THE PHOSPHORYLASE KINASE REACTION ON A PEPTIDE DERIVED FROM GLYCOGEN PHOSPHORYLASE*

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SUMMARY

The phosphorylation of the peptide, Ser Asp Gln Glu Lys Arg Lys Gln-Ile Ser Val Arg Gly Leu, at the seryl residue between isoleucine and valine by phosphorylase kinase is described. No phosphorylation occurs at the seryl residue at the amino terminus. With this peptide as a substrate, EGTA was found to inhibit enzyme activity and activity was restored by Ca^{++}. The ratio of activities at pH's (6.8/8.2) was higher than with phosphorylase b. The K_m was found to be 4×10^{-4} M and the catalytic efficiency is reduced from that observed with phosphorylase b. Arginine methyl ester was found to be a competitive inhibitor with respect to phosphorylase b suggesting that an arginyl residue in the sequence is important for substrate binding.

INTRODUCTION

Phosphorylase kinase, in response to various physiological stimuli, catalyzes the phosphorylation of phosphorylase b to make phosphorylase a. This reaction scheme which leads to glycogen degradation is controlled by a variety of factors, e.g., Ca^{++}, Mg^{++}, glycogen, and H^{+}. Since the substrate for phosphorylase kinase is a protein, control of the reaction by such factors can occur by binding to the substrate, enzyme, or both. In order to distinguish various mechanisms, an alternative substrate that does not bind

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modifier can be used. The action of phosphorylase kinase on a peptide containing the seryl residue phosphorylated in the conversion of phosphorylase b to a is the subject of this communication.

MATERIALS AND METHODS

Crystalline phosphorylase b was prepared according to the procedure of Fischer and Krebs (1). ^{32}P-labelled phosphorylase a was prepared using ^{32}P-ATP (2) with purified phosphorylase kinase (3). The isolation of a ^{32}P-labelled peptide was accomplished by digesting ^{32}P-labelled phosphorylase a with chymotrypsin, gel filtration on Sephadex G-25, and ion exchange chromatography on Dowex-50 (4). The phosphorylated peptide was dephosphorylated with purified bacterial alkaline phosphatase purchased from Worthington and the dephosphorylated peptide was obtained by chromatography on Dowex-50 using a linear gradient between 2 M pyridine adjusted to pH 5.0 with acetic acid and 8.5 M pyridine adjusted to pH 5.6 with acetic acid. Amino acid analysis showed that the peptide was pure and contained the amino acids of a tetradecapeptide whose sequence was previously determined (4).

Phosphorylation of the peptide by phosphorylase kinase was determined by an assay involving ion exchange chromatography. The kinase reaction mixtures (50 µl) were stopped by the addition of 200 µl of pyridine-acetate buffer, pH 3.1. This was applied to a small column of Dowex-50 (approximately 0.5 ml resin) in a Pasteur pipet. The column was then washed with 4 mls of the pyridine-acetate buffer, pH 3.1 and 1 ml of pyridine-acetate buffer, pH 5.0 to remove ^{32}P-ATP. High voltage electrophoresis of the eluate showed that no phosphorylated peptide was removed. The phosphorylated peptide was eluted by 2 mls of 4 N NH_4OH and was detected by scintillation counting in Bray's solution. The conversion of phosphorylase b to a was measured by the incorporation of ^{32}P from ^{32}P-ATP (5) or by measurement of enzyme activity (3).

RESULTS AND DISCUSSION

Incubation of phosphorylase kinase with the tetradecapeptide, Ser Asp-

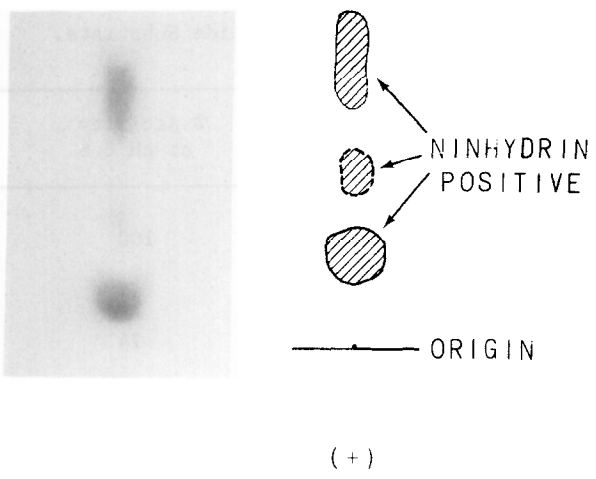

Figure 1. Electropherogram of trypsin digested ^{32}P-phosphorylated peptide and authentic phosphorylated peptide obtained from phosphorylase a. Electrophoresis was for one hour at 2000 V. Radioactive components were located by radioautography and peptides by their reaction with ninhydrin.

Gln Glu Lys Arg Lys Gln Ile Ser Val Arg Gly Leu, and ^{32}P-ATP results in an incorporation of ^{32}P as previously indicated (4). In the conversion of phosphorylase b to phosphorylase a, phosphorylation occurs in the protein at the seryl residue between isoleucine and valine. In order to determine that this residue is the site of phosphorylation in the reaction with the peptide substrate, the phosphorylated product was compared with the phosphorylated peptide derived directly from phosphorylase a. Figure 1 shows the results of tryptic digestion of these two peptides. Radioautography showed that the ^{32}P components obtained from the peptide experiment corresponded exactly with ninhydrin staining materials obtained from the tryptic digestion of peptide from phosphorylase a. If the seryl residue at the amino terminus or if both seryl residues were phosphorylated, different or additional ^{32}P components should have been seen. In a separate experiment, the phosphorylation of the n-terminal seryl residue was attempted using phosphorylated peptide derived from phosphorylase a. No phosphorylation could be detected. This result along with the tryptic finger-

Table I. Effect of pH, EGTA, and Ca^{++} on Phosphorylase Kinase Activity with a Peptide Substrate.

Additions	% Activity at pH 8.6
None	100
10^{-3} M EGTA	7
10^{-3} M EGTA + 10^{-3} M Ca^{++}	74
pH 6.8	18
	50*

* The kinase used in this experiment was activated by partial digestion with trypsin (8).

prints suggest that the high degree of specificity of the phosphorylase kinase reaction is retained on a low molecular weight substrate.

The effect of various effectors on the phosphorylase kinase reaction was tested using the peptide substrate. Before doing this it was established that at pH 8.6, the formation of product was linear up to 30 minutes of reaction and was proportional to enzyme concentration from 0.5 to 2 μg/ml. In Table I, it can be seen that the chelating agent, EGTA, is a potent inhibitor of inactive phosphorylase kinase and that a significant recovery of enzymic activity occurs upon addition of an equimolar amount of Ca^{++}. The specific effect of EGTA and Ca^{++} on phosphorylase kinase (6,7) demonstrates that phosphorylation of the peptide is occurring by a reaction mediated by phosphorylase kinase. The activity at pH 6.8 was 18% of that obtained at 8.6 which is significantly higher than that seen with the substrate, phos-

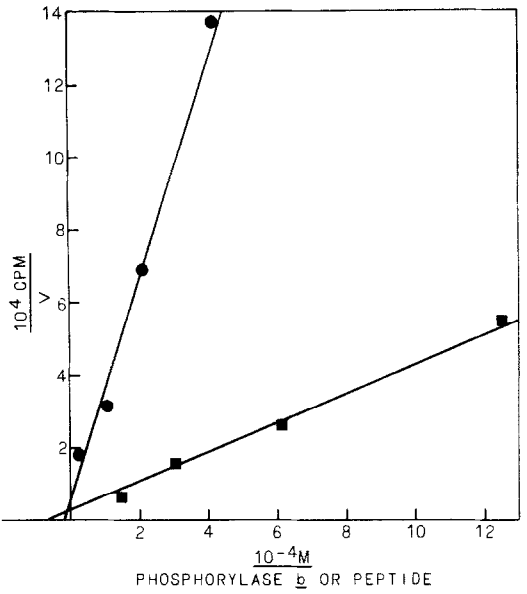

Figure 2. Reciprocal plot for peptide and phosphorylase b in the phosphorylase kinase reaction. The reaction mixtures contained inactive phosphorylase kinase (2 μg/ml), 6 mM Mg^{++}, 1.8 mM ^{32}P-ATP, 25 mM GP - 25 mM Tris, pH 8.6, and varying amounts of peptide (●) or phosphorylase b (■). The reaction was for 30 minutes at 30° C. Incorporation of ^{32}P was determined as described in Methods.

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phorylase b. Our preparations of phosphorylase kinase at pH 6.8 showed 5% of the activity seen at pH 8.6. In a recent report, Krebs et al. demonstrated that phosphorylase kinase can phosphorylate troponin and in this case the ratio of activities (6.8/8.2) was also higher than with phosphorylase b. Also shown in the table are results obtained with phosphorylase kinase activated by trypsin (8). This treatment increases the ratio of activities (6.8/8.2) with phosphorylase b. Our preparation showed a ratio of 0.7 with phosphorylase b as a substrate and 0.5 with the peptide. The significance of the differences in activities of untreated phosphorylase kinase at the two pH's for the protein and the peptide is not understood. Since changes in activities occur upon activation, the peptide will be useful in studying the catalytic properties of the different molecular forms of phosphorylase kinase.

A comparison of the effectiveness of the peptide to serve as a substrate relative to phosphorylase b was undertaken. Figure 2 shows double reciprocal

plots of the kinetic experiments. With the peptide or phosphorylase b, less than 5% of the starting material was utilized. A K_m of 4 x 10^{-4} M was obtained for the peptide and 1.6 x 10^{-4} M for phosphorylase b using a molecular weight of 100,000 for the monomeric form. The K_m for phosphorylase b agrees approximately with a previously reported value (8). Similar results for K_m were obtained for the peptide and the protein in an additional experiment. The ratio of maximal velocities in two experiments were compared and it was found that the catalytic efficiency is lower with the peptide by a factor of 4 to 9. These data therefore, show that the tetradecapeptide is a reasonably good substrate for phosphorylase kinase.

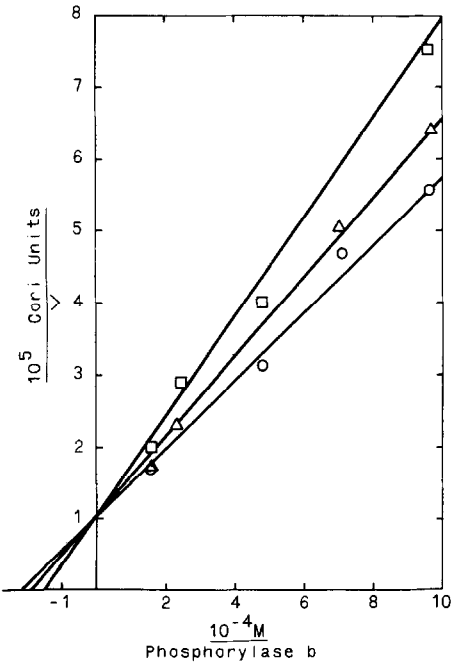

Figure 3. Reciprocal plot for phosphorylase b in the presence of arginine methyl ester. The reaction mixtures contained inactive phosphorylase kinase (0.5 µg/ml), 8.6 mM Mg^{++}, 2.6 mM ATP, 36 mM GP - 36 mM Tris, pH 8.6 and varying amounts of phosphorylase b. No additions, open circles; with 5.7 mM arginine methyl ester, open triangles, with 11.4 mM arginine methyl ester, open squares. Velocity was measured by phosphorylase a formation (3).

It had been previously shown that the phosphorylated tetradecapeptide is a good substrate for phosphorylase phosphatase (4,9) and that the arginyl residue is essential for the action of phosphorylase phosphatase (10). In

order to determine whether phosphorylase kinase has a similar specificity, the action of arginine methyl ester, a competitive inhibitor of phosphorylase phosphatase (11), was tested with phosphorylase kinase. At 10 mM arginine methyl ester and 4 mM peptide, 50% inhibition was obtained. In order to ascertain the nature of the inhibition, further studies were conducted with the more readily available substrate, phosphorylase b. The results of Fig. 3 show that arginine methyl ester is a weak competitive inhibitor with respect to phosphorylase b. A K_i of 33 mM was determined. Thus, it appears that both phosphorylase kinase and phosphorylase phosphatase have some similarity in specificity. Both of these enzymes have a high degree of specificity and are perhaps related to the contribution the arginyl residue has in this sequence for substrate binding. The synthesis of different seryl peptides and the action of phosphorylase kinase on these should provide a test for this hypothesis and give some interesting information on the regulatory and catalytic properties of phosphorylase kinase.

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