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., NOVA, 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 monomer of glycogen phosphorylase b by phosphorylase kinase (2-4). The formation of the phosphoenzyme, phosphorylase a, leads to an enzyme form which has increased stability (5, 6), has a high affinity for substrate (7, 8), does not respond to the allosteric effectors glucose-6-P (9), and does not bind AMP cooperatively (10). Upon conversion to phosphorylase a, phosphorylase b, normally present as a dimer, undergoes association to a tetramer (11) and loses the requirement of AMP for catalytic activity (12). Also, the addition of AMP to phosphorylase b tends to promote a form of the enzyme with properties resembling those described above for phosphorylase a (5, 6). These alterations in the enzymatic properties of phosphorylase a, preferentially in forms of the enzyme which are not present. It is known that the site of phosphorylation is at a serine residue 14 amino acids from the NH₂ terminus of the protein (13). We have undertaken experiments using a phosphorylated tetradecapeptide obtained by proteolytic digestion of phosphorylase b. The phosphorylated tetradecapeptide is derived from amino acids beginning at the 5th residue from the NH₂ terminus (13) and is released from phosphorylase a by chymotryptic attack (14). The peptide is highly basic, contains the important serine residue, and has the following sequence: Ser-Asp-Glu-Lys-Arg-Lys-Gln-Ile-Ser(p)-Val-Arg-Gly-Leu (14). In addition to phosphorylase b, we have used a modified form of the enzyme called phosphorylase b'. Phosphorylase b' is prepared by limited digestion of phosphorylase a by trypsin, a treatment which removes a peptide segment near the NH₂ terminus containing the phosphorylated serine residue (4). One of the products has been identified as the heptapeptide Lys-Gln-Ile-Ser(p)-Val-Arg (4). Although nonlabeled peptides result from the tryptic attack on [³²P]phosphorylase a, the extent of cleavage is believed to be small since 95% of the protein still precipitates in trichloroacetic acid (4). Like phosphorylase b, phosphorylase b' is catalytically active in the presence of AMP (15).

In this communication, we report results which show that the phosphorylated tetradecapeptide can induce the enzymatic properties of phosphorylase a in phosphorylase b and phosphorylase b'. Studies such as these in which a protein molecule is reconstituted with a small complementary peptide segment have been
carried out previously. For instance, the catalytic properties of ribonuclease A can be restored by mixing ribonuclease B with the NH₂-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 effectively reivate 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 phosphorylase.

RATHER, it is concerned with the regulatory properties of the enzyme.

MATERIALS AND METHODS

Phosphorylase b was isolated from mature rabbit skeletal muscle (Pel-Freeze Biologicals, Inc.) according to the method of Fiegener and Krebs (20). 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). [³²P]Phosphorylase a (22) was prepared from [γ-³²P]ATP (23) and phosphorylase b kinase (24, 25), a gift from Mr. Gerald M. Carson of this laboratory. The completion of this reaction was confirmed by equivalence of the specific radioactivity of ATP and phosphorylase a, and an enzyme activity ratio of 0.75 in the absence and presence of AMP (26, 27). Phosphorylase b' was prepared from [³²P]phosphorylase a by limited attack with trypsin in the presence of glucose (28).

A residual amount of 5 to 6% phosphorylase a 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 [³²P]phosphotetradecapeptide was isolated after digestion of [³²P]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 dipotassium 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 commerically.

Phosphorylase was assayed in the direction of glycogen synthesis (32). AMP-independent activity is the amount of enzyme which will release 1 μmol of Pi from glucose-1-P per min. The amount of P, was determined by the Finke–Subballow method (32); for reaction mixtures of 0.08 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 dimethylsulfoxide. 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 in the presence of spermine tetrahydrochloride which 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 commerically.

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° 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° (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 μM 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 like salmine and polylysine when AMP was present in limiting amounts. Kinetic analyses had demonstrated that salmine 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 105 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 phosphorylase b. This concentration of AMP is 10-fold higher than the level of residual AMP carried into the reaction mixture.
The enzyme is inhibited by glucose-6-P and binds AMP cooperatively. On the other hand, phosphorylase a is desensitized with respect to glucose-6-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 \( \rightleftharpoons \) tetramer equilibrium of phosphorylase b is displaced in favor of the tetrameric form. In order to determine the effect of the phosphopeptide tetradecapeptide on this equilibrium, ultracentrifugal experiments were carried out. As presented in Fig. 2A, the native enzyme at 9° 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 \( s_{20,w} \) values 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 \( \rightleftharpoons \) 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, \( s_{20,w} \) 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°. At 23°, 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

### Table II

**Lower limit of AMP required to activate phosphorylase b with phosphopeptide**

<table>
<thead>
<tr>
<th>Additions to reaction</th>
<th>AMP 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

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

The reaction mixture consisted of 0.018 mg/ml of phosphorylase b, 16 mM glucose-1-P, 1% 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° for 5 min. The amount of Pi 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>109</td>
</tr>
</tbody>
</table>
FIG. 2. Ultracentrifugal patterns of phosphorylase b. A, with phosphopeptide at 9°. Enzyme (4.0 mg/ml) in 50 mM sodium β-glycerol-P buffer (pH 6.8) containing 50 mM β-mercaptoethanol. Upper curve, no additions; lower curve, with 1 mM phosphopeptide. The photograph was taken 50 min after attainment of full speed at 52,000 rpm. B, with dephosphopeptide at 11°. Upper curve, enzyme (4.7 mg/ml) in 40 mM sodium β-glycerol-P buffer (pH 6.8) containing 30 mM β-mercaptoethanol. Lower curve, enzyme (5.4 mg/ml) plus 1 mM peptide under the same conditions. The photograph was taken 50 min after attainment of full speed at 52,000 rpm.

C, with spermine at 11°. Upper curve, enzyme (4.5 mg/ml) in 40 mM sodium β-glycerol-P buffer (pH 6.8) containing 30 mM β-mercaptoethanol. Lower curve, enzyme (5.5 mg/ml) plus 5 mM spermine under the same conditions. The photograph was taken 52 min after attainment of full speed at 60,000 rpm. D, with phosphopeptide at 23°. Same conditions as described in Fig. 2A. The photograph was taken 52 min after attainment of full speed at 52,000 rpm.

TABLE IV

AMP-independent activation of phosphorylase b' as function of phosphopeptide concentration

<table>
<thead>
<tr>
<th>Phosphopeptide</th>
<th>Per cent total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>8.3</td>
</tr>
<tr>
<td>0.6</td>
<td>21.0</td>
</tr>
<tr>
<td>1.1</td>
<td>46.5</td>
</tr>
<tr>
<td>1.7</td>
<td>63.0</td>
</tr>
<tr>
<td>2.2</td>
<td>86.0</td>
</tr>
</tbody>
</table>

\[ a \] The amount of AMP remaining with the enzyme was estimated to be at an upper limit of 2.6 × 10^{-8} M.

\[ b \] 100% = activity in presence of 1 mM AMP.

The individuality of the effect exerted on the enzyme by phosphopeptide is further emphasized by the response of phosphorylase b' to activation in the presence of IMP. Wang et al. (35) first reported, and we have confirmed here (Table V) that spermine is inactive in enhancing IMP-dependent activity in phosphorylase b'. On the other hand, the activation of phosphorylase b' to IMP is enhanced by the tetradecapeptide as shown in Table V. No further activation is observed with spermine and phosphopeptide together.

On the basis of these results, we conclude that when the phosphopeptide binds to its complementary region on the protein, phosphorylase undergoes a structural change similar to that
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**

<table>
<thead>
<tr>
<th>Addition to reaction</th>
<th>P₂ 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
dimer form of phosphorylase a has been found to be more active than the tetramer form (38, 40, 41). By an analogous series of reactions (Scheme 1, lower path), we propose that the phos-
P phospheptide ( ) binds to its complementary region on phospho-
ylase b and b' (note that the binding is indicated by the fact that the peptide is not attached to the circle). In doing so, it may displace the corresponding dephosphorylated region of the protein to form a species of enzyme which we call "pseudo phosphorylase a" (PSEUDO-PHOS 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 covalent modification through the introduction of phosphate (42-44). It remains to be seen if there is some common theme in terms of protein structure required for the change in enzyme properties upon phosphorylation.

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