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Quantitative Structure–Activity Relationships in the Protein Kinase C Reaction with Synthetic Peptides Derived from Myelin Basic Protein

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A set of peptides, Lys-Arg-Pro-Ser-X-Arg-Ala-Lys-Ala, where X stands for Ala, Val, Leu, Ile, Phe, Pro, Lys, Arg, Asp, Glu, Asn, Gln, and His, was synthesized and the kinetics of their phosphorylation by protein kinase C was studied. All compounds, except the peptide with Pro at the position X, were effectively phosphorylated by this enzyme, and for these substrates the kinetic constants K_m, maximal velocity constants V, and second-order rate constants k_{II} were determined. 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 molecular refractivity constants MR, and ionic status of these substituents by using an independent variable $+1$ for cationic, -1 for anionic, and 0 for nonionic substituents. These structural factors influenced the K_m values, while the maximal velocity of phosphorylation depended mostly on the ionic status of the variable amino acid. The latter effect seems to characterize electrostatic interaction between the substrate molecule and some negative charge located in the enzyme active center.

INTRODUCTION

The basic amino acids on both sides of the phosphorylatable Ser/Thr residues in peptide or protein substrates seem to constitute an important recognition determinant for protein kinase C (*1*), as well as for several other protein kinases (*2*). On the other hand, specificity of all these enzymes is not related to some absolutely fixed amino acid sequence, and rather large variations in these structures can be observed even in the case of endogenous selective substrates. For example, in myelin basic protein the phosphorylatable serine residues are followed in the major phosphorylation sites by asparagine and lysine (*3*), but also by glutamine (*3, 4*). In the brain-specific protein neurogranin, which is also a physiologically relevant substrate for protein kinase C, the phosphorylatable serine residue is followed by phenylalanine (*5*). These amino acids are very different in terms of their chemical structure, hydrophobicity, and charge-distribution character, but despite that they all must fit the recognition motif for this enzyme.

In the present study the specificity of protein kinase C for amino acid structure in this position, adjacent to the phosphorylation site, was studied in detail by using

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the synthetic peptides Lys-Arg-Pro-Ser-X-Arg-Ala-Lys Ala with various amino acids in the position X. These peptides were derived proceeding from the amino acid sequence surrounding Ser(8) of myelin basic protein (*3*) and therefore can be treated as congeneric analogs of the peptide Gln-Lys-Arg-Pro-Ser(8)-Gln-Arg-Ser-Lys-Tyr-Leu, synthesized proceeding from this sequence by Nishizuka and coworkers (*4*) and recommended by these authors as a specific substrate for the assay of activity of protein kinase C.

The variable amino acids in the position X included hydrophobic (Gly, Ala, Val, Leu, Ile, Phe, Pro), cationic (Lys, Arg), anionic (Asp, Glu), and polar (Asn, Gln, His) functional groups to provide a systematic overview of the specificity-determining factors in this particular position of protein kinase C substrates.

EXPERIMENTAL

Materials. Fmoc amino acids were obtained from Millipore (USE). Side-chain protecting groups were OtBu for Asp and Glu, tBu for Ser, Trt for Asn, Gln, and His, Boc for Lys, and Pmc for Arg. Fmoc-L-Ala-Resin was obtained from Calbiochem–Novabiochem AG (Switzerland). [γ-^{32}P]ATP was obtained from Amersham (Great Britain). 1,2-Diolein and L-α-phosphatidyl-α-serine were products of Sigma (St. Louis, MO). Phosphocellulose paper P81 was from Whatman (UK).

Synthesis and purification of peptides. The peptides were prepared by the solid phase method, using either Boc amino acids as described in (*6, 7*) (peptides 9, 10, and 12 in Table 1), or Fmoc amino acids (peptides 1–8, 11, 13, and 14 in Table 1).

TABLE 1
Kinetic Parameters for Phosphorylation of Peptides Lys-Arg-Pro-Ser-X-Arg-Ala-Lys Ala by Protein Kinase C

X	10^7 V (mol/min mg)	K_m (μM)	10^4 k_{II} (liter/min mg)
Alanine	0.89 ± 0.1	324 ± 74	2.7
Arginine	2.6 ± 0.2	22 ± 6	117
Asparagine	1.2 ± 0.2	312 ± 58	3.7
Aspartic acid	0.28 ± 0.06	1106 ± 408	0.26
Glutamine	1.3 ± 0.1	59 ± 10	21
Glutamic acid	0.53 ± 0.09	896 ± 110	0.59
Glycine	0.35 ± 0.01	161 ± 8	2.2
Histidine	1.6 ± 0.1	60 ± 15	24
Isoleucine	0.91 ± 0.08	20 ± 5	46
Leucine	0.86 ± 0.06	16 ± 3	54
Lysine	2.6 ± 0.2	18 ± 4	146
Phenylalanine	0.96 ± 0.05	20 ± 4	47
Proline[a]	Not determined	Not determined	Not determined
Valine	1.2 ± 0.1	18 ± 3	66

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

In the latter case the couplings were done with a fourfold excess of Fmoc amino acids during 1 h, initiating the reaction by the addition of a fourfold molar excess of HOBt and HBTU and an 8-molar excess of DIEA. For removal of Fmoc groups, the resin was treated twice with 20% piperidine in DMF for 5 min each. Cleavage from the resin and deprotection was achieved by treatment with a mixture of TFA/phenol/thioanisole/ethanedithiol/water (v/v 82.5/5/5/2.5/5).

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.

Using Boc chemistry the side-chain protecting groups were 2-chlorobenzyloxycarbonyl for Lys and benzyl for Ser. Couplings were performed with the aid of dicyclohexylcarbodiimide using a 2.5-fold excess of Boc amino acid. Deprotection was done by treatment with 30% trifluoroacetic acid in dichloromethane, and 10% triethylamine in dichloromethane was used for neutralization. The peptides were cleaved from the resin by liquid HF in the presence of anisole at 0°C, extracted with 10% acetic acid, and lyophilized. Purification was done by ion-pair liquid chromatography as described in (*8*).

The purity of the products was confirmed by amino acid analyses and mass spectra (Bioion 20 Nordic AB).

Enzyme purification. Protein kinase C was prepared from pig spleen by the method of Parker *et al.* (*9*), except for the final chromatography on phenyl–Sepharose, which was excluded because of low recovery. The product obtained consisted mainly of the β-isozyme with traces of the α-isozyme and had a specific activity 80 U/mg, where 1 unit was defined as the amount of enzyme that transferred 1 nmol of phosphate from ATP to histone H1 (1 mg/ml in the incubation mixture) in 1 min.

Assay of peptide phosphorylation. Phosphorylation of synthetic peptides by protein kinase C was carried out at 25°C in a reaction mixture composed of 115 μl substrate solution in 50 mM Tris–HCl buffer, pH 7.5; 30 μl 150 mM Tris–HCl buffer, pH 7.5, with 5 mM calcium acetate; 30 μl 20 mM Tris–HCl buffer, pH 7.5, containing 450 μg/ml phosphatidylserine and 8 μg/ml diolein; 20 μl of protein kinase C in 20 mM Tris–HCl buffer, pH 7.5, with 2 mM EDTA and 0.5 dithiothreitol; 30 μl of 0.75 mM [^{32}P]ATP in 37.5 mM $MgCl_2$ with specific radioactivity of about 100 cpm/pmol.

The phosphorylation reaction was initiated by the addition of [^{32}P]ATP solution and monitored by transferring 30-μl aliquots of the reaction mixture onto 2 × 2-cm pieces of phosphocellulose paper at appropriate time intervals. The papers were then immersed in ice-cold 75 mM phosphoric acid to stop the reaction, washed four times with this acid for 10 min, and dried at 80°C, and the radioactivity attached was measured as Cerenkov radiation.

The initial rates of the phosphorylation reaction were calculated from the cpm vs time plots and then used to estimate the parameters of the Michaelis–Menten rate equation (V and K_m) by a nonlinear regression method (Enzfitter program package).

RESULTS

Peptide Phosphorylation

The peptides Lys-Arg-Pro-Ser-X-Arg-Ala-Lys Ala, where X stands for Gly, Ala, Val, Leu, Ile, Phe, Gln, Asn, His, Glu, Asp, Lys, and Arg, were phosphorylated by protein kinase C. The phosphorylation reactions followed the Michaelis–Menten rate equation and allowed calculation of the kinetic parameters V and K_m for these substrates. These constants are listed in Table 1 together with the second-order rate constants k_{II}, calculated as ratio of the former parameters, V/K_m.

In contrast to the compounds mentioned above, the peptide Lys-Arg-Pro-Ser-Pro-Arg-Ala-Lys-Ala with a proline residue in the position adjacent to the phosphorylatable serine was phosphorylated at a very low velocity. This hampered a reliable determination of the V and K_m values under the experimental conditions used in this study. The low reaction rate can be related to the specific implication of proline residues on the conformation of proteins and peptides. Therefore the structural and chemical properties of the latter peptide may not fit those of the other compounds studied and the kinetics of its phosphorylation will be analyzed elsewhere.

Variation of the amino acid structure near the phosphorylatable serine altered mainly the K_m values, while these effects were less significant in the case of V (Table 1). It can be seen that the maximal velocity of peptide phosphorylation varied less than 10 times within the whole reaction series, but even less within the subgroup of substrates with nonionic side groups at the position of the variable amino acid. The Gly-containing peptide was an exception from this regularity, as the V value for this substrate remained three to four times below the mean level of the maximal velocities for other nonionic substrates (Table 1). At the same time the K_m values varied approximately 70 times, including peptides with both ionic and nonionic side groups at the position of the variable amino acid.

It is noteworthy that the cationic amino acids Lys and Arg, while located next to the phosphorylatable serine, improved the reactivity of the substrates mainly by increasing the V value, while the K_m values for these peptides were comparable with substrates carrying aliphatic groups in this position. On the other hand, the carboxylic side groups of Glu and Asp decreased the V and increased the K_m for these substrates.

These regularities can be more clearly followed in the case of the second-order rate constants k_{II}, calculated from the V and K_m values and listed in Table 1. It can be seen that the peptides with anionic and cationic charges on the side group of the amino acid, following the phosphorylatable serine, represented indeed the best and worse substrates within this series. Substrates with polar groups belonging to the side chains of Asn, Gln, and His in this position were phosphorylated even more slowly than several peptides with aliphatic (hydrophobic) groups in this position.

All together, the kinetic analysis of phosphorylation of peptides Lys-Arg-Pro-Ser-X-Arg-Ala-Lys Ala with various types of amino acids in the position denoted by X revealed that protein kinase C was rather tolerant against variation of substrate structure in this area of the phosphorylatable peptide or protein.

Structure–Activity Relationships

For a more systematic understanding of the molecular mechanism of recognition of substrates by protein kinase C, an attempt was made to analyze the kinetic data of phosphorylation of the peptides Lys-Arg-Pro-Ser-X-Arg-Ala-Lys Ala by means of quantitative structure–activity relationships. In this analysis the second-order rate constants k_{II} should be preferred, as the physical meaning of this parameter does not depend on the rate-limiting step of the enzyme reaction, as happens in the case of V and K_m. Moreover, the meaning of the latter parameters may change due to variation of substrate structure and reactivity within the reaction series.

As usual, the π and MR constants, characterizing hydrophobicity and bulkiness of amino acid side chains, were used to analyze the interactions of these groups with the enzyme active center. The values of these parameters, compiled from (*10*) and (*11*), are listed in Table 2. It should be emphasized that due to the presence of electronegative (polar) and ionic groups in the present set of substituents, there was no significant correlation between the π and MR constants within the reaction series ($r = 0.0876$). Therefore both parameters can be used in the same correlation equation.

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

$$\log k_{II} = -(4.18 \pm 0.41) + (0.25 \pm 0.17)\pi - (0.07 \pm 0.02)MR, \quad n = 13, s = 0.635, r = 0.758. \qquad [1]$$

In this and the following correlations n is the number of data points, s is the standard

TABLE 2
Substituent Constants for Amino Acid Side Groups Used in the Correlation Analysis

Amino acid	π (*10*)	MR (*11*)	Ind
Alanine	0.31	5.65	0
Arginine	−1.01	30.05	1
Asparagine	−0.60	14.46	0
Aspartic acid	−0.77	11.58	−1
Glutamine	−0.22	19.11	0
Glutamic acid	−0.64	16.23	−1
Glycine	0	1.03	0
Histidine	0.13	23.79	0
Isoleucine	1.80	19.59	0
Leucine	1.70	19.59	0
Lysine	−0.99	25.05	1
Methionine	1.23	23.12	0
Phenylalanine	1.79	30.01	0
Proline	0.72	13.95	0
Serine	−0.04	11.82	0
Threonine	0.26	11.82	0
Tryptophan	2.25	39.81	0
Tyrosine	0.96	31.83	0
Valine	1.22	14.96	0