Bioorganic Chemistry **27,** 189–196 (1999)
Article ID bioo.1998.1124, available online at http://www.idealibrary.com on IDEAL®

Differential Specificity of Protein Kinases A and C in Reaction with Synthetic Peptides[1]

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Received June 9, 1998

A set of peptides Lys-Arg-Pro-Ser-X-Arg-Ala-Lys-Ala, where X stands for Gly, Ala, Val, Leu, Ile, Phe, Lys, Glu, and Gln was studied as protein kinase A substrates. Although the lead peptide of this series was designed as a specific substrate for protein kinase C, all the compounds listed were also phosphorylated by protein kinase A. The data were analyzed by means of quantitative structure–activity relationships, taking into account hydrophobicity of the variable amino acids, bulkiness of their side-groups quantified by the molecular refractivity constants MR. Differently from similar correlation, obtained previously for protein kinase C, there was no influence of the ionic status of the variable amino acid on peptide reactivity in reaction with protein kinase A. The results of correlation analysis were used to compare substrate specificity patterns of protein kinase A and protein kinase C, focusing on details of the molecular recognition of peptide structure in vicinity of the phosphorylatable serine residue. A quantitative structure–activity relationship was formulated to characterize differences in specificity of these enzymes at the peptide position +1.

INTRODUCTION

The sequence of amino acids on both sides of the phosphorylatable Ser/Thr residues in substrates of protein kinases A and C is undoubtedly an important recognition determinant for these enzymes (*1–4*). On the other hand, specificity of these enzymes is not related to some absolutely fixed amino acid sequence and rather large variation can be observed even in the case of the endogenous substrates, where the phosphorylatable residues are surrounded by amino acids of different chemical structure, hydrophobicity, and charge distribution (*5*). Therefore, for more adequate description of substrate specificity of protein kinases quantitative structure–activity relationships were applied to quantify the specificity-determining factors at different positions of amino acids

[1] This work was supported by grants from International Science Foundation LCI 100, from Estonian Science Foundation ETF-3348 and Swedish Medical Research Council Project No 13X-00050.

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0045-2068/99 $30.00

around the phosphorylatable residue (*6*). These quantitative structure–activity relationships can be used to predict reactivity of peptides in the phosphorylation reaction and for design of optimal substrates (*7*).

On the other hand, the quantitative structure–activity relationships can also be used to compare the substrate specificity determinants of different enzymes. This is important, for example, for finding correlates between catalytic properties and structure of the enzymes, but may also have a practical value if the peptide substrates are used for selective assay of particular protein kinases in crude enzyme preparations or cell extracts containing several enzymes.

Our previous study on the "cross-specificity" of protein kinases A and C (*8*) has pointed to the possibility that only a short part, mostly the sequence of 1 to 2 amino acids around the phosphorylatable serine residue, were important for differentiation of substrates by these enzymes. The remaining part of the peptides had a rather similar influence on reactivity of substrates of these two enzymes. Therefore it was proposed that the active center of these enzymes can conventionally be divided into two parts, responsible for selectivity and effectiveness of the phosphorylation reaction, respectively. In the present study the former factors, governing selectivity of substrate differentiation, have been analyzed in more detail. For this purpose the specificity of protein kinase A for amino acids adjacent to the phosphorylation site was studied by using synthetic peptides Lys-Arg-Pro-Ser-X-Arg-Ala-Lys-Ala with various amino acids in the position X. The variable amino acids were Gly, Ala, Val, Leu, Ile, Phe, Lys, Glu, and Gln.

EXPERIMENTAL

Chemicals. [γ-^{32}P]ATP was purchased from Amersham, Great Britain. The peptides were synthesised by the Merryfield solid phase synthetic procedure (*9*). Phosphocellulose paper P81 was from Whatman (UK). Purification of the peptides was done by RP-HPLC chromatography using a 10 mm diameter Vydac column. Elution was performed by a linear gradient of 0–40% acetonitrile in 0.1% aqueous trifluoroacetic acid for 60 min. The flow rate was 4 ml/min. The peptides were detected at 230 nm. Fractions were combined after analysis by plasma desorption mass spectrometer and lyophilized.

Enzyme purification. Catalytic subunit of protein kinase A was purified from pig heart according to the previously published protocol (*10*). The enzyme stock solution with protein concentration 0.4 mg/ml was stored at −75°C and was diluted immediately before the kinetic assay in 20 mM MES (2-(*N*-morpholino)-ethanesulfonic acid) buffer (pH 6.5, 2 mg/ml bovine serum albumin, 1 mM DTT).

Kinetics of peptide phosphorylation. The phosphorylation of peptides was carried out at 30°C. The reaction mixture (200 μl) contained 50 μl 150 mM Tris–HCI buffer (pH 8.5); 75 μl of peptide stock solution in 50 mM Tris–HCL buffer containing 0.005% Triton X-100 (pH 7.5), 25 μl of solution of 37.5 mM $MgCl_2$ and 0.75 mM [γ-^{32}P]ATP (specific radioactivity 100 cpm/pmol); and 50 μl of solution of protein kinase A catalytic subunit. The enzyme concentration in the reaction mixture was 3–7 U/ml.

The phosphorylation reaction was started by the addition of catalytic subunit of the enzyme and monitored by transferring aliquots of 25 μl onto pieces of phosphocellulose paper. The reaction was stopped by immersing the papers in ice-cold 75 mM H_3PO_4. The papers were washed four times with ice-cold 75 mM H_3PO_4 (10 min each time) and dried at 80°C for 30 min. The radioactivity bound to paper was measured as Cerenkov radiation.

The initial rates of the phosphorylation reaction were calculated from cpm *vs* time plot using the least square linear regression analysis. The parameters of Michaelis–Menten rate equation (V and K_m) were calculated by using a nonlinear regression analysis program package (Enzfitter). In the case of peptides of low reactivity only the second order rate constants k_{II} were calculated from the slope of initial velocity *vs* peptide concentration.

RESULTS

Peptide Phosphorylation by Protein Kinase A

The peptides Lys-Arg-Pro-Ser-X-Arg-Ala-Lys-Ala, where X stands for Gly, Ala, Val, Leu, Ile, Phe, Gln, Glu, and Lys were phosphorylated by protein kinase A and linear plots of the initial rate *vs* enzyme concentration were observed in all cases. This was taken as an additional indication that the reaction followed was catalyzed by the enzyme. If the initial rate *vs* substrate concentration plots were hyperbolic, the Michaelis–Menten rate equation parameters V and K_m were calculated by means of the conventional nonlinear least-squares method following the equation:

$$v = \frac{k_{cat}[E][S]}{K_m + [S]}, \qquad [1]$$

where $[E]$ and $[S]$ denote the enzyme and substrate concentrations, respectively. The results of these calculations are listed in Table 1.

TABLE 1

Kinetic Parameters for Phosphorylation of Peptides Lys-Arg-Pro-Ser-X-Arg-Ala-Lys-Ala by Protein Kinase A

X	10^7 V mol/min mg	K_m μM	10^4 k_{II} l/min mg
Alanine	n.d.[a]	n.d.[a]	0.074 ± 0.006
Glutamine	0.27 ± 0.04	941 ± 230	0.29
Glutamic Acid	n.d.[a]	n.d.[a]	0.061 ± 0.007
Glycine	n.d.[a]	n.d.[a]	0.043 ± 0.006
Isoleucine	15.3 ± 4.4	1845 ± 665	8.29
Leucine	n.d.[a]	n.d.[a]	5.56 ± 0.19
Lysine	0.088 ± 0.005	258 ± 40	0.34
Phenylalanine	1.9 ± 0.6	170 ± 126	11.4
Valine	9.7 ± 1.0	2321 ± 304	4.18

[a] Due to the low rate of phosphorylation the kinetic parameters could not be determined under the experimental conditions used in this study.

However, in several cases the peptide concentrations accessible for experimental investigation were obviously too low to exceed the K_m value and the initial rate versus substrate concentration plots observed were linear, following the simplified version of the rate equation [1] at $[S] < K_m$:

$$v = \frac{k_{\text{cat}}}{K_m}[E][S]. \qquad [2]$$

From these linear plots the second order rate constants of the enzyme reaction, $k_{\text{II}} = k_{\text{cat}}/K_m$, were calculated for all substrates and listed in Table 1. As stressed before (*8*), the latter rate constants should be considered as meaningful parameters for QSAR analysis, as their physical meaning does not depend on the rate-limiting step of the reaction.

Influence of Amino Acid X on Peptide Phosphorylation by Protein Kinase A

Variation in structure of the amino acid X in peptide series Lys-Arg-Pro-Ser-X-Arg-Ala-Lys-Ala altered both the K_m and V values of the phosphorylation reaction by protein kinase A (Table 1). As a result of this variation only the k_{II} values were measured for four of the less reactive peptides.

It can be seen in Table 1 that the most effective substrates were peptides with hydrophobic side groups of the amino acid X. On the other hand, location of ionic charge of both negative and positive signs in position X considerably decreased the reactivity of peptides. Most clearly these regularities can be followed in the case of the second-order rate constants k_{II}, which values covered more than two powers of magnitude within the present reaction serie.

For a more systematic analysis of these regularities the rate constants k_{II} were analyzed by means of quantitative structure–activity relationships, taking into account hydrophobicity (π) and bulkiness (MR) of the side chains of amino acids X. The values of these structural parameters were compiled from (*10*) and (*11*) and were the same as used in our previous studies (*6*,*8*). It should be emphasized that there was no significant correlation between the π and MR constants within the present reaction series ($r = 0.087$) and both parameters can be used in the same correlation equation.

For the log k_{II} values this analysis yielded the following relationship:

$$\log k_{\text{II(PKA)}} = -(4.7 \pm 0.2) + (0.65 \pm 0.09)\,\pi + (0.05 \pm 0.01)\,\text{MR}, \qquad [3]$$

where $n = 9$, $s = 0.281$, $r = 0.969$.

In this and the following correlations n is the number of data points, and quality of the correlation was described by the standard error of estimation (s) and the correlation coefficient (r). The 95% confidence intervals of the parameters are given.

Omission of either π or MR from this analysis significantly lowered the quality of the correlation, pointing to significance of both parameters used. On the other hand, however, addition of the independent variable Ind to take into consideration the ionic status of substituents (see in (*7*)) did not improve the correlation and yielded the correlation coefficient 0.960. This means that the ionic charge, if located in the