Use of an Antibody Probe to Study Regulation of Glycogen Phosphorylase by Its NH$_2$-terminal Region

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Antibodies that are specific for the NH$_2$-terminal region of rabbit muscle glycogen phosphorylase were isolated. Studies, using synthetic peptides representing different segments of the NH$_2$-terminal region of muscle phosphorylase, indicated the antibodies are highly specific for the first 4 NH$_2$-terminal residues of the enzyme. The molecular weight of the complex formed between dimeric phosphorylase and the antibodies estimated by gel filtration suggests that only 1 molecule of antibody binds per dimer of phosphorylase. The antibodies were strongly inhibitory to both phosphorylase kinase and phosphorylase phosphatase. Apparent binding constants for glucose 1-phosphate and AMP and inhibition by compounds that bind at or near the glucose 1-phosphate and AMP sites were not affected by the antibodies. The apparent $K_m$ for the high molecular weight substrate, glycogen, was lowered 2-fold by the presence of the antibodies. The primary binding site for maltoheptaose, and presumably for glycogen, recently has been shown to be a site separate from the active site (Kasvinsky, P. J., Madsen, N. B., Fletterick, R. J., and Sygusch, J. (1978) J. Biol. Chem. 253, 1290-1296). The improved binding affinity for glycogen, induced by the antibodies, is consistent with regulation of this glycogen site by the NH$_2$-terminal region. The binding of the antibodies to phosphorylase $b$ completely stabilized the enzyme to loss of its cofactor, pyridoxal 5'-phosphate, under conditions in which the cofactor is normally completely resolved. Because the antibodies did not affect the apparent binding affinities for compounds (glucose, glucose 1-phosphate, and caffeine) that bind in the same hydrophobic active site crevice as pyridoxal phosphate, the suggestion is made that the dramatic effect of the antibodies on the pyridoxal 5'-phosphate site is quite specific.

The specific antibodies against muscle phosphorylase were able to bind to the liver isozyme of phosphorylase. When antibodies were bound to the liver isozyme, the apparent affinity ($K_m$) for glucose 1-phosphate was improved by 4.1-fold at saturating AMP. At concentrations of glucose 1-phosphate lower than the $K_m$, the antibodies increased enzyme activity by more than 10-fold. The conclusion is made that the structural character of the NH$_2$-terminal regions of liver and muscle phosphorylase $b$ isozymes may be, at least partially, responsible for their differing affinities for glucose 1-phosphate.

The NH$_2$-terminal region of rabbit skeletal muscle glycogen phosphorylase is a primary locus for dictating the catalytic potential of this enzyme. The physical and catalytic properties of the enzyme are heavily dependent on whether serine-14 is phosphorylated or dephosphorylated (1). A variety of approaches has been utilized in attempts to gain some understanding of the mechanism involved in regulation by phosphorylation. Comparisons of the physical parameters and structural features of phosphorylase $a$ (phosphorylated form) and phosphorylase $b$ (dephosphorylated form) have indicated only subtle differences in the monomeric unit of these two forms (1). Fletterick et al. (2) used a difference Fourier electron density map to compare the crystalline structures of phosphorylase $a$ and $b$. They found only localized differences in the protein chain. The most dramatic structural difference was in the NH$_2$-terminal segment itself. Although the NH$_2$-terminal region of phosphorylase $a$ is easily discernible by x-ray diffraction (3), the first 17 NH$_2$-terminal residues of phosphorylase $b$ are not visible on the x-ray map (4).

Helmreich and co-workers used an immunochemical approach to the study of the regulatory phenomena of glycogen phosphorylase (5, 6). The antibodies used in their experiments were directed against many antigenic determinants on phosphorylase; thus, the effects of antibodies on properties of phosphorylase could not be related to their binding at specific regions of the enzyme molecule. Utilization of antibodies that bind specifically to a given region of an enzyme molecule should greatly improve the usefulness of an immunochemical approach to studying regulation of enzymes. Antibodies to specific regions of protein molecules have been used to predict structural conformation (7). In an earlier publication, we described the preparation of antibodies that specifically interact with the NH$_2$-terminal region of muscle phosphorylase (8). In the present communication, we describe the use of this antibody probe for the NH$_2$-terminal region to study the involvement of this important site in regulating the properties of the enzyme. It was our hope that the binding of the antibody would induce changes in those physical and catalytic properties most intimately controlled by the NH$_2$-terminal region.

Antibodies have been an important tool for studying structural homology of different glycogen phosphorylases (9-15). There is a wide range in the degree of immunochemical cross-reactivity between glycogen phosphorylases from different species (9-12), as well as phosphorylases from different organs of the same species (13-15). In spite of major differences in immunochemical, in addition to physical and catalytic, properties of different glycogen phosphorylases, evidence exists...
that suggests there may be considerable structural homology in the NH$_2$-terminal regions of the different isozymes. The amino acid sequence surrounding the phosphorylatable NH$_2$-terminal serine residue is highly conserved for phosphorylases from a variety of sources (16-20). Glycogen phosphorylase kinases are highly specific for phosphorylation of NH$_2$-terminal regions of glycogen phosphorylases; yet they phosphorylate phosphorylase isozymes from a variety of species and organs (1). Because of this evidence for structural homology, we decided to analyze what effect, if any, our specific antibody would have on a rabbit phosphorylase isozyme that has properties very different from the muscle isozyme. Liver phosphorylase has almost no immunological cross-reactivity with muscle phosphorylase (13) and differs from muscle phosphorylase in physical and catalytic properties (1). One of the major differences is that the dephosphorylated form of the liver isozyme was reported to be insensitive to the allosteric activator AMP of the muscle isozyme (21). The primary cause for the observed insensitivity to AMP was a very low affinity for substrate, glucose 1-phosphate, and an inadequate substrate concentration during assay (22). The most dramatic effect of the specific antibody on the liver isozyme was on its AMP-dependent activity.

**EXPERIMENTAL PROCEDURES**

**Preparation of Antibody**—Hyperimmune serum, containing anti-phosphorylase a antibodies, was obtained from the same goat used in a previous study (8). After the initial immunization (9), the goat was boosted with soluble phosphorylase a six times over a period of 7 months. A 2000-ml blood sample was extracted at the end of this period; experiments described in this paper were performed by using antibodies isolated from this blood sample. The γ-globulin fraction was prepared (8), and this γ-globulin fraction should contain primarily protein. The second (3.0 to 3.6 M guanidine HCl) was eluted following amino acid sequence: NHL-Ser.Arg-Pro-Ieu-Srr-Asl.-Glu-Lys-Arg-Lys-Gln-Ile-Ser(I)~)-Val-Arg-Gly-Leu-COOH. A gradient of 0.0 to 4.0 M guanidine HCl was used to elute anti-(1-18)p from the affinity resin. Two major protein components were eluted from the column, and both were dialyzed against 0.15 M NaCl to remove guanidine HCl and stored in the freezer. The first (1.1 to 2.3 M guanidine HCl) was eluted from the column, and both were dialyzed against 0.15 M NaCl to remove guanidine HCl and stored in the freezer. The first (1.1 to 2.3 M guanidine HCl) contained 82% of the eluted anti-phosphorylase a activity, as measured by radioimmunoassays (4), and 55% of the eluted protein. The second (3.0 to 3.6 M guanidine HCl) contained only 8% of the eluted anti-phosphorylase a activity and 26% of the eluted protein. The first component, anti-(1-18)p, was used for the experiments described in this paper because of its greater specific immunochemical activity.

Normal goat γ-globulin was obtained from a goat that had not been injected with phosphorylase. Antibody was prepared against goat γ-globulin in rabbit (rabbit anti-goat γ-globulin) as previously described (8).

**Preparation of Enzymes**—Rabbit skeletal muscle glycogen phosphorylase b and rabbit liver glycogen phosphorylase b were isolated by Fischer and Krebs (24) and Appleman et al. (21), respectively. Muscle phosphorylase a and liver phosphorylase a were prepared from their respective phosphorylase b forms by phosphorylation with muscle phosphorylase kinase (25). [3H]Phosphorylation a was obtained in an identical manner except [3H]ATP, prepared by the method of Glynn and Chappell (26), was used as substrate for kinase. Phosphorylation b was obtained from rabbit muscle phosphorylase a by limited proteolysis with trypsin (27). Rabbit muscle phosphorylase kinase and rabbit liver phosphorylase phosphatase were purified as described by Brostrom et al. (28) and Brandt et al. (29), respectively. The protein concentration of phosphorylase was measured spectrophotometrically by using the extinction coefficient E$_{260}$ nm at 280 nm of 13.2 (30). For calculation of moles of phosphorylase, a monomer of phosphorylase was assumed to have a molecular weight of 97,400 (31). The specificity of the antibodies described in this paper because of its greater specific immunochemical activity.

**RESULTS**

**Specificity of Anti-(1-18)p**—A study, analogous to the one performed on the first preparation of anti-(1-18)p (8), was conducted on the new anti-(1-18)p preparation described in this communication. The results of these experiments are vital to the understanding of other experiments. Because these results are very similar to those obtained for the first anti-(1-18)p preparation, only a brief summary of these experiments is presented. They are almost identical in protocol with those published for the first preparation of anti-(1-18)p. The best evidence for the specificity of anti-(1-18)p for the NH$_2$-terminal region was obtained from experiments with phosphorylase b', which is missing the first 16 NH$_2$-terminal residues, using radioimmunoassays similar to those previously described (8). Phosphorylase b' did not bind to anti-(1-18)p and could not inhibit the interaction of anti-(1-18)p with the synthetic NH$_2$-terminal peptide, Ac(1-18)p, nor with native phosphorylase a or b. Ac(1-18)p is the same as (1-18)p except that its NH$_2$ terminus is N-acetylated. In contrast, anti-(1-18)p could bind phosphorylase a and b equally well, and phosphorylase a and b could completely prevent binding of anti-(1-18)p to Ac(1-18)p. In addition, Ac(1-18)p caused 90 to 100% inhibition of binding of anti-(1-18)p to phosphorylase a and b. Therefore, anti-(1-18)p is highly specific for determinants located within the NH$_2$-terminal region. The amount of binding of anti-(1-18)p to phosphorylase and peptides was not affected by 2-mercaptoethanol. Titers for IgM are known to be sensitive to 2-mercaptoethanol. However, for IgG, precipitin complexes of anti-(1-18)p with either phosphorylase or peptides were never observed. A second antibody against normal goat γ-globulin prepared in rabbit (rabbit anti-goat γ-globulin) was needed to precipitate complexes of anti-(1-18)p with phosphorylase or with peptides.

Experiments were conducted to ascertain which portions of the NH$_2$-terminal region of native phosphorylase a are necessary for interaction with anti-(1-18)p. Peptides representing different segments of the NH$_2$-terminal region of phosphorylase were surveyed as inhibitors of the binding of anti-(1-18)p to the tritiated NH$_2$-terminal peptide, [3H]Ac(1-18)p (Table I). [3H]Ac(1-18)p is the same peptide Ac(1-18)p except its N-acetylated NH$_2$ terminus was labeled with tritiated acetic anhydride, as previously described (8). The NH$_2$ terminus of native phosphorylase a is N-acetylated (16).

For the first preparation of anti-(1-18)p, data suggested that a phosphate group on the NH$_2$-terminal serine-14 was not necessary for interaction with anti-(1-18)p (8). This conclusion also can be made for the new preparation of anti-(1-18)p from the data in Table I. The ratio of dephosphopeptide Ac(1-18) to phosphorylase [3H]Ac(1-18)p was 2.2, and this amount of Ac(1-18)p displaced 79% of [3H]Ac(1-18)p from anti-(1-18)p. This is close to what one would expect (69%) if anti-(1-18)p bound equally well to the phosphorylated dephosphopeptide. The acetylated NH$_2$ terminus was not vital for...
inhibition of binding of anti-(1-18)p to [3H]Ac(1-18)p because nonacetylated peptide (1-18)p displaced 64% of [3H]Ac(1-18)p. Peptide (2-18)p, which is missing serine-1, displaced 52% of [3H]Ac(1-18)p. Therefore, serine-1 was not necessary for good displacement of [3H]Ac(1-18)p from anti-(1-18)p. However, residues 2 to 4 were extremely important since peptide (5-18)p caused only 6% displacement. The acetylated analog of peptide (5-18)p, Ac(5-18)p, was also a very poor inhibitor. Other short peptides, (7-18)p, (9-18)p, and (11-18)p, were equally poor in displacing [3H]Ac(1-18)p. The necessity for residues 1 to 4 was confirmed by the ability of peptide, Ac(1-4), at a high concentration, to displace [3H]Ac(1-18)p. An equally high concentration of another short peptide fragment, peptide Ac(9-11)Gly, was not a good inhibitor. An experiment was conducted to ascertain whether residues 1 to 4 also are important in the binding of anti-(1-18)p to phosphorylase (Table II). Peptide (5-18)p was ineffective, relative to Ac(1-18)p, in displacing [3H]phosphorylase from anti-(1-18)p. Peptide Ac(1-4) was able to cause significant displacement. From these studies, we concluded that anti-(1-18)p has a high degree of specificity for the first 4 NH₂-terminal residues.

Estimated Molecular Weight of Rabbit Muscle Phosphorylase (anti-(1-18)p) Complex—At the concentrations (3 μg/ml) of phosphorylase used to study the effects of anti-(1-18)p on the properties of the muscle enzyme, both phosphorylase a and phosphorylase a were present as dimers (35). It is possible that anti-(1-18)p could bind to both NH₂-terminal regions of a phosphorylase dimer. In addition, each molecule of anti-(1-18)p has two combining sites for interaction with phosphorylase. Therefore, high molecular weight complexes of anti-(1-18)p and phosphorylase could form.

Experiments were conducted to estimate the molecular weight of these complexes (Fig. 1). [32P]Phosphorylase a was preincubated with anti-(1-18)p under conditions similar to those used to study the effects of anti-(1-18)p on the properties of the enzyme. The preincubation mixture was chromatographed on a Sepharose 4B column to estimate the molecular weight of the complexes generated. Of the radioactivity that was applied, 88% was recovered. A small amount (7.9% of recovered radioactivity) of high molecular weight material was eluted as a peak just after the void volume (molecular weight greater than 10²). A peak containing 71% of the recovered radioactivity was eluted in a position corresponding to a relatively low molecular weight. Arrow B, Fig. 1, indicates the elution position for dimeric phosphorylase a (M, = 195,000) when no γ-globulin was present and also when anti-(1-18)p was substituted with normal goat γ-globulin. The column was further calibrated by determining the elution position of a tetramer of phosphorylase a (M, = 390,000). This was accomplished by applying phosphorylase a to the column at a much higher concentration (9.3 mg/ml) because, at high concentrations, phosphorylase a is known to exist as tetramer (35). Arrow A indicates the elution position of tetrameric.

### Table I

<table>
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<tr>
<th>Abbreviation</th>
<th>Sequence</th>
<th>Amount</th>
<th>% Displacement</th>
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<tr>
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<td>6</td>
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<tr>
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<tr>
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<td>500</td>
<td>6</td>
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* The peptides were synthesized, phosphorylated, and purified by the methods of Tessmer et al. (34). [H]Ac(1-18)p was prepared as described in a previous communication (8).

### Table II

<table>
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<th>Abbreviation</th>
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<tr>
<td>(1-18)p</td>
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<td>4</td>
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<tr>
<td>Ac(1-4)</td>
<td>Acetyl NH₆-Ser-Ac(1-4)p</td>
<td>500</td>
<td>30</td>
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</table>
phosphorylase a. The major peak of radioactivity, representing the complex of anti-(1-18)p and dimeric phosphorylase a, eluted at a position corresponding to a molecular weight slightly less than the molecular weight of a tetramer of phosphorylase a. Since anti-(1-18)p is probably IgG (Mr = 160,000), it is likely that this peak represents a complex of one dimer of phosphorylase a and 1 molecule of anti-(1-18)p. Addition of larger quantities of anti-(1-18)p to the same quantity of phosphorylase (peak 390 µg of phosphorylase a) was substituted for 5 µg of phosphorylase a and when normal goat γ-globulin was substituted for anti-(1-18)p in the sample mixture. The above experiments were repeated at least three times each, and the elution profiles were always the same.

Resolution of Pyridoxal 5′-Phosphate—Pyridoxal 5′-phosphate can be resolved from phosphorylase b in the presence of the deforming buffer, imidazole, and the trapping agent, cysteine (36). The effect of anti-(1-18)p on resolution is presented in Fig. 1. In the absence of anti-(1-18)p, a first order loss of enzyme activity, due to resolution, was observed. When anti-(1-18)p was preincubated with phosphorylase b, there was a 39% drop in the reaction velocity before introduction of the resolving conditions; however, the remaining activity was completely stabilized to the conditions of resolution. The decrease in reaction velocity is due to an effect of anti-(1-18)p on the maximal velocity, and this effect is discussed in another section. When anti-(1-18)p was added to the resolution mixture that was not preincubated with antibody (arrow), there was an immediate 43% drop in reaction velocity, followed by complete stabilization of enzymic activity. These results indicate that binding of anti-(1-18)p to the NH2-terminal region prevents resolution of pyridoxal 5′-phosphate. Anti-(1-18)p had no effect on resolution of pyridoxal 5′-phosphate from phosphorylase b. Because anti-(1-18)p does not bind to phosphorylase b as indicated in the section on specificity of anti-(1-18)p, the effect of anti-(1-18)p on resolution of pyridoxal 5′-phosphate from phosphorylase b is a specific effect on the enzyme itself.

To completely prevent resolution of 6 µg of phosphorylase b, 120 µg of antibody was required (data are not presented). When lesser amounts of anti-(1-18)p were used, some resolution was observed. Assuming anti-(1-18)p is primarily IgG (Mr = 160,000), this represents a molar ratio of anti-(1-18)p to phosphorylase b as 125. The molar ratio of anti-(1-18)p to phosphorylase in the experiments described by Fig. 2 was 25, or twice what is necessary to afford protection against resolution. In all other experiments to survey the effect of anti-(1-18)p on phosphorylase b, the molar ratio of anti-(1-18)p to phosphorylase was 25. This should also be enough to saturate phosphorylase a with anti-(1-18)p because phosphorylase a and phosphorylase b bound equally well to anti-(1-18)p.

Effect of Anti-(1-18)p on Interconversion Reactions—The effect of anti-(1-18)p on phosphorylation of the NH2-terminal region of phosphorylase b is represented by Fig. 3A. Conversion of phosphorylase b to phosphorylase a was measured, after different periods of incubation with phosphorylase kinase, by assay of phosphorylase activity in the absence of AMP. Activities in the presence of anti-(1-18)p have been corrected for inhibition of the maximal velocity of phosphorylase a (Table III). When phosphorylase b was preincubated with anti-(1-18)p, there was an 88% reduction in the extent of conversion to phosphorylase a. Therefore, in the presence of anti-(1-18)p, phosphorylation may occur only on NH2-terminals that are not bound or weakly bound to anti-(1-18)p.

The phosphorylase phosphatase reaction, dephosphorylation of phosphorylase a, was also inhibited by anti-(1-18) p (Fig. 3B). However, the type of inhibition by anti-(1-18)p was very different for the phosphatase reaction than for the kinase reaction. The rate of the phosphatase reaction was greatly reduced by anti-(1-18)p, but the extent of the reaction was the same as when anti(1-18)p was not present. When the phosphatase reaction was allowed to proceed for longer periods or with more phosphatase than indicated in the legend.
Specific Antibody Probe for Glycogen Phosphorylase

Effect of anti-(1-18)p on kinetic constants of rabbit muscle phosphorylase

Phosphorylase (0.3 μg) was preincubated in 0.02 ml at 30°C and pH 6.8 with either (i) 12 μg of anti (1-18)p and 25 μg of normal goat γ-globulin or (ii) 57 μg of normal goat γ-globulin in 40 mM β-glycerophosphate, 30 mM 2-mercaptoethanol, and 75 mM NaCl. After 10 min, 80 μl of substrate mixture was added and phosphorylase activity was measured as described in the text. The standard assay mixture (100 μl, pH 6.8) contained 16 mM [U-14C]glucose-1-phosphate, 1.0% (w/v) glycogen, 1 mM AMP, 20 mM β-glycerophosphate, 15 mM 2-mercaptoethanol, and 15 mM NaCl. When the kinetic constants for a given substrate were determined, the concentration of that substrate was varied. The V_{max} and K_{m} were estimated from double reciprocal plots of initial velocity versus substrate concentration and the K_{m} and the Hill coefficient (n) were estimated from a Hill plot.

### Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Anti-(1-18)p</th>
<th>K_{m} (μM)</th>
<th>Hill coefficient (n)</th>
<th>V_{max} (μmol/min/mg)</th>
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<tbody>
<tr>
<td>Glucose-1-P</td>
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<td>1.5</td>
<td>46</td>
</tr>
<tr>
<td>Glycogen</td>
<td>-</td>
<td>0.02%</td>
<td>1.6</td>
<td>46</td>
</tr>
<tr>
<td>AMP</td>
<td>-</td>
<td>0.17</td>
<td>1.0</td>
<td>46</td>
</tr>
</tbody>
</table>

* K represents the apparent K_{m} for glucose 1-phosphate and glycogen and the apparent K_{m} for AMP.

Fig. 3. Influence of antibody on enzymic interconversion. A, a preincubation mixture (20 μl, pH 6.8), containing 0.8 μg of phosphorylase b, 40 mM β-glycerophosphate, 30 mM 2-mercaptoethanol, 75 mM NaCl, and either (i) 12 μg of anti-(1-18)p and 10 μg of normal goat γ-globulin (C) or (ii) 22 μg of normal goat γ-globulin (C), was incubated at 30°C. After 30 min, a solution (30 μl, pH 6.8), containing 1.0 μg phosphorylase kinase, 0.5 mg/ml of normal goat γ-globulin, 2.5 mM MgCl₂, 1.17 mM CaCl₂, 1.67 mM ATP, 40 mM β-glycerophosphate, and 30 mM 2-mercaptoethanol, was added and incubated at 30°C. At different times during phosphorylation, the activity in the absence of AMP (i.e. phosphorylase a activity) was measured by addition of 50 μl of a solution, containing 2.0% glycogen and 32 mM [U-14C]glucose 1-phosphate, and incubation for 15 min at 30°C. Incorporation of 14C into glycogen was determined as described in the text. B, a preincubation mixture (20 μl, pH 7.4) containing [3H]phosphorylase a (0.3 μg, 20,000 cpm), 25 mM Tris, 0.5 mM dithioerythritol, 75 mM NaCl, and either (i) 12 μg of anti-(1-18)p and 10 μg of normal goat γ-globulin (C) or (ii) 22 μg of normal goat γ-globulin (C), was incubated at 30°C. After 30 min, a solution (30 μl, pH 7.4), containing 1.0 μg phosphorylase kinase, 0.5 mg/ml of normal goat γ-globulin, 2.5 mM MgCl₂, 0.17 mM CaCl₂, 1.67 mM ATP, 40 mM β-glycerophosphate, and 30 mM 2-mercaptoethanol, was added and incubated at 30°C. At different times during dephosphorylation, the protein was precipitated by addition 10 μl of 15% trichloroacetic acid and 10 μl of 1 mg/ml bovine serum albumin, and the amount of acid-soluble 32P was measured.

Effect of Anti-(1-18)p on Kinetic Constants—The effect of anti-(1-18)p on the binding sites for the substrates, glucose 1-phosphate and glycogen, and the binding site for the activator, AMP, was surveyed by measurement of their kinetic constants with and without anti-(1-18)p bound to the NH₂-terminal region (Table III). Anti-(1-18)p did not affect the apparent K_{m} for glucose 1-phosphate with muscle phosphorylase b nor with muscle phosphorylase a. The binding of high molecular weight antibodies (M₅ = 130,000) to phosphorylase might interfere with the binding of the high molecular weight substrate, glycogen (9). Surprisingly, the binding of anti-(1-18)p did not have an adverse effect on the apparent K_{m} for glycogen. The apparent K_{m} for glycogen was decreased by anti-(1-18)p for both phosphorylase b and phosphorylase a. In the case of phosphorylase a, the K_{m} for glycogen was decreased 2-fold by anti-(1-18)p, both in the presence and absence of AMP.

Binding of AMP to phosphorylase b was not affected by anti-(1-18)p. The apparent affinity (K_{a}) at the AMP site of a monomer of dimeric phosphorylase b and the homotetramer cooperativity between the AMP sites (Hill coefficient, n) were not significantly affected by anti-(1-18)p. In addition, anti-(1-18)p did not affect the degree of inhibition by inhibitors (ATP, glucose 6-phosphate, and glucose) that bind at the AMP site or glucose 1-phosphate site of phosphorylase b. Recently, Madson and co-workers (37) reported that caffeine can competitively inhibit binding of glucose 1-phosphate to phosphorylase a. Caffeine binds to a site just 10 Å from the glucose 1-phosphate site (37). Independently, we have found caffeine to be a competitive inhibitor for phosphorylase b and b', as well as phosphorylase a. The K for caffeine was unaffected by anti-(1-18)p.

Although the apparent affinity for substrates and activator (AMP) was not reduced by anti-(1-18)p, there was a large decrease in the V_{max} when anti-(1-18)p was bound to the NH₂-terminal region. Anti-(1-18)p reduced the V_{max} for phosphorylase b by 35%; the V_{max} for phosphorylase a in the presence of AMP was reduced by 47%, and in the absence of AMP, the V_{max} was reduced by 70%. Addition of larger quantities of anti-
(1-18)p did not increase the inhibition by anti-(1-18)p for either phosphorylase b or phosphorylase a. Therefore, the amount of anti-(1-18)p used was saturating. For phosphorylase a, the lower amount of inhibition of the \( V_{\text{max}} \), in the presence than in the absence of AMP is not because AMP can displace anti-(1-18)p from phosphorylase. In a radioimmunoassay similar in protocol to that described in Table II, AMP, as well as glucose 1-phosphate, glucose 6-phosphate, and ATP had no effect on the binding of anti-(1-18)p to phosphorylase. Because the AMP-dependent activity of phosphorylase b was less inhibited by anti-(1-18)p than the AMP-independent activity of phosphorylase a, the absence of AMP might be expected to decrease the inhibition of phosphorylase a. Possible interpretations of the inhibition of \( V_{\text{max}} \) are presented in the discussion.

Effect of Anti-(1-18)p on Rabbit Liver Glycogen Phosphorylase—The liver isozyme of glycogen phosphorylase differs from the muscle isozyme in immunological (38) as well as other physical and catalytic properties (1). However, muscle phosphorylase kinase will catalyze phosphorylation of the N-terminal region of the liver phosphorylase b isozyme (21). Using a radioimmunoassay similar to that described in Table II, we have found that anti-(1-18)p binds equally well to both the phosphorylated (a form) and dephosphorylated (b form) forms of liver phosphorylase. However, a given quantity of anti-(1-18)p bound 10-fold less liver phosphorylase than muscle phosphorylase a.

Liver phosphorylase b has a much lower affinity for glucose 1-phosphate than muscle phosphorylase b (22). Liver phosphorylase b has, relatively, little enzymatic activity in the presence of 16 mM glucose 1-phosphate. The effect of different amounts of anti-(1-18)p on the activity of liver phosphorylase b in the presence of 16 mM glucose 1-phosphate is presented in Fig. 4. At the highest level of anti-(1-18)p tested, a greater than 4-fold increase in enzyme activity was observed. When normal goat \( \gamma \)-globulin was substituted for anti-(1-18)p, there was no change in activity. When liver phosphorylase b was converted to phosphorylase a by rabbit muscle phosphorylase kinase and assayed under the same conditions as in Fig. 4, the specific activity was 32 \( \mu \)mol/min/mg. Unlike liver phosphorylase b, when anti-(1-18)p was added to the liver phosphorylase a, there was little or no effect on the activity of liver phosphorylase a.

A common population of antibodies may bind to both liver phosphorylase and muscle phosphorylase since the liver isozyme displaced the muscle isozyme from anti-(1-18)p in radioimmunoassays. The population of anti-(1-18)p that catalytically activates liver phosphorylase is probably specific for the N-terminal region because the muscle phosphorylase phosphopeptide Ac(1-18)p completely blocked the activation by anti-(1-18)p. In the absence of anti-(1-18)p, Ac(1-18)p slightly stimulated liver phosphorylase b.

Anti-(1-18)p greatly improved the affinity for glucose 1-phosphate and removed most of the cooperativity seen in the double reciprocal plot (Fig. 5). The ratio of the apparent \( K_a \), for glucose 1-phosphate in the absence of anti-(1-18)p to the \( K_a \) in the presence of anti-(1-18)p was 4.1 (Table IV). At the lowest concentrations of glucose 1-phosphate tested, the activity was increased by anti-(1-18)p by more than 10-fold. The apparent \( K_a \) for AMP was dependent on the concentration of glucose 1-phosphate. At saturating levels of glucose 1-phosphate, the ratio of the \( K_a \) for AMP in the absence of anti-(1-18)p.

![FIG. 4. Activation of rabbit liver phosphorylase b by anti-(1-18)p.](http://www.jbc.org/)

![FIG. 5. Effect of anti-(1-18)p on double reciprocal plot of initial velocity versus glucose 1-phosphate concentration for rabbit liver phosphorylase b.](http://www.jbc.org/)

### Table IV

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration of glucose-1-P</th>
<th>( K_a )</th>
<th>( V_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-1-P</td>
<td>Varying</td>
<td>+</td>
<td>13</td>
</tr>
<tr>
<td>Glucose-1-P</td>
<td>Varying</td>
<td>+</td>
<td>63</td>
</tr>
<tr>
<td>AMP</td>
<td>50</td>
<td>+</td>
<td>0.57</td>
</tr>
<tr>
<td>AMP</td>
<td>50</td>
<td>+</td>
<td>1.82</td>
</tr>
<tr>
<td>AMP</td>
<td>100</td>
<td>-</td>
<td>0.91</td>
</tr>
</tbody>
</table>

\( K_a \) represents the apparent \( K_a \) for glucose 1-phosphate, and the apparent \( K_a \) for AMP.
Specific Antibody Probe for Glycogen Phosphorylase

18p to the \( K_a \) in the presence of anti-(1-18)p was 1.6 (Table IV). The maximal velocity was not affected by anti-(1-18)p. Thus, the primary reason for catalytic activation of liver phosphorylase \( b \) is an increased affinity for glucose 1-phosphate. In the absence of AMP, anti-(1-18)p had no effect on the activity of liver phosphorylase \( b \).

DISCUSSION

The difference in the effect of anti-(1-18)p on the interconversion reactions may be explained by differences in the degree of steric interference by anti-(1-18)p with the binding of the interconverting enzymes to phosphorylase. Binding of phosphorylase phosphatase (\( M_r = 35,000 \)) (29) probably would be affected less than binding of phosphorylase kinase (\( M_r = 1,300,000 \)) (29) by steric interference from anti-(1 18)p. Phosphorylase kinase may not bind to phosphorylase \( b \) containing bound anti-(1-18)p; the 12% conversion that did occur may reflect the percentage of NH2-terminal regions that were not bound to anti-(1-18)p. The phosphorylated seryl residue (serine-14) seems to be accessible to phophatase when anti (1 18)p is combined with phosphorylase. This is consistent with the observed high specificity of anti-(1-18)p for a determinant located within the first 4 NH2-terminal residues of the enzyme.

An estimation of the molecular weight of the complex of muscle phosphorylase and anti-(1-18)p was made by gel filtration chromatography. The complex eluted from the column in a position that corresponded to a molecular weight slightly less than tetrameric phosphorylase \( a \) (\( M_r = 390,000 \)). Anti-(1-18)p is most probably IgG (\( M_r = 160,000 \)) because of the injection schedule used to prepare the goat hyperimmune serum and its insensitivity to 2-mercaptoethanol. The low molecular weight of the complex between anti-(1-18)p and phosphorylase cannot be explained by proteolysis of the IgG that composes anti-(1-18)p. The conditions used to isolate anti (1-18)p are similar to conditions that have been used previously to isolate intact, unproteolyzed goat IgG antibodies (23, 40). The major complex formed between anti-(1-18)p and phosphorylase \( a \) is, likely, made up of one dimer of phosphorylase \( a \) and 1 molecule of anti-(1-18)p. It is possible that the binding of 1 anti-(1-18)p molecule at one NH2-terminal region of the dimer precludes the binding of a 2nd molecule of anti-(1-18)p at the other NH2-terminal region of the same dimer. Both combining sites of the bivalent anti-(1-18)p molecule must be tied up in interaction with the dimer; otherwise, complexes of higher molecular weight would be possible. The most probable explanation for the stoichiometry is that 1 molecule of anti-(1-18)p bridges the two NH2-terminal regions of phosphorylase \( a \). Each monomer of dimeric phosphorylase \( a \) has overall dimensions of 85 \( \times \) 75 \( \times \) 55 \( \AA \), with the NH2-terminal regions located very near the subunit interface (3). From x-ray crystallographic data, the distance between the variable region combining sites of a human myeloma IgG was found to be 142 \( \AA \) (41). Therefore, it is certainly a physical possibility for anti-(1-18)p to span the two NH2-terminal regions of a phosphorylase dimer. This interpretation of the data would also explain why precipitin complexes are not formed when anti-(1-18)p combines with phosphorylase.

Tzartos and Evangelopoulos (9) studied the effects of antibodies, directed against multiple determinants of pig muscle glycogen phosphorylase \( b \), on the kinetic constants for the enzyme. They also found that the apparent affinities for glucose 1-phosphate and AMP were unaffected by their nonspecific antibodies. However, the apparent affinity for glycogen was reduced when the pig enzyme was complexed with antibodies. When glycogen fragments were used instead of the intact macromolecular substrate, glycogen, the antibodies did not affect the apparent affinity for these lower molecular weight substrates. They suggested that this observation was consistent with steric hindrance by the antibodies. In contrast, anti-(1-18)p improved the apparent affinity for the macro-molecular substrate, glycogen, for both rabbit muscle phosphorylase \( a \) and \( b \). Therefore, steric hindrance to binding of glycogen did not exist when anti-(1-18)p was bound to the NH2-terminal region. The apparent \( K_a \) for glycogen may be influenced by binding of glycogen at the “glycogen storage site” (3), which is located 25 \( \AA \) from the active site (3, 42). The improved affinity for glycogen in the presence of anti-(1-18)p may be related to binding of glycogen at either the glycogen storage site or the active site, or both. The dissociation constant for oligosaccharide at the active site is at least 20-fold greater than that at the storage site (42). The dissociation constant at the storage site is similar to that reported for glycogen (43). Because of the better binding affinity at the “storage site” and the observation that anti-(1-18)p does not affect apparent binding constants for other compounds that bind in or near the active site, the improved apparent affinity for glycogen by anti-(1-18)p could be due to an effect on the glycogen storage site. Therefore, the NH2-terminal region may regulate what occurs at the glycogen storage site, as well as its well known regulation of activities at the catalytic site.

The reduction in \( V_{max} \) of phosphorylase could be due to steric interference, by anti-(1-18)p, with the interaction of phosphorylase with glycogen during catalysis, after binding of glycogen at the storage site. In the experiments for determination of kinetic constants we observed that the percentage of inhibition by anti-(1-18)p was the same at all concentrations of AMP and glucose 1-phosphate, including concentrations that gave less than half the maximal velocity. In contrast, as the concentration of glycogen decreased below its \( K_m \), the percentage of inhibition by anti-(1-18)p decreased. Activation by anti-(1-18)p of the initial velocity was observed at concentrations of glycogen that were less than half the apparent \( K_m \). Presumably, at these glycogen concentrations, the improved affinity for glycogen becomes more important than the reduced catalytic turnover when anti-(1-18)p is bound.

Anti-(1-18)p may lower the \( V_{max} \) because of some effect on the structure or conformational mobility of phosphorylase. An antibody molecule bound to phosphorylase could interfere with necessary movements of the protein chain during catalysis. Freedom of movement of the NH2-terminal region may be important for catalysis, and the binding of large antibody molecules may hinder that movement. This latter explanation is supported by a larger reduction in the \( V_{max} \) for phosphorylase \( a \) (70% inhibition) in the absence of AMP (Table III). In the case of phosphorylase \( a \), the NH2-terminal phosphate has a definite role in catalytic activation. The NH2-terminal region of phosphorylase \( b \) is not directly involved in activation by AMP, but some effect by anti-(1-18)p might be expected because of the close proximity of a segment of the NH2-terminal region to the AMP site (4).

The pyridoxal 5'-phosphate site is located very close to the binding site for glucose 1-phosphate (32, 44). This important cofactor site was dramatically affected by anti-(1-18)p. Under conditions in which complete loss of activity could be obtained in the absence of anti-(1-18)p, the binding of anti-(1-18)p to the NH2-terminal region of phosphorylase \( b \) provided complete protection against loss of enzymatic activity due to loss of pyridoxal 5'-phosphate. The AMP binding site and the NH2-terminal region are close to one another, but they are about 30 \( \AA \) from the glucose 1-phosphate site (catalytic site) and the pyridoxal 5'-phosphate site (44). Nevertheless, phosphorylation of the NH2-terminal region (45) or binding of AMP (46) will also protect the enzyme from loss of pyridoxal 5'-phosphate, as well as increase affinity for substrates and
differential changes in the affinity for inhibitors (caffeine, glucose, glucose 6-phosphate, and AMP) (1). Because anti-(1-18)p has little or no effect on the apparent affinity for substrates and inhibitors, its stabilization of the pyridoxal 5'-phosphate site is particularly interesting. The data support the hypothesis that close communication may exist between the NH$_2$-terminal region and the pyridoxal 5'-phosphate site. Graves et al. (47) showed that the pyridoxal 5'-phosphate in phosphorylase had an important effect on the enzymatic interconversion and tryptic attack of the NH$_2$-terminal region.

Pyridoxal 5'-phosphate is located in the same hydrophilic pocket as the binding site for glucose 1-phosphate (44). Phosphorylase is completely inactive when pyridoxal 5'-phosphate is removed (48). Because anti-(1-18)p had no effect on the binding affinity for compounds (glucose-1-P, glucose, and caffeine) that bind in the same hydrophilic active site crevice as pyridoxal 5'-phosphate, but had a major effect on the stability of the pyridoxal 5'-phosphate site, communication between the NH$_2$-terminal region and the hydrophobic active site region may be mediated through pyridoxal 5'-phosphate.

Another interpretation of the effect of anti-(1-18)p on resolution of pyridoxal 5'-phosphate should be considered. When dimeric phosphorylase b was subjected to a deforming buffer (imidazolium citrate, pH 6.0) used for resolution, the enzyme was reversibly dissociated into monomers (48). During this treatment enzyme-bound pyridoxal 5'-phosphate may become more exposed, since it will now exchange with free radiolabeled pyridoxal 5'-phosphate (47). As previously discussed, anti-(1-18)p may cross-link the monomeric units of dimeric phosphorylase. Monomerization could be a necessary step for exposure of pyridoxal 5'-phosphate by the deforming buffer, and anti-(1-18)p might prevent exposure of pyridoxal 5'-phosphate by linking the monomers of dimeric phosphorylase b.

The data on the activation of liver phosphorylase b in the presence of AMP give strong support for a structuring role for anti-(1-18)p. When anti-(1-18)p binds to liver phosphorylase b, the apparent $K_m$ for AMP, at saturating concentrations of glucose 1-phosphate, was decreased slightly; in contrast, the apparent $K_m$ for glucose 1-phosphate was decreased to one-fourth of the control value. The large effect of anti-(1-18)p on the glucose 1-phosphate site of liver phosphorylase b is not inconsistent with the absence of an effect on the affinity of muscle phosphorylase b for glucose 1-phosphate. Muscle phosphorylase b is receptive to AMP activation without any additional activating factors. Liver phosphorylase a is inactive in the absence of AMP, and anti-(1-18)p did not affect its activity. Therefore, anti-(1-18)p is activating the liver enzyme to bind glucose 1-phosphate, probably because of an indirect effect on the NH$_2$-terminal region, rather than a direct effect on the glucose 1-phosphate site. High concentrations of SO$_4^{2-}$ or F$^-$ will stimulate activity of liver phosphorylase b in the absence and in the presence of AMP (49). In addition, we have observed that certain organic solvents will activate liver phosphorylase b in the absence and the presence of AMP to a greater extent than Na$_2$SO$_4$ and NaF. However, anti-(1-18)p had no effect on the activity of liver phosphorylase b in the absence of AMP. Therefore, stimulation by anti-(1-18)p may have a more specific effect on the structure of the enzyme. The structural integrity of the NH$_2$-terminal region of liver phosphorylase may determine the enzyme’s ability to bind glucose 1-phosphate. Further, the differences in the magnitude of AMP activation for liver and muscle phosphorylase b may be, at least partially, due to differing structural features of their respective NH$_2$-terminal regions.

Previous reports have been made of antibodies causing activation of enzymes. Antibodies prepared against native, active enzymes have been used to enhance enzyme activity in partially active enzymes (50, 51) or to induce enzyme activity in inactive enzymes (52-54). Because of the rigid specificity of antibody combining sites, antibodies may induce activation by causing a conformation change in that region of the protein molecule that interacts with the antibody, which may lead to conformation changes in other regions of the protein molecule. This mechanism of antibody-induced effects on specific proteins has been reviewed by Crumpton (55) and Melchers et al. (54). Anti-(1-18)p could activate by inducing and stabilizing a conformation of the NH$_2$-terminal region of liver phosphorylase b that is similar to that of muscle phosphorylase.

Anti-(1-18)p was unable to affect the $V_{max}$ for liver phosphorylase b (Table IV), but the $V_{max}$ for muscle phosphorylase b and a was reduced. As previously discussed, the amount of reduction of $V_{max}$ was very different for the a and b forms of the muscle isozyme. The variation in the degree of effect of anti-(1-18)p on the $V_{max}$ for different phosphorylases could be related to differences in the nature of the interaction of anti-(1-18)p with the different phosphorylases.

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Specific Antibody Probe for Glycogen Phosphorylase

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