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PHOSPHORYLATION OF SYNTHETIC PEPTIDE ANALOGS OF THE PHOSPHORYLATABLE SITE OF PHOSPHORYLASE b WITH PHOSPHORYLASE KINASE*

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SUMMARY

A synthetic octapeptide of the phosphorylatable site of phosphorylase and its analogs were used to determine the specificity of nonactivated phosphorylase kinase. By substitution of each of six amino acid residues (lysine$_{11}$, glutamine$_{12}$, isoleucine$_{13}$, serine$_{14}$, valine$_{15}$, and arginine$_{16}$), it was found that these residues were all important in the enzyme recognition. Valine$_{15}$ was more important than isoleucine$_{13}$, when either valine$_{15}$ or isoleucine$_{13}$ was substituted by glutamic acid. A peptide containing two isoleucyl residues (surrounding serine$_{14}$) had a better phosphorylation rate than a peptide containing two valyl residues. A peptide with a threonine residue instead of serine could be phosphorylated but with a low reaction rate.

INTRODUCTION

Phosphorylation, a covalent modification of proteins, is known to be an important regulatory mechanism in biological systems. In glycogen metabolism, phosphorylase b kinase (ATP: phosphorylase phosphotransferase, ED 2.7.1.38) catalyzes the phosphorylation of phosphorylase b to phosphorylase a in the presence of divalent metal ions and ATP (1,2,3). In this reaction, the terminal phosphate group of ATP is transfered to a specific seryl residue located in the N-terminal portion of phosphorylase molecule (1,4). The enzyme will also phosphorylate the tetradecapeptide, Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu, containing the phosphorylatable seryl residue (4,5). Tessmer et al. (6) synthesized a series of peptides, analogs of the tetradecapeptide, to determine which amino acids were necessary for recognition by

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phosphorylase kinase. By reduction of the peptide length, they found that the first six residues in the sequence were not important while substitution of residues in the sequence, Lys-Gln-Ile-Ser-Val-Arg, had a large effect. This sequence is present in human, rat, and dogfish phosphorylases (7,8,9). All of these phosphorylases can be phosphorylated by rabbit muscle phosphorylase kinase. However, yeast phosphorylase has a different sequence and cannot be phosphorylated (10). To further study the significance of the preceding sequence for recognition by nonactivated phosphorylase kinase, a series of peptides was synthesized and tested as substrates.

MATERIALS AND METHODS

Nonactivated phosphorylase kinase was prepared from white New Zealand rabbit back and hind leg muscles by the method of Brostrom et al. (11). The concentration was determined spectrophotometrically with an absorbance index of 12.0 for a 1.0% protein solution at 280 nm (12). The specific activity of the kinase was 70,000 units/mg as determined by the method of Brostrom et al. (11). Preparation of [γ-^{32}P]-ATP was done according to the method of Glynn and Chappell (13).

Peptides were synthesized on a Beckman model 990 automated peptide synthesizer, using the solid-phase method of Merrifield (14). Attachment of leucine to the resin was done by using cesium bicarbonate according to the method of Gisin (15). Peptides were cleaved from the resin and deprotected with hydrogen fluoride by the method of Lenard and Robinson (16). Peptides were purified by gel filtration and ion exchange chromatography.

The purity of the peptides was determined by high voltage electrophoresis on Gilson high voltage electrophorator by using 10% pyridine-0.5% acetic acid buffer pH 6.4 for one and a half hours at 2,000 volts and by amino acid analysis. Each peptide was analyzed on a Durrum amino acid analyzer model D-400 after hydrolysis in constant boiling 5.7 N CHl in vacuo at 110° for 22 hours. The concentration of each peptide was determined by amino acid analysis.

Each peptide was phosphorylated by nonactivated phosphorylase kinase at a saturating level of Mg-ATP. The reaction mixture, having a final volume of 120 µl, contained 60 µl of each peptide at various concentrations, 20 µl of 0.25 M β-glycerophosphate-0.25 M Tris-0.6 mM calcium chloride pH 8.6, and 20 µl of phosphorylase kinase. After 15-minute preincubation at 30°C, 20 µl of a Mg-^{32}P-ATP mixture ([Mg^{++}] = 60 mM, [ATP] = 18 mM) was added to start the reaction. An aliquot of 25 µl was taken at several time points, added to 0.5 ml of 30% acetic acid to stop the reaction, and applied to a 6.5-cm column of AG1x8 anion exchange resin (Biorad Laboratories) in a disposable pasteur pipette. The phosphorylated peptide was eluted from the column with 9.5 ml of 30% acetic acid directly into a scintillation vial. Radioactivity was determined in a Beckman scintillation spectrometer in the tritium channel (17).

Initial rate data were analyzed according to the weighted least-square method by a computer program written in the Omnitab language as described by Siano et al. (18). The kinetic parameters and their standard deviations were calculated by using standard formulas as described by Meyer (19).

Table I. K_m and V_m values for synthetic peptides

	Ac-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu[a] (11 12 13 14 15 16 17 18)		
Peptide no.	Substitution	K_m (mM)	V_m (μmole/min/mg)
1	11 NH_2-Lys	1.48 ± 0.36	1.84 ± 0.25
2	none	1.56 ± 0.03	3.09 ± 0.11
3	11 Ac-Glu	3.86 ± 1.10	0.23 ± 0.06
4	12 Asn	0.21 ± 0.05	0.07 ± 0.01
5	13 Glu	2.18 ± 0.39	0.88 ± 0.42
6	13 Val	0.30 ± 0.06	0.41 ± 0.05
7	15 Ile	0.57 ± 0.12	2.33 ± 0.29
8	15 Glu	3.23 ± 0.65	0.06 ± 0.01
9	14 Thr	0.70 ± 0.30	0.04 ± 0.01
10	16 Lys	2.37 ± 0.44	0.14 ± 0.02
11	16 Glu	n.d.[b]	n.d.[b]

[a]The residues are numbered according to their actual sequence in phosphorylase b.

[b]Not determined because of the low rate of phosphorylation.

RESULTS

The octapeptide (peptide number 1, Table I) was chosen to be a model for this specific study. Substitution of each amino acid residue in the octapeptide was made as shown in Table I. Acetylation of the octapeptide was done to neutralize the positive charge at the end of these peptides and to improve phosphorylation. Peptide number 2, an acetylated analog of peptide number 1, has the same K_m value but the V_m is increased 1.7-fold. Therefore, all the remaining peptides are acetylated. All peptides end in the sequence, GlyLeu. It was previously shown that peptides ending in arginine were poor substrates (6). The K_m and V_m values for these peptides are summarized in Table I.

Peptide number 3 whose $lysine_{11}$ was substituted by glutamic acid has 2.5-fold higher K_m value and 13.6-fold lower V_m value as compared with peptide

number 2. The positive charge in this position seems important. Peptide number 4 contains asparagine in place of glutamine. The side chain substituted in this peptide contains one less methylene group and suggests that a length of a side chain containing amide group is quite important for phosphorylase kinase recognition. The K_m and V_m values are, respectively, 7.6- and 47-fold lower than those of peptide number 2.

The importance of the hydrophobic amino acid surrounding $serine_{14}$ was examined using peptides in which the two hydrophobic groups were individually substituted by glutamic acid. When either $isoleucine_{13}$ or $valine_{15}$ was replaced by glutamic acid, both peptide number 5 and 8 were poorer substrates with higher K_m values and lower V_m values, compared with peptide number 2. Peptide number 5 is a better substrate than peptide number 8. Therefore, it is suggested that $valine_{15}$ might be more important than $isoleucine_{13}$. Also valine was changed to isoleucine in one peptide and $isoleucine_{13}$ replaced by valine in another peptide, peptide 6, to test the effect of two identical hydrophobic amino acids surrounding $serine_{14}$. Peptide number 6 with two valines surrounding $serine_{14}$ has a lower K_m value than peptide number 7. The increase in apparent binding affinity of peptide number 6 might be explained by a less bulky side chain of valine. However, the V_m value of peptide number 7 was higher than that of peptide number 6. The substitution of hydrophobic amino acids also has another important effect on the kinetics of the reaction. When $valine_{15}$ was substituted by glutamic acid, a linear double reciprocal plot was obtained (Fig. 1A). Linear kinetics were also seen with peptide 2 (data not shown), but when $valine_{15}$ was substituted by isoleucine, considerable substrate inhibition was evident. Similar substrate inhibition was seen with peptide 6 but not with peptide 5.

The threonine containing peptide (peptide number 9) was shown to be phosphorylated by nonactivated phosphorylase kinase. Although the phosphorylation rate was reduced 88-fold, the K_m value was lower as compared with the serine peptide (number 2). The reason for the lower K_m value is not understood.

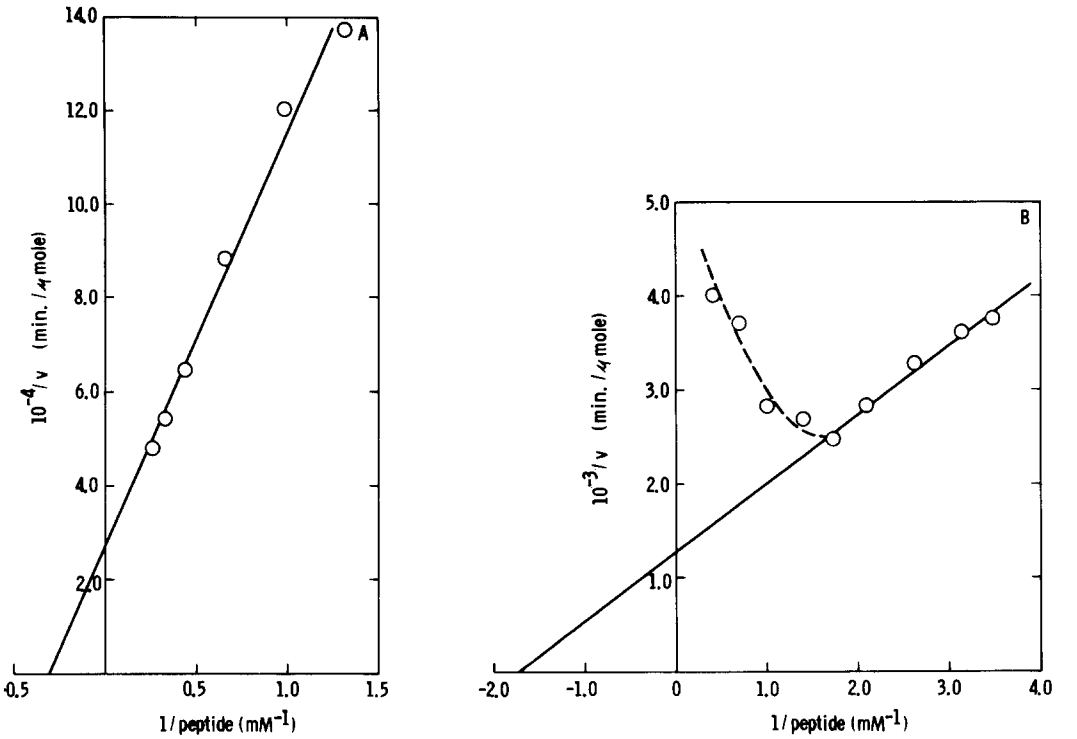

Figure 1. Lineweaver-Burk plot for peptide number 8 (A) and peptide number 7 (B). Assay conditions are listed in the section on experimental procedures. The final protein concentrations of the reaction mixture for peptides number 8 and 7 are 25.8 and 13.5 μg/ml, respectively. The linear lines were obtained by using intercepts and slopes calculated by a least square method using the Omnitab computer program.

The role of $arginine_{16}$ was tested by substituting it with another basic amino acid, lysine (peptide number 10) and an acidic glutamic acid (peptide number 11). Peptide number 10 is not a good substrate. Peptide 11 was phosphorylated as detected by high voltage electrophoresis. The rate of phosphorylation is very low, and it was not possible to determine kinetic parameters for this peptide. Therefore, the K_m and V_m values of this peptide are not reported. Although these studies show that arginine is important, it is clear that other residues are also important.

DISCUSSION

A synthetic tetradecapeptide and other peptides resembling the phosphorylase sequence have been used in studies of the specificity of phosphoryl-

ase kinase. Reduction of peptide size and substitution of amino acid residues were done (6). The first six residues of the tetradecapeptide, SerAspGlnGlu-LysArgLysGlnIleSerValArgGlyLeu, were determined to be unimportant. By substitution of arginine$_{16}$ in an octapeptide (lysine$_{11}$ to leucine$_{18}$) with glycine or alanine, it was found that arginine$_{16}$ is an important residue. Secondly, serine$_{14}$ must be adjacent to at least one hydrophobic amino acid on each side (6).

Study of the specificity of phosphorylase kinase presented here also indicates that hydrophobic residues are very very important. This was evident by effects of replacement of individual residues by glutamic acid and by peptides with two identical hydrophobic amino acid residues surrounding serine$_{14}$. Surprisingly, a peptide with two isoleucyl residues had a better catalytic reaction rate and lower K_m than a peptide with the sequence of muscle phosphorylase. The peptide with two isoleucyl residues is similar to the phosphorylated region of rabbit liver phosphorylase, which has an amino acid sequence of Arg-Gln-Ile-Ser(P)-Ile-Arg as determined by Wolf et al. (20). These results suggest that rabbit liver phosphorylase b might be a better substrate than rabbit muscle phosphorylase b. Arginine$_{16}$ is again found to be an important residue by substitution with lysine. The guanidino group itself might be important in addition to the presence of the positive charge. Tessmer et al. (6) showed that threonine peptides sequences 10 to 16 or 11 to 16 cannot be phosphorylated by nonactivated phosphorylase kinase. When threonine substituted serine$_{14}$, peptide 9, we found the peptide can be phosphorylated but poorly in comparison with the seryl analog. The peptides Tessmer et al. (6) used did not contain an acetyl group or residues 17 and 18. Glycine$_{17}$ and leucine$_{18}$ might be important in the enzyme recognition. Phosphorylase kinase is known to phosphorylate a threonyl site, Ala-Ile-Thr-Ala-Arg-Arg-Gln, found in troponin I (21). Troponin I, however, is not a good substrate for phosphorylase kinase (21) and no studies have been done with peptides of this sequence.

The K_m values for phosphorylase kinase with synthetic peptides reported here and elsewhere (6,22) are considerably higher than that reported for phosphorylase b but yet the V_m values are quite good (23). These differences suggest to us that phosphorylase kinase might require considerable structure in its substrate and that with peptide substrates some of the binding energy is utilized with these substrates to orient the peptide properly for reaction. Hence, higher K_m values are obtained. Since substitution of any of the six residues in the sequence, LysGlnIleSerValArg, affects the reaction, these results might be explained by important conformational differences in the free substrates or their conformations on the enzyme surface.

Some peptides in this study showed substrate inhibition at high concentration of substrate. In 1930, Haldane (23) explained substrate inhibition by a model as follows:

$$E + S \rightleftharpoons ES \xrightarrow{k_1} E + P$$

$$ES + S \rightleftharpoons ES_2 \xrightarrow{k_2} E + 2P$$

It is assumed that k_1 is greater than k_2. If phosphorylase kinase contained a second catalytic or regulatory site as suggested by studies with troponin I (24) and with effects of peptides on autophosphorylation (25,26), substrate inhibition could be explained by a similar model. Another possibility exists in that many of the peptides used are quite apolar. If aggregation of these substrates occurs at high concentration and these bind to the enzyme, but are acted upon more poorly than uncomplexed substrate, inhibition will occur.

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