

Comparison of Substrate Specificities of Protein Kinases A and C Based on Peptide Substrates

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Peptides, obtained by gradual removal of amino acids from both ends of pEKRPQRSKYL, and stereoisomeric nonapeptides KRPSQRAKY with one D-amino acid residue successively in each position, were tested as substrates for protein kinase A. All these compounds were phosphorylated but at quite different rates by the enzyme. Comparison of the kinetic data with the appropriate results for protein kinase C, measured earlier, was used to analyze and compare the specificity determining factors of these enzymes. The analysis of the cross-specificity points to the possibility that only a short part, mainly the sequence of 1 to 2 amino acids around the phosphorylatable serine residue, is important for differentiation of substrates by these enzymes, while the remaining part of the peptide structure has similar influence on their reactivity in the case of these two protein kinases. Thus, the active center of these enzymes can be conventionally divided into two parts, which are responsible for selectivity and effectiveness of the phosphorylation reaction, respectively. © 1994 Academic Press, Inc.

INTRODUCTION

In many biological systems extracellular signals are transformed into intracellular effects via phosphorylation of different cell proteins by second-messenger-dependent protein kinases (1). The target sites of phosphorylation are determined, in their turn, by the substrate specificity of the intracellular enzymes (2, 3). Therefore, the selectivity of the signaling process and the following changes in the cell functions depend on the exactness of recognition of the phosphorylatable sites.

For protein kinase A the target site recognition seems to be determined by the amino acid sequence around the phosphorylatable amino acid residue (2, 3). This conclusion has been reached by analysis of the primary structures of the phosphorylatable sites of intact proteins (4) and confirmed by studies with synthetic peptide substrates, analogous to the peptide sequences around these sites (3). Such studies have also provided the main body of information about the structural requirements for substrates of different protein kinases (2).

On the other hand, the site specificity of protein kinases is not absolute, as the same sites of proteins can be phosphorylated by different enzymes in some cases (5). Consequently, there should also be synthetic peptides, which act as substrates

for two or even more protein kinases. Systematic kinetic analysis of phosphorylation of such peptides by different enzymes and comparison of the appropriate data should provide information about cross-specificity of protein kinases and allow estimation of common specificity determinants of these enzymes. Such data 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. In spite of that, the cross-specificity of protein kinases has not been systematically analyzed.

In the present paper we have studied the kinetics of protein kinase A-catalyzed phosphorylation of two sets of peptides, both related to peptide pEKRPQRSKYL which was initially designed as a specific substrate for protein kinase C (6). The results obtained together with the appropriate data, obtained previously for protein kinase C for these substrates (8), provide a unique opportunity to compare the substrate specificities of these two enzymes.

MATERIALS AND METHODS

[γ - ^{32} P]ATP was purchased from Amersham, Great Britain. The bovine serum albumine (BSA), fatty acid-free, was obtained from Sigma (U.S.A.). All other reagents were of highest commercially available grade. Phosphocellulose paper P81 was from Whatman (Great Britain). All buffer and washing solutions were prepared using Seralpur UP-50 deionized water.

Peptides were prepared by the solid-phase method of Merrifield (7) using Boc-amino acids. Experimental details have been given elsewhere (8, 9).

The catalytic subunit of protein kinase A was purified from porcine heart as described by Zoller *et al.* (10). The enzyme was stored at -75°C in 400 mM potassium phosphate buffer, pH 6.5, with 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol (v/v). This enzyme stock solution contained 0.39 mg protein/ml and had a sp act of 400 U/mg protein where 1 unit was defined as the amount of enzyme that transferred 1 nmol of phosphate from ATP to mixed histone (1 mg/ml of incubation mixture) in 1 min.

The phosphorylation of peptides by protein kinase A was carried out at 30°C . The reaction mixture (200 μl) contained 50 μl 150 mM Tris-HCl buffer (pH 8.5); 75 μl of the 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 protein kinase A solution. This enzyme solution was made immediately before the experiment by diluting the stock solution in 20 mM Mes buffer (pH 6.5), containing 0.5 mM dithiothreitol, 0.1 mM EDTA, and 0.05 mg/ml bovine serum albumin. The enzyme concentration in the reaction mixture was 3–7 U/ml.

The phosphorylation reaction was started by the addition of the enzyme into the reaction mixture and the peptide phosphorylation was followed by removal of 25- μl aliquots of the reaction mixture onto pieces of phosphocellulose paper. Thereafter the reaction was immediately stopped by immersing the papers in ice-cold 75 mM H_3PO_4 . The papers were washed four times with ice-cold 75 mM

TABLE 1
Phosphorylation of Synthetic Peptides by Protein Kinase A

Peptide	$10^4 k_{\text{II}}^{\text{a}}$ l/min * mg	$k_{\text{II}}^{\text{rel}}$	$10^7 V_{\text{max}}$ mol/min * mg	$10^6 K_{\text{m}}$ M
pEKRP <u>S</u> QRSKYL I	14.6 ± 4.8	1	—	—
KRP <u>S</u> QRSKYL II	19.6 ± 8.8	1.34	2.65 ± 0.67	135 ± 28
RPSQ <u>R</u> SKYL III	0.58 ± 0.05	0.04	—	—
KRP <u>S</u> QRAKY IV	1.83 ± 0.41	0.125	1.66 ± 0.12	898 ± 138
KRP <u>S</u> QRSKY V	2.90 ± 0.89	0.20	1.67 ± 0.16	574 ± 124
RPSQ <u>R</u> SKY VI	0.50 ± 0.01	0.034	—	—
PSQ <u>R</u> SKY VII	0.010 ± 0.002	0.0007	—	—
KRP <u>S</u> RSK VIII	2.07 ± 0.59	0.14	2.51 ± 0.27	1209 ± 217
RPSQ <u>R</u> SK IX	0.113 ± 0.004	0.0077	—	—
RRAS <u>V</u> A	4920 ± 522	—	73.8 ± 9.0	15 ± 1.8

^a Calculated from v vs $[S]$ plots according to Eq. [2].

H₃PO₄ (10 min each time) and dried at 80°C for 30 min. The radioactivity bound to the paper was measured as Cerenkov radiation.

The initial rates of the phosphorylation reaction were calculated from the slopes of the counts per minute (cpm) versus time plots using linear regression with statistical weighting (experimental error structure assumed to be proportional to the square root of counts per minute). No phosphorylation of bovine serum albumin was noted in these experiments. The background of [γ -³²P]ATP binding to the phosphocellulose paper was measured in the absence of the enzyme and this value was taken as the intercept in the cpm vs time plots.

Data processing was performed using the Enzfitter program package (Elsevier).

RESULTS

Kinetic Analysis of Peptide Phosphorylation

The protein kinase A-catalyzed incorporation of radioactive phosphorus into the peptides studied (see Tables 1 and 2) was followed for 10–15 min by taking aliquots from the reaction mixture at intervals of 1–3 min. During the incubation no inactivation of the enzyme was observed and the concentration of the reaction product showed linear dependence upon time. This allowed reliable determination of the values of the reaction initial rates (v).

The kinetic measurements were made at different concentrations of protein kinase A, as some substrates were phosphorylated at a low rate and meaningful results were obtained only at a relatively high enzyme concentration. The plot of the initial rate versus enzyme concentration was checked also and its linearity was taken as an additional indication that the process observed was catalyzed by the enzyme added (Fig. 1A).

TABLE 2
Phosphorylation of Diastereomeric Peptides by
protein Kinase A^a

Peptide	$k'_{II} \cdot 10^4$ liter/min $\cdot$ mg	k'_{II} rel
KRPSQRAKY	1.83 ± 0.52	1
kRPSQRAKY	1.10 ± 0.40	0.60
KrPSQRAKY	0.084 ± 0.021	0.046
KRpSQRAKY	0.035 ± 0.019	0.019
KRP _s QRAKY	0.022 ± 0.007	0.012
KRP _s qRAKY	0.92 ± 0.13	0.50
KRP _S QrAKY	0.98 ± 0.18	0.54
KRP _S Qr _a AKY	1.51 ± 0.344	0.83
KRP _S QRA _k Y	1.29 ± 0.31	0.70

^a The peptides contain *D*-amino acids indicated by lower case letters.

For several peptides studied the plot of the initial rate (v) versus substrate (S) concentration was hyperbolic and followed the Michaelis–Menten rate equation:

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

For these peptides the kinetic parameters K_m and $V = k_{\text{cat}}[E]$ were calculated by means of the conventional nonlinear least-squares method and listed in Table 1.

For other peptides the K_m values were obviously too high to be practically reached by the compound concentration. Therefore the v versus $[S]$ plots were linear within the whole peptide concentration interval accessible for investigation.

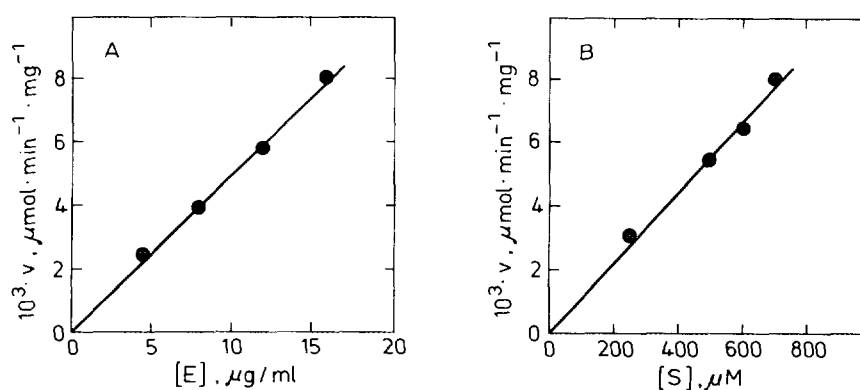


FIG. 1. Phosphorylation of peptide RPSQRSK by protein kinase A. (A) The dependence of the phosphorylation rate upon the enzyme concentration (peptide concentration $700 \mu\text{M}$), and (B) the dependence of the reaction rate upon the peptide concentration.