Regulation of Glycogen Phosphorylase

ROLE OF THE PEPTIDE REGION SURROUNDING THE PHOSPHOSERINE RESIDUE IN DETERMINING ENZYME PROPERTIES*

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SUMMARY

A phosphopeptide which contains 14 residues including phosphoserine and which is derived from the NH₂-terminal region of skeletal muscle glycogen phosphorylase (NOLAN, C., NOOVA, W. R., KEERS, E. G., AND FISCHER, E. H. (1964) Biochemistry 3, 542–551) has been shown to induce the enzymic properties of phosphorylase a in phosphorylase b and b'. When phosphorylase b is incubated with the phosphorylated tetradecapeptide, the following changes occur: (1) the enzyme becomes partially catalytically active in the absence of AMP; (2) the allosteric interactions of the enzyme are altered, as evidenced by the fact that phosphorylase b does not bind AMP cooperatively, and is no longer inhibited by glucose-6-P; and (3) the enzyme, normally present as a dimer, associates to a tetramer. Phosphorylase b' is a modified form of phosphorylase in which the phosphorylation site has been removed by limited tryptic attack. In the presence of phosphopeptide, 86% of the total enzyme activity can be induced in the absence of AMP. The properties of phosphorylases b and b' with phosphopeptide, cited above, are all characteristics of the phosphoenzyme, phosphorylase a. In addition, evidence is presented that these effects are specific. They are not the result of the polycationic nature of the peptide since they cannot be duplicated by spermine, and the phosphate group must also be present for the peptide to effect changes on the enzyme.

One of the well known events of biochemical control in skeletal muscle is the phosphorylation of a single seryl residue per mono-

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carried out previously. For instance, the catalytic properties of ribonuclease A can be restored by mixing ribonuclease B with the NH$_2$-terminal S-peptide$^2$ either derived from the enzyme itself (17) or prepared synthetically (18). A recent report has shown that a synthetic COOH-terminal tetradecapeptide can fully reactivate ribonuclease A from which the last six amino acids had been removed (19). The phosphopeptide described here is different from ribonuclease peptides in one significant aspect in that it does not comprise the active site of phosphorlyase. Rather, it is concerned with the regulatory properties of the enzyme.

MATERIALS AND METHODS

Phosphorylase $b$ was isolated from mature rabbit skeletal muscle (Pel-Freez Biologicals, Inc.) according to the method of Flesher and Krebs (30). All preparations of the enzyme were recrystallized three times and were treated twice with acid-washed Norit (1 mg/mg of protein). The resulting enzyme had a specific activity of 58.0 to 41.0 units/mg under the 10 mM glucose-1-P assay condition of Illingworth and Cori (21). $^{32}$PPhosphorylase a (22) was prepared from $[y-^{32}$P]ATP (23) and phosphorylase $b$ kinase (24, 25), a gift of Mr. Gerald M. Carlson of this laboratory. The completeness of this reaction was confirmed by equivalence of the specific radioactivity of ATP and phosphorylase a, and an enzyme activity ratio of 0.78 in the absence and presence of AMP (26, 27). Phosphorylase $b'$ was prepared from $[^{32}$P]phosphorylase a by limited attack with trypsin in the presence of glucose (28). A residual amount of 5 to 6% phosphorylase $b$ was determined by enzyme activity without AMP and by the specific radioactivity of the phosphorylase $b'$ solution. The specific activity of the phosphorylase $b'$ preparations ranged from 39 to 41 units/mg measured as indicated above (21). The $[^{32}$P]phosphotetradecapeptide was isolated after digestion of $[^{32}$P]phosphorylase a by chymotrypsin (14, 29). Amino acid composition, used as a criterion of purity, was determined by amino acid analysis on a Beckman model 120C analyzer after hydrolysis for 24 hours in constant boiling HCl.

The phosphopeptide was dephosphorylated according to a published procedure (30). Inosine monophosphate (IMP) and AMP were obtained from Sigma. Glucose-1-P diopotassium salt and spermine tetrahydrochloride were purchased from Calbiochem. Shellfish glycogen was obtained from Sigma and was purified further by the method of Anderson and Graves (31). All other reagents were of the highest purity obtainable commercially.

Phosphorylase was assayed in the direction of glycogen synthesis (21). Units of activity is the amount of enzyme which will release 1 mmol of Pi from glucose-1-P per min. The amount of P, was determined by the Fiske-Subbarow method (32); for reaction mixtures of 0.8 ml, the assay was scaled down appropriately. Incubations were stopped by the addition of the ammonium molybdate-sulfuric acid reagent; in experiments in which a peptide was present, the "stopping reagent" was made 12% in dimethylosulfoxide. Color was measured at 750 nm in the spectrophotometer. For studies in which AMP-independent enzyme activity was measured, both phosphorylase $b$ and phosphorylase $b'$ were dialyzed against 40 mM sodium $\beta$-glycerol-1-P buffer (pH 6.8) containing 30 mM $\beta$-mercaptoethanol, the enzyme was treated twice with acid-washed Norit (1 mg/mg of protein) and dialyzed again. The amount of AMP remaining in phosphorylase $b$ was measured to be 0.05 nmol/mg of enzyme. The residual AMP in phosphorylase $b'$ was determined to have an upper limit of 0.2 nmol/mg of enzyme. The apparent difference in the amounts of AMP independence enzyme activity which we had observed in the absence of AMP might be explained by an action of the phosphopeptide to increase the binding of small amounts of AMP present with the enzyme. As described under "Materials and Methods," the phosphorylase $b$ solution contained 0.005 nmol of AMP/nmol of enzyme (assuming a molecular weight of 10$^4$ per monomer). Also, Table II shows that amounts of AMP less than 10$^{-7}$ M added in the presence of 1.5 mM phosphorylase give no additional activation of enzyme activity which we had observed in the absence of AMP.

Radiochemical assays were performed by liquid scintillation counting with Bray's solution (34) with the use of a Packard Tri-Carb (model 3320) liquid scintillation spectrometer. Sedimentation velocity experiments were carried out with a Spinco model E analytical ultracentrifuge. Sedimentation coefficients were measured with the aid of a Nikon model 6C microcomparator and were corrected for viscosity of the buffer to water at 20°C.

RESULTS

Effect of Phosphopeptide on Phosphorylase $b$—We have examined the capacity of the phosphorylated tetradecapeptide to induce enzyme activity in the absence of AMP. Incubation of phosphorylase $b$ with phosphopeptide at 10$^{-6}$ leads to induction of 10% of the total enzyme activity as shown in Table I. This activation appears to be temperature-dependent since little or no activity can be observed at 30°C (not shown). Phosphorylase $b$ surprisingly reveals similar induction of enzyme activity in the presence of spermine at the low temperature (Table I). In a separate experiment, we confirmed that a concentration of 12 mM spermine is saturating, which had been reported previously (35). When phosphopeptide and spermine are added to the enzyme together, the amount of activation is found to be additive (Table I).

In interpreting these results, an important question arises. In early studies (36), Krebs had shown that the catalytic activity of phosphorylase $b$ was increased considerably with polycations such as saline and polylysine when AMP was present in limiting amounts. Kinetic analyses had demonstrated that saline increased the binding of AMP for phosphorylase $b$. Some time later Wang et al. (35) observed that polyamines spermine, spermidine, and putrescine in the presence of either AMP or IMP could activate phosphorylase $b$ but not phosphorylase $a$. In view of these studies and the fact that the phosphotetradecapeptide is a polycation, we recognized the possibility that the induction of enzyme activity which we had observed in the absence of AMP might be explained by an action of the phosphopeptide to increase the binding of small amounts of AMP present with the enzyme. As described under "Materials and Methods," the phosphorylase $b$ solution contained 0.005 nmol of AMP/nmol of enzyme (assuming a molecular weight of 10$^4$ per monomer). Also, Table II shows that amounts of AMP less than 10$^{-7}$ M added in the presence of 1.5 mM phosphorylase give no additional activation of enzyme activity. This concentration of AMP is 10-fold higher than the levels of residual AMP carried into the reaction mixture.

TABLE I

<table>
<thead>
<tr>
<th>Additions to reaction</th>
<th>$P_i$ formed (nmol/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.02</td>
</tr>
<tr>
<td>Phosphopeptide (1.5 mM)</td>
<td>0.13</td>
</tr>
<tr>
<td>Spermine (12.5 mM)</td>
<td>0.15</td>
</tr>
<tr>
<td>Phosphopeptide (1.5 mM) + spermine (12.5 mM)</td>
<td>0.32</td>
</tr>
<tr>
<td>AMP (1.5 mM)</td>
<td>1.05</td>
</tr>
</tbody>
</table>

1 Ribonuclease $S$ refers to subtilisin-modified beef ribonuclease $A$.

2 S-peptide is the peptide obtained from ribonuclease $S$, and is believed to be a mixture of three peptides, probably S-peptide$^{1-20}$, S-peptide$^{1-31}$, and S-peptide$^{1-42}$ (16).
Table II

Lower limit of AMP required to activate phosphorylase b with phosphopeptide

Reaction mixtures contained 7.3 units/ml (approximately 2.4 X 10^{-4} m) of phosphorylase b, 16 mM glucose-1-P, 1% glycogen, 14 mM sodium β-glycerol-P buffer (pH 6.8), 0.2 mM EDTA, and 0.3 mM β-mercaptoethanol in a volume of 0.08 ml. Other components were added as indicated. The reaction was initiated by the addition of enzyme, and was conducted at 10°C for 10 min (1 to 2 min for the mixture containing AMP). The amount of P_i formed was assayed as described under "Materials and Methods." 

<table>
<thead>
<tr>
<th>Additions to reaction</th>
<th>P_i formed µmol/10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.02</td>
</tr>
<tr>
<td>Phosphopeptide (1.5 mm)</td>
<td>0.12</td>
</tr>
<tr>
<td>Phosphopeptide (1.5 mm) + AMP (0.3 X 10^{-3} m)</td>
<td>0.11</td>
</tr>
<tr>
<td>Phosphopeptide (1.5 mm) + AMP (0.9 X 10^{-3} m)</td>
<td>0.12</td>
</tr>
<tr>
<td>Phosphopeptide (1.5 mm) + AMP (2.8 X 10^{-3} m)</td>
<td>0.16</td>
</tr>
<tr>
<td>Phosphopeptide (1.5 mm) + AMP (8.3 X 10^{-3} m)</td>
<td>0.25</td>
</tr>
<tr>
<td>AMP (1 mm)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* The concentration of residual AMP present with the enzyme is estimated to be 1.2 X 10^{-4} m.

Table III

Influence of phosphorylated tetradecapeptide on inhibition of phosphorylase b by glucose-6-P

The reaction mixture consisted of 0.018 mg/ml of phosphorylase b, 15 mM glucose-1-P, 0.9 mM AMP, 0.9% glycogen, 19 mM sodium β-glycerol-P buffer (pH 6.8), and 14 mM L-cysteine-HCl in a volume of 0.425 ml. Glucose-6-P and phosphopeptide were present as indicated. Reactions were initiated by the addition of enzyme or enzyme containing glucose-6-P, and were carried out at 30°C for 5 min. The amount of P_i, formed was assayed as described under "Materials and Methods." 

<table>
<thead>
<tr>
<th>Additions</th>
<th>Percent enzymic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Glucose-6-P (7.5 mm)</td>
<td>50</td>
</tr>
<tr>
<td>Peptide (0.12 mm)</td>
<td>77</td>
</tr>
<tr>
<td>Peptide (0.24 mm)</td>
<td>92</td>
</tr>
<tr>
<td>Peptide (0.48 mm)</td>
<td>100</td>
</tr>
</tbody>
</table>

by the enzyme. The importance of an AMP contamination having thus been excluded, we conclude from Table I that both the phosphopeptide and spermine can induce AMP-independent enzyme activity in phosphorylase b. Since an optimal concentration of spermine was present, these results further suggest that the phosphorylated tetradecapeptide and spermine probably act differently to effect activation.

Phosphorylase b is recognized as an allosteric enzyme. This enzyme is inhibited by glucose-6-P and binds AMP cooperatively. On the other hand, phosphorylase a is desensitized with respect to glucose-4-P, and exhibits a hyperbolic saturation curve for the binding of AMP. The data presented in Table III show that the phosphopeptide reverses the inhibition of phosphorylase b caused by glucose-6-P. At a peptide concentration of 0.48 mM, inhibition is completely released. To obtain additional information for the effect of the phosphopeptide on the allosteric interactions of phosphorylase b, we studied the binding of AMP. The Lineweaver-Burk plot presented in Fig. 1 shows the expected cooperative binding of AMP on phosphorylase b. When the phosphopeptide is added at the low concentration of 0.12 mM, however, the kinetics change to simple Michaelis-Menten type (Fig. 1). Further, Wang et al. (35) have shown that spermine did not alter the homotropic interactions with respect to AMP. We have found this to be true at low concentrations of spermine. Nevertheless, at a concentration of 2 mM, we have observed that spermine does remove the cooperative binding of AMP (not shown). Therefore, the loss of allosteric cooperativity of phosphorylase b, induced by the phosphopeptide, is not unique. The low concentration required suggests an efficiency of the phosphopeptide in effecting this change, however. Together with the reversal of the glucose-6-P inhibition, this evidence is consistent with the idea that the binding of phosphopeptide to phosphorylase b leads to formation of an enzyme species which, like phosphorylase a, is devoid of these allosteric interactions.

At protein concentrations used in ultracentrifugation, phosphorylase b and a sediment as a dimer and a tetramer, respectively. These two forms correspond to s_20,w values of 8.8 and 13.5 (37–39). In the presence of the nucleotide activator, AMP the dimer = tetramer equilibrium of phosphorylase b is displaced in favor of the tetrameric form. In order to determine the effect of the phosphorylated tetradecapeptide on this equilibrium, ultracentrifugal experiments were carried out. As presented in Fig. 2A, the native enzyme at 9°C sediments as a dimer with s_20,w of 7.9 S (upper curve). Incubation of the enzyme with phosphopeptide leads to a pronounced shift from the dimer to the tetramer. The s_20,w were 8.0 and 12.9 S for the slow and fast moving components, respectively (Fig. 2A, lower curve). Yet, when the phosphate group is removed from the peptide by alkaline phosphatase, the peptide loses its ability to affect the dimer = tetramer equilibrium. The single sedimenting species shown in Fig. 2B, lower curve, has an s_20,w of 8.9 S equivalent to that of the native enzyme itself, s_20,w of 8.8 S (Fig. 2B, upper curve). An identical result was observed with the use of 5 mM spermine (Fig. 2C, lower curve). It should be noted that these studies were carried out at 11°C. At 23°C, phosphorylase b sedimented as a dimer, s_20,w 8.1 S regardless of whether the phosphopeptide was present (Fig. 2D, lower curve) or not (upper curve). From this evidence
we conclude that the phosphorylated tetradecapeptide effects a change in enzyme structure which allows tetramer formation to occur. In addition, these sedimentation velocity studies show that the effect of phosphopeptide on the polymeric nature of the enzyme is temperature-dependent, is specific for the presence of the phosphate group, and cannot be duplicated by spermine.

Effect of Phosphopeptide on Phosphorylase b'—Under the assumption that the peptide region at the NH₂ terminus containing the phosphorylation site possibly could compete with the phosphopeptide for binding, we prepared phosphorylase b', a form of the enzyme in which this region has been removed. Phosphorylase b' was tested for AMP-independent enzyme activity at various concentrations of the phosphorylated tetradecapeptide. The results are presented in Table IV. As shown, addition of phosphopeptide to phosphorylase b' results in activation of the enzyme up to 86% of the amount observed with AMP. Clearly, the absence of the peptide fragment at the NH₂ terminus has permitted considerably greater activation by the phosphopeptide. Again, these results were obtained at 10°C; at higher temperatures, the capacity of the phosphorylated tetradecapeptide to activate was reduced (not shown). In addition, the induction of enzyme activity without AMP required the use of the tetradecapeptide in its phosphorylated form. Incubation of phosphorylase b' with 1.5 mM dephosphopeptide under the same conditions described in Table IV results in no activation whatever.

To rule out the possibility that residual AMP present with the phosphorylase b' preparation might be in part or totally responsible for the results presented in Table IV, we performed an experiment similar to that described in Table II. The results indicated that, at a concentration less than 0.8 × 10⁻⁶ M, AMP gave no enhancement of the activation by phosphopeptide. The upper limit of AMP present (see under "Materials and Methods") was determined to be 0.02 nmol/nmol of enzyme (assuming a molecular weight of 10⁸ per monomer). Therefore, the upper limit of AMP present with the enzyme is 3 times lower (Table IV) than that amount found to affect activation.
which occurs upon phosphorylation of the intact enzyme, phosphorylase b, by phosphorylase kinase and ATP.

**DISCUSSION**

The data reported here show that a phosphopeptide of 14 residues containing a phosphate ester of serine and derived from the NH2-terminal region of phosphorylase modifies the regulatory properties of phosphorylase b and b'. Incubation with the phosphopeptide influences a change in enzyme structure which leads to (a) desensitization of allosteric interactions toward AMP and glucose-6-P; (b) establishment of proper residue contacts to allow association of the enzyme; and (c) induction of AMP-independent enzyme activity. Based on these experimental results, we suggest that the phosphotetradecapeptide binds to phosphorylase b and phosphorylase b' at a specific site on the surface of the enzyme normally occupied by the homologous region of the enzyme after phosphorylation by phosphorylase kinase. Because of the accessibility of the phosphopeptide region on the protein as a substrate for phosphorylase phosphatase and the ease with which it can be cleaved by trypsin, it has long been thought that this region at the NH2 terminus is exposed (4). In addition, we believe that the interaction of the phosphopeptide is likely to be through noncovalent binding.

**TABLE V**

**Effect of phosphopeptide and spermine on IMP-dependent activity of phosphorylase b**

<table>
<thead>
<tr>
<th>Addition to reaction</th>
<th>P; produced (µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.34</td>
</tr>
<tr>
<td>Phosphopeptide (0.17 mM)</td>
<td>1.53</td>
</tr>
<tr>
<td>Spermine (2.5 mM)</td>
<td>0.39</td>
</tr>
<tr>
<td>Phosphopeptide (0.17 mM) + spermine (2.5 mM)</td>
<td>1.45</td>
</tr>
</tbody>
</table>

The ability of phosphorylase to respond to the phosphopeptide for the association reaction and the induction of catalytic activity was observed to be dependent on temperature. Association of phosphorylase b (Fig. 1) in the presence of phosphopeptide could be demonstrated at 9° but not at 23°. In contrast to the results obtained at 10°, the induction of enzyme activity was reduced in magnitude at 30°. We interpret this observation to suggest that at higher temperatures the enzyme is being interconverted in equilibria between different conformational states, which either cannot bind the phosphopeptide, or cannot be influenced by it. At low temperature, we postulate, a structure of the enzyme is favored which can be influenced by the phosphopeptide to permit association and induction of AMP-independent activity.

The specificity of the interaction of the phosphopeptide with phosphorylase is emphasized by the fact that the phosphate group must be present for these effects to occur. The dephosphorylated tetradecapeptide was totally inactive in inducing enzyme activity in phosphorylase b' as well as in affecting the dimer = tetramer equilibrium in phosphorylase b. Also, spermine was shown to exert its effect differently from that of phosphopeptide (Table I). Thereby, we suggest that spermine binds at some site on the enzyme other than that occupied by the phosphopeptide. Further, because of its inability to activate phosphorylase b' with respect to AMP or IMP, Wang et al. (35) has proposed that spermine, in its activation of phosphorylase b to these nucleotides, does not bind to the site normally occupied by the polycationic NH2-terminal region. The uniqueness of the phosphopeptide effect is, therefore, seen in its capacity to activate phosphorylase b' to IMP (Table V) and in the absence of AMP (Table IV).

We tentatively interpret our results according to Scheme 1. The NH2-terminal region of phosphorylase b (PHOS b) is depicted as extending within the circle to show that this region is surface-exposed; the notch in the line refers to the β-hydroxyl group of the seryl residue which becomes phosphorylated by phosphorylase b kinase (PBK). Phosphorylation (upper path) results in the formation of phosphorylase a (PHOS a) which is shown as a dimer. This dimer undergoes a change in conformation (from circles to squares) and in turn associates to a tetramer. It should be pointed out that tetramer formation is not a prerequisite for catalytic activity independent of AMP since the
mon theme in terms of protein structure required for the change in the properties of phosphorylase a. This form of the enzyme then undergoes a conformational change. This conformational change may arise from the fact that in the non-phosphorylated state certain regions of the enzyme may be denied proximity because they are repelled by a charge effect. When this charge is partially neutralized by the introduction of a phosphate group, as proposed by Fischer et al. (4), the conformation change takes place. Such a change in enzyme structure leads to the enzyme properties of phosphorylase a.

Glycogen phosphorylase is only one of a growing number of enzymes (e.g. glycogen synthase, pyruvate dehydrogenase complex, acetyl-CoA carboxylase) which are now known to be regulated by it may displace the corresponding dephosphorylated region of the protein to form a species of enzyme which we call “pseudo phosphorylase a” (PEUSO-PHOS a). This form of the enzyme lower path), we propose that the phosphate (42-44). It remains to be seen if there is some com-plexes (e.g. glycogen synthase, pyruvate dehydrogenase complex, acetyl-CoA carboxylase) which are now known to be regu-

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