A, protein kinase activity measured using the synthetic peptide substrate Lys-Arg-Arg-Ala-Lys-Asp-Leu-Leu (50 µM). Panel B, protein kinase activity measured using the synthetic peptide substrate Lys-Arg-Arg-Ala-Lys-Asp-Leu-Leu (50 µM).
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