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Phosphorylation of Sepharose-Coupled Peptides by Protein Kinase A

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The kinetics of phosphorylation of the Sepharose-coupled peptide RRASVA by catalytic subunit of protein kinase A, and diastereomers of this peptide, containing D-amino acids successively in each position, were studied. Coupling of these peptides with the amino and carboxyl termini to CH- and AH-Sepharoses had similar effects on the phosphorylation reaction, increasing the K_m and decreasing the V values, respectively. The diastereomeric peptides were also phosphorylated by the enzyme and the rate of this reaction depended on the position of substitution of L-amino acids with their D-analogs. However, this dependence was much less pronounced if compared with stereoselectivity of the enzyme in reactions with these peptides in solution: the K_m values for the Sepharose-coupled peptides were almost insensitive to the replacement of L-amino acids with D-analogs and moderate stereoselectivity was revealed in the maximal velocity of the reaction. The Sepharose-coupled peptide containing D-serine was also phosphorylated by protein kinase A while the same peptide in solution did not interact with the enzyme. Consequently, the polymer, enveloping the phosphorylatable peptide, may remarkably influence the recognition of the reaction site, altering both V and K_m values.

INTRODUCTION

Protein kinases phosphorylate Ser/Thr or Tyr residues in soluble and membrane-integrated proteins (*1*, *2*) and most often the specificity of these enzymes is discussed with regard to the amino acid sequence around the phosphorylatable site (*3*). However, it cannot be excluded that additional features of the three-dimensional structure of substrate may also influence the reaction. Therefore it seemed to be of interest to extend the specificity studies of peptide substrates to heterogeneous systems, containing polymeric structures and surfaces which might modify the peptide–enzyme interactions.

In this study such a heterogeneous substrate system for protein kinase A (EC 2.7.1.37, ATP : protein phosphortransferase) was designed by covalent coupling of the hexapeptide RRASVA,[2] and its diasterometric derivatives, containing successively D-amino acids in each position, to a Sepharose matrix. These peptides reveal

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[2] RRASVA, hexapeptide Arg-Arg-Ala-Ser-Val-Ala, and the lower case letters are used to denote D-amino acid in its diastereomers.

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a rather characteristic stereoselectivity pattern in the reaction with protein kinase A in solution (*4*) and therefore were selected as tools in this study.

Sepharose is specially designed for affinity chromatography and therefore it has a relatively small void volume for proteins. On the other hand, its polymeric structure immobilizes the peptide and may support the peptide–protein interactions via binding of the enzyme molecule, thus providing the possibility to analyze the sorption effect on recognition of the substrate structure.

MATERIALS AND METHODS

Chemicals. CH-Sepharose 4B and AH-Sepharose 4B were purchased from Pharmacia Fine Chemicals. 1-ethyl-3(3-dimethylaminopropyl)carbodiimide HCl (EDAC) was obtained from Bio-Rad Laboratories [γ^{32}P]ATP was from Amersham. Other commercially obtained chemicals were of highest available grade from Sigma. The peptides were synthesized by the Merrifield solid phase synthetic procedure (*5*) as previously described (*4*, *6*). The chemical and stereochemical purity of the peptides was checked using the ion-pair chromatography as described (*5*, *6*).

Coupling of peptides to Sepharose. Water-soluble carbodiimide EDAC was used for peptide coupling to CH- and AH-Sepharoses according to Pharmacia instructions (Affinity Chromatography. Principles and Methods. Pharmacia Fine Chemicals, 1979). The molar ratio of the spacer groups of the gel, peptide, and carbodiimide in most experiments was 1:3:60. The peptide was dissolved in water and mixed with washed and preswollen Sepharose to obtain 1.5 ml of gel suspension in water (liquid/gel ratio 2:1). The pH of this mixture was checked and adjusted with 0.5 M HCl to 5.5 when necessary. Then an excess of EDAC was added as free powder, the pH of the mixture checked and adjusted when necessary, and the suspension slowly rotated at 4°C for 5 h. The peptide content of the gel was 2–4 μmol/ml in the case of CH-Sepharose 4B and around 1 μmol/ml in the case of AH-Sepharose, as calculated from amino acid analysis data.

Enzyme. Protein kinase A catalytic subunits were prepared from pig heart essentially according to the previously published procedure (*7*). The enzyme stock solution (0.21 mg protein/ml) was kept at −70°C and diluted with 20 mM MES (2-(*N*-morpholino)-ethanesulfonic acid) buffer (pH 6.5, 2 mg/ml bovine serum albumine, 1 mM DTT) before the experiments.

Kinetics of peptide phosphorylation. All kinetic studies were made at saturating ATP concentration (0.1 mM). The reaction of peptide phosphorylation was carried out at 25°C in 225 μl of reaction mixture, containing 75 μl of 150 mM Tris–HCl buffer (pH 8.5), 100 μl of substrate solution or suspension in 50 mM Tris–HCl buffer (pH 7.5, 0.005% Triton X-100), 30 μl of 0.75 mM [γ^{32}P]ATP with specific radioactivity around 100 cpm/pmol, and 20 μl of the enzyme solution, diluted as described above. The reaction was initiated by the addition ATP and the course of the peptide phosphorylation was followed by analysis of six 30-μl samples removed during 5–10 min.

In the case of Sepharose-coupled peptides these aliquots were transferred to Whatman glass microfiber filters (GF/A) and the reaction was stopped by immediate

extensive washing with salt solution (0.5 M NaCl and 0.1 M $MgSO_4$, 3 × 20 ml). Afterwards the filters were dried and the Cherenkov radiation was measured.

In the case of soluble peptide these aliquots were transferred onto 2 × 2-cm pieces of phosphocellulose paper (Whatman 81) and the reaction was stopped by immersing these papers into ice-cold 75 mM phosphoric acid. After this the papers were washed four times with the same acid solution and dried at 80°C before measurement of the Cherenkov radiation.

Data processing. The initial rates of the phosphorylation reaction were calculated from the slopes of the cpm vs time plots, which were linear during the 5–10 min used for monitoring the process. In the case of the Sepharose-coupled peptides the intercept of these plots, characterizing the nonspecific adsorption of radioactive compounds on the filter, could be decreased by extensive washing of the samples during the filtration procedure. Calculations were carried out on an IBM PS/1 computer using the Enzfitter program package (Elsevier).

RESULTS

Phosphorylation of Sepharose-Coupled Peptides

The hexapeptide RRASVA was coupled to 6-carbon spacer arms with COOH and NH_2 groups of CH- and AH-Sepharoses 4B, respectively. This provided coupling of the peptide via its terminal amino and carboxyl groups, respectively. Both products were substrates for protein kinase A. The phosphorylation of the immobilized substrates was carried out as for the soluble peptides except that continuous agitation of the reaction mixture was necessary to avoid precipitation of the Sepharose beads during the reaction.

With CH-Sepharose 4B, higher yields and better reproducibility of peptide coupling were obtained. Therefore, in the following systematic study of phosphorylation of diastereomeric peptides, only this matrix was used.

The product of the phosphorylation was assayed through the mechanical separation of the Sepharose beads from other components of the reaction mixture. Therefore extensive washing of the filters could be used to reduce the filter-adsorbed radioactivity without loss of the reaction product. On the other hand, at high enzyme concentrations (above 0.01 mg protein/ml) some phosphorylation of the Sepharose matrix was observed. This time-dependent nonspecific binding of radioactive phosphorous could not be removed by washing. But in the presence of substrates the rate of this background reaction was below 0.1% of the phosphorylation rate of the least reactive peptides.

Kinetics of Immobilized Peptide Phosphorylation

The initial velocities of phosphorylation of the immobilized peptides were proportional to the enzyme concentration (Fig. 1). Thus the entire amount of the phosphorylation product detected was formed via the enzyme-catalyzed process. At saturating ATP concentration the dependence of the initial rate of phosphorylation upon

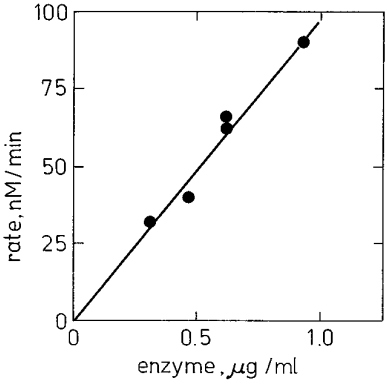

FIG 1. Effect of concentration of protein kinase A on the initial rate of phosphorylation of CH-Sepharose 4B-coupled peptide RRASVA at substrate concentration 7.5 μM.

peptide concentration was hyperbolic (Fig. 2), following the common Michaelis–Menten rate equation

$$v = \frac{V[S]}{K_m + [S]}, \qquad [1]$$

which allowed calculation of the V and K_m values. The constants obtained are listed in Table 1.

As the substrate concentration in these kinetic experiments was calculated from the data of amino acid analysis, the net amount of the immobilized peptide per unit of the reaction volume was used in these plots. The variation in peptide concentration was achieved in two different ways. First, the suspension of the Sepharose beads was simply diluted in the reaction mixture that reduced the amount

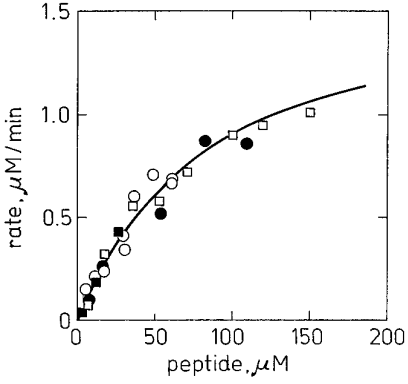

FIG. 2. Kinetics of phosphorylation of hexapeptide RRASVA, coupled to CH-Sepharose 4B. Gel preparations of different coupling density were used: ○, 2.2 μmol/ml gel; ●, 4.1 μmol/ml gel; □, 3.0 μmol/ml gel; ■, 1.5 μmol/ml gel.