Activation of Skeletal Muscle Phosphorylase Kinase by Adenosine Triphosphate and Adenosine 3',5'-Monophosphate*

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SUMMARY

Rabbit skeletal muscle phosphorylase kinase has been obtained as a nearly homogeneous protein showing a single peak on electrophoresis and in the ultracentrifuge. Incubation of the purified kinase with γ32P-ATP in the presence of Mg++ ions activates the enzyme; this process is accompanied by phosphorylation of the protein. Activation and phosphorylation of phosphorylase kinase occur more rapidly when cyclic 3',5'-AMP or glycogen is present in activation reaction mixtures, but these two substances apparently act by different mechanisms since their effects are additive. Cyclic 3',5'-AMP and glycogen stimulate activation of the kinase more than they affect phosphorylation. No evidence was obtained to indicate that cyclic 3',5'-AMP undergoes any modification when it exerts its effect on phosphorylase kinase activation. Activation of the kinase was shown to be autocatalytic, but the possibility that cyclic 3',5'-AMP acts through stimulation of a second enzyme involved in the system was suggested by the failure to find any evidence for a significant degree of binding of this nucleotide to purified phosphorylase kinase.

Phosphorylase kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.37) catalyzes the conversion of phosphorylase b to phosphorylase a, a reaction in which the terminal phosphate group of ATP is transferred to a specific serine residue in phosphorylase (1-4). This reaction is irreversible, but phosphorylase b can be re-formed through the action of a second enzyme, phosphorylase phosphatase, which brings about a hydrolytic cleavage of the bound phosphate in phosphorylase a (5, 6). In skeletal muscle the conversion of phosphorylase b to phosphorylase a constitutes an important step in the coupling of contraction to glycogenolysis (7). In muscle, as well as in other tissues, phosphorylase a is also formed as a result of hormonal action (8, 9). From kinetic data Danforth, Helmreich, and Cori (10) have concluded that the increased amounts of phosphorylase a formed under these conditions occur as a result of increased activity of phosphorylase kinase rather than decreased activity of phosphorylase phosphatase. Furthermore, studies on the purified kinase have revealed properties in keeping with the idea that its activity is readily subject to control (11-13). It is also possible, however, that regulation of phosphorylase phosphatase activity may be involved in determining the relative levels of phosphorylases b and a existing at any given time. Recently it has become apparent that the existence of phospho-dephospho hybrid phosphorylase molecules will have to be taken into account in any complete description of the b to a and a to b interconversion reactions (14).

Early work on phosphorylase kinase showed that this enzyme, like phosphorylase itself, is capable of existing in more than one molecular form (11); these forms have been referred to as nonactivated and activated forms of the enzyme, and differ in affinity for the substrate (12). This difference is especially marked at pH 7 and below, where the nonactivated form of the enzyme has such a low affinity for phosphorylase b as to be essentially inactive under ordinary assay conditions (11). An increase in the ratio of activity at pH 6.8 to activity at pH 8.2 has been used as an indication of kinase activation, which has been shown to occur in intact tissue (15-19) as well as with the isolated enzyme. Nonactivated phosphorylase kinase can be converted to the activated form in vitro by any of three seemingly diverse procedures: (a) preliminary incubation with Ca++ ions plus a protein factor from muscle (11-13, 20), (b) treatment with trypsin (12), and (c) preliminary incubation with ATP plus Mg++ ions (11-13). The last type of activation is accelerated by adenosine 3',5'-monophosphate (cyclic 3',5'-AMP) (21) pre-
sumably accounting for the role of this nucleotide in the ensemble-like series of reactions depicting the mechanism of action of epinephrine on glycogenolysis (22, 23).

In preliminary accounts (22, 24) it has been reported that activation of phosphorylase kinase by ATP is accompanied by phosphorylation of the enzyme. This observation is described in more detail in the present paper. Experiments relating to the mechanism of action of cyclic 3', 5'-AMP in the activation of phosphorylase kinase are also described.

**EXPERIMENTAL PROCEDURE**

**Materials**

**Preparation of Phosphorylase b—**Phosphorylase b was prepared essentially as described previously (25) except that frozen rabbit skeletal muscle from mature rabbits, obtained from Pel Freez Biologicals, Inc., Rogers, Arkansas, was used instead of fresh tissue. The muscle was thawed in a plastic bag under cool (15-20°) running tap water and ground while still cold. Water used for the initial extraction was warmed to 40° in advance so that the final temperature was 25-30° after mixing with the ground muscle. 2-Mercaptoethanol was substituted for cysteine in the preparation. An additional step was introduced to ensure that any phosphorylase a present would be converted to phosphorylase b. This was accomplished by adding 2-mercaptoethanol to the neutral, dialyzed, ammonium sulfate precipitate to a final concentration of 0.03 M and incubating for 1 hour at 30° just prior to the alkaline heat treatment. The final crystallization was carried out in 0.1 mM AMP as described before (12).

Solutions of phosphorylase b were treated with charcoal to remove AMP (25).

**Preparation of Cyclic 3', 5'-AMP Phosphodiesterase—**Rabbits were anesthetized with Nembutal and immediately bled through the jugular veins. The hind leg and back muscles were excised, ground with a meat grinder in the cold room, and homogenized with 3 volumes of neutral 4 mM EDTA at 0° for 1 min with a Waring Blender. The homogenate was centrifuged at 50,000 × g for 30 min at 4°, and the supernatant solution was decanted through glass wool. The extract was adjusted to pH 7.5 with 1 M acetic acid. The resulting suspension was centrifuged at 50,000 × g for 30 min at 4°, and the pellet was suspended in 300 ml of 50 mM Tris-HCl-2 mM EDTA, pH 7.5, per kg of muscle, and the fraction was adjusted to pH 7.5 by the addition of 1 M Tris. This suspension was centrifuged for 180 min at 40,000 rpm with the use of the No. 40 rotor in the Spinco preparative ultracentrifuge. The supernatant was decanted and adjusted to 35% saturation by the addition of solid ammonium sulfate. After standing for 30 min, the suspension was centrifuged and the pellet was collected. The fraction was dialyzed overnight against 50 mM glycerol-2 mM EDTA, pH 6.1. The dialyzed enzyme was centrifuged to remove any precipitated protein and adjusted to pH 7.0. This preparation, which represents a 25- to 35-fold purification over the initial extract, could be stored frozen for a period of months without appreciable loss in activity.

**Other Materials—**32P-Pi in weak HCl (usually 60 mC) was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee. It was passed through a column, 0.9 × 9.0 cm, of Dowex 50-X2 in the H+ form to remove contaminating metal ions and then lyophilized to dryness over NaOH. γ-32P-ATP was prepared by a procedure combining features of the methods of Tanaka, Mano, and Shimazono (26) and Jones (27). Flaked KCNO (1.6 moles) was added to 0.8 ml of 1 M KH2PO4 containing 60 mC of 32P-Pi, and the solution was warmed at 30° for 1 hour to prepare 32P-carmabyl phosphate. The latter was not isolated but was used immediately to form γ-32P-ATP in a reaction mixture containing 0.01 M magnesium acetate, 0.015 M ADP, and 0.2 m potassium acetate buffer, pH 5.5, with a final volume of 74 ml to which was added 1 ml of a crude sonic extract of Streptococcus faecalis R. (27) as a source of carbamyl phosphokinase. After 20 min at 30° the mixture was heated to 95° for 1 min, collod, and centrifuged to remove denatured protein. The pH of the supernatant solution was adjusted to 7.0 with KOH and the γ-32P-ATP was purified on Dowex 1 as described by Hurwitz et al. (28), except that following elution of ADP with 4 M formic acid the resin was converted to the carbonate form as described by Cohn (29) and the γ-32P-ATP was eluted with 0.8 M NH4HCO3. The NH4HCO3 was readily removed by lyophilization. β, γ-32P-ATP was prepared by oxidative phosphorylation in the reaction mixture described by Kielley and Kielley (30) and purified as above. α-32P-ATP was a commercial sample obtained from International Chemical and Nuclear Corporation, City of Industry, California.

**H-Cyclic 3', 5'-AMP** was obtained from Schwarz Bio-Research and was purified by thin layer chromatography before use as follows. The material was spotted on a thin layer plate covered with MN cellulose powder 300 (Macherey, Nagel and Company, Düren, Germany). Development was carried out with isopropyl alcohol-NH4OH-0.1 M boric acid (7:1:2); the cyclic 3', 5'-AMP spot, detected under ultraviolet light, was scraped off and the nucleotide was eluted with water.

Shellfish glycogen, obtained from Krishi Laboratories, Inc., Portland, Oregon, was purified by the Somogyi procedure (31) before use.

**Methods**

Unless noted otherwise, activation of phosphorylase kinase was carried out by incubating the enzyme at 30° in reaction mixtures containing 0.1 to 10.0 mg of nonactivated phosphorylase kinase per ml, 17 mM glycerol-P, 0.7 mM EDTA buffer, pH 6.8, 12 mM magnesium acetate, and 3.6 mM ATP or γ-32P-ATP. When cyclic 3', 5'-AMP was included in the activation reaction, its concentration was 0.1 mM unless noted otherwise. Aliquots were removed at intervals and diluted 1:30 or more in neutral 15 mM cysteine, which stopped the activation process. Kinase activity determinations at pH 6.8 and 8.2 (12) were either carried out immediately or delayed (determinations could be delayed up to 1 hour with identical results provided that dilution tubes were kept at 0° during this time). Protein-bound 32P in phosphorylase kinase was determined on 0.1- to 1.0-mg samples of the enzyme which were added to 1.0 ml of 0.2% bovine serum albumin and immediately precipitated with an equal volume of 10% trichloroacetate acid. The mixtures were allowed to stand at 0° for 15 min, then centrifuged, and the supernatants were decanted. The pellets were dissolved in 1.0 ml of 0.1 N NaOH and the protein was reprecipitated with trichloroacetic acid. Centrifugation and decantation were repeated. The pellets were then washed with 3 ml of 5% trichloroacetic acid and again collected by centrifugation. The well drained pellets were dissolved in 1 ml of 23 M formic acid and aliquots were counted. Control experiments showed that a third precipitation with trichloroacetic acid caused no further reduction in the amount of radioactivity per mg of protein.
Phosphorylase activities were determined by the method of Cori, Cori, and Green (32). Phosphofructokinase assays were carried out by the method described earlier (33), phosphorylase phosphatase assays by the method of Hurd (34), and glycogen synthetase assays by the method of Kornfeld and Brown (35).

Cyclic 3',5'-AMP phosphodiesterase activities were measured by an assay involving the production of 3H-adenosine from 3H-carried out by the method described earlier (33), phosphorylase nucleotidase. Reaction mixtures contained 0.2 ml of 0.2 M Tris cyclic 3',5'-AMP by the action of diesterase and snake venom moles of 3H-cyclic 3',5'-AMP hydrolyzed, based on the specific activity. The reaction was allowed to proceed for 10 min at 30°C; it was stopped by placing the sample in a boiling water bath for exactly 1 min. The sample was cooled and applied to a column, 4 x 0.5 cm, of Dowex 1-Cl⁺ previously washed with water. The column was then washed with sufficient water to give a final volume of 12 ml of combined initial effluent and wash; 1 ml of this was counted with the scintillation counter. The radioactivity in a blank fraction was carried out as reported (12) with the following exceptions. (a) The acid precipitate fraction was brought to a final volume of 200 ml/1,000 g of muscle used in the preparation instead of 165 ml. (b) The 30-supernatant fraction was not frozen. When it was necessary to interrupt the preparation at this point, the fraction could be stored unfrozen at 0° overnight before proceeding. (c) The 40-precipitate fraction was brought into solution gently at 0° over an 8- to 12-hour period rather than dissolving the enzyme by incubating for several hours at 20-25° as originally described.

Additional purification of phosphorylase kinase beyond the 40-precipitate stage was achieved by the use of ammonium sulfate fractionation and filtration through Sephadex G-200. Because the kinase proved to be somewhat labile at high salt concentrations, the first procedure was carried out quickly under carefully controlled conditions as follows. Cold, neutral, saturated ammonium sulfate was added dropwise to the 40-precipitate fraction with continuous stirring at 0° to a final concentration of 30% saturation. The mixture was allowed to stand at 0° for 30 min and then centrifuged at 15,000 x g to collect the precipitated protein. This was taken up in small amounts of 50 mM glycerol-P·2 mM EDTA buffer, pH 6.8, to a final volume of 6 ml/1,000 g of muscle, and this solution was applied to a column, 2.5 x 80 cm, of Sephadex G-200 previously equilibrated with the same buffer; the enzyme was then eluted with this buffer at a rate of 8 to 15 ml per hour. Fractions of 4 ml were collected, and those which contained the first protein peak coming through the column were pooled; a small, retarded peak and the trailing edge of the main peak were discarded.

The additional purification steps increased the specific activity of phosphorylase kinase 1.5- to 2-fold over the 40-precipitate stage, i.e., to 45,000 to 60,000 units per mg at pH 8.2; occasionally, higher specific activities were achieved. The ratio of activity at pH 6.8 to activity at pH 8.2 was between 0.02 and 0.05, indicating that the enzyme had remained in the non-activated form (12); after storage for several weeks this ratio often increased to 0.1 to 0.2. The free boundary electrophoretic pattern showed only one major peak in 0.1 ionic strength phosphate buffer at pH 6.3, pH 6.9, and pH 7.8 or in 0.02 ionic strength buffer at pH 6.9 (illustrated for one of these conditions in Fig. 1). On ultracentrifugation (Fig. 2) the enzyme sedimented as a single, somewhat asymmetric peak except for a small amount of heavier material. One of the major contaminants of

![Image](http://www.jbc.org/)

**RESULTS**

**Purification of Phosphorylase Kinase**—The method for purification of phosphorylase kinase was modified and extended from the procedure given earlier (12); some of these modifications have already been described (40). All steps through the second ultracentrifugation step, i.e., those leading to the 40-precipitate fraction, were carried out as reported (12) with the following exceptions. (a) The acid precipitate fraction was brought to a final volume of 2 ml/1,000 g of ground muscle used in the preparation instead of 165 ml. (b) The 30-supernatant fraction was not frozen. When it was necessary to interrupt the preparation at this point, the fraction could be stored unfrozen at 0° overnight before proceeding. (c) The 40-precipitate fraction was brought into solution gently at 0° over an 8- to 12-hour period rather than dissolving the enzyme by incubating for several hours at 20-25° as originally described.

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1 In the published procedure for preparing phosphorylase kinase (12) two differential centrifugation steps are carried out. One of these is performed at 30,000 rpm and the supernatant fluid (30-supernatant fraction) is saved. The other centrifugation step is carried out at 40,000 rpm and the precipitate (40-precipitate fraction) was saved.

2 Although freezing of the 30-supernatant fraction should be avoided in preparing pure phosphorylase kinase, the fraction can be stored frozen when used for assaying cyclic 3',5'-AMP (41). For the latter use, it is advantageous to divide a preparation into a number of individual tubes, each of which is thawed when needed.
the 40-precipitate fraction, phosphofructokinase, which constitutes about one-fourth to one-third of the total protein at that stage (42), was greatly reduced in the purified preparation, i.e. to approximately 1% of the total protein based on a specific activity of 160 for pure phosphofructokinase (33). Another enzyme contaminant, cyclic 3',5'-AMP phosphodiesterase, which was detectable at the 40-precipitate state, was not reduced in amount by the subsequent treatment; its activity in these fractions was generally in the range of 0.5 to 1.0 unit per mg of protein. Phosphorylase b activity was reduced from about 100 units per mg in the 40-precipitate fraction to 5 units per mg in the Sephadex fraction, i.e. to 0.3% of the total protein on the basis of a specific activity of 1,600 units per mg for pure phosphorylase b (25). The purified kinase was essentially free of phosphorylase phosphatase, amylo-1,6-glucosidase, and glycogen synthetase.

Activation and Phosphorylation of Phosphorylase Kinase—Activation of purified phosphorylase kinase by ATP was studied with the use of γ-32P-ATP to determine whether or not the enzyme is phosphorylated in the reaction. Fig. 3 gives the result of a typical experiment carried out in the presence and in the absence of cyclic 3',5'-AMP, which is known (12, 13) to accelerate the activation reaction. It can be seen that 32P was incorporated into the enzyme in a trichloroacetic acid-precipitable form and that the rate of incorporation was increased by cyclic 3',5'-AMP (Curves A' and B'). The effect of this nucleotide on 32P incorporation was not as great, however, as that which it had on kinase activation (Curves A and B). Activation of the kinase showed an initial lag period in the absence of cyclic 3',5'-AMP; this was apparent in Curve B of Fig. 3 and was confirmed in other experiments in which more early points were taken. No lag was apparent in the uptake of 32P by the enzyme. Treatment of the kinase with cyclic 3',5'-AMP phosphodiesterase prior to activation had no effect on the reaction when the final Sephadex G-200 fraction was used as the source of enzyme. In experiments with the less pure, 40-precipitate fraction, however, diesterase treatment diminished the activation rate in the absence of added cyclic 3',5'-AMP, showing that this fraction was contaminated with traces of the nucleotide.

The maximum level of phosphate incorporation observed in activation experiments with labeled ATP was approximately 3

![Fig. 1. Free boundary electrophoresis of phosphorylase kinase in 0.1 ionic strength phosphate buffer, pH 6.9, after 8700 sec at 6.1 volts per cm. Mobility was -6.5 × 10^-4 cm^2 volt^-1 sec^-1.](image)

![Fig. 2. Ultracentrifuge pattern of phosphorylase kinase. Protein concentration was 8.4 mg per ml. Solvent was 50 mM glycerol-P-2 mM EDTA, pH 6.8. Sedimentation is from left to right; pictures were taken at 24, 32, and 40 min. Bar angle, 70°; temperature, 4°; s20,w, 22.4.](image)

![Fig. 3. Activation and phosphorylation of phosphorylase kinase. Phosphorylase kinase, at a concentration of 4.0 mg per ml, was incubated with γ-32P-ATP and magnesium acetate in the presence (Curves A and A') or absence (Curves B and B') of cyclic 3',5'-AMP as described in "Methods." At intervals aliquots were removed and assayed for kinase activity at pH 6.8 (O--O) and for incorporation of 32P into protein (O- - -O).](image)

moles/10⁵ g of phosphorylase kinase. No radioactive label was incorporated during activation of the kinase with α-32P-ATP, but the same amount of phosphate incorporation was observed with either β, γ-32P-ATP or γ-32P-ATP, showing that only the terminal phosphate group of ATP was transferred to the enzyme. Evidence is presented in the following paper (43) that serine residues in phosphorylase kinase constitute the site (or sites) of phosphorylation in the enzyme.

Significance of Bound Phosphate in Phosphorylase Kinase—It appeared possible that all or a part of the phosphate transferred from ATP to phosphorylase kinase might have a direct role in the kinase reaction itself, i.e. in the phosphorylation of phosphorylase b by ATP. Several experiments were carried out to gain information on this point. In one of these, the kinase was first labeled with 32P by preliminary incubation with γ32P-ATP, as in the experiment of Fig. 3, after which the excess labeled ATP was removed by the use of Sephadex G-25 column. This enzyme, 6.4 mg containing 1.9 moles of bound 32P per 10⁵ g of protein, was then incubated with 16 mg of phosphorylase b in 5 ml of 36 mM glycerol-P-1.4 mM EDTA-4 mM cysteine, pH 6.8, for 2 hours at 30°. At the end of this period the phosphorylase was separated
Activation of Phosphorylase Kinase by ATP

The rate of activation was found to be dependent on the concentration of ATP. The phosphorylase a was separated from the kinase and analyzed for protein-bound $3^P$; no significant radioactivity was found. In experiments similar in design to the above it was determined that the protein-bound phosphate of phosphorylase kinase does not exchange with the phosphate of ATP, ADP, cyclic 3',5'-AMP, or inorganic phosphate.

Kinetics of Phosphorylase Kinase Activation Reaction—A detailed analysis of the kinetics of the phosphorylase kinase activation reaction could not be carried out readily because of the margin of error inherent in the multistep assay system. Instability of the enzyme in the presence of Mg$^{2+}$ ions (12) presented another problem. Nevertheless, limited studies were done in an attempt to clarify the mechanism of the reaction. The rate of activation was found to be dependent on the concentration of phosphorylase kinase in the activation reaction mixture. This is shown in the experiment of Fig. 4, depicting the time course of activation at four phosphorylase kinase concentrations with the other components fixed at high levels. Since the ordinate of Fig. 4 is in terms of specific enzyme activity, the experiment can be interpreted as indicating that progressively smaller proportions of the kinase present are activated in a given period of time as the enzyme concentration decreases. This result supports the concept that the activation reaction involves more than simple interaction between ATP and the kinase, since such a reaction would probably be first order with respect to phosphorylase kinase when ATP is in excess. It is more likely that the reaction is enzyme catalyzed and that dilution results in a simultaneous lowering of substrate (phosphorylase kinase) and enzyme concentrations. The enzyme involved could be phosphorylase kinase itself or a contaminant in the preparation.

As noted earlier (Fig. 3), and as is evident in the experiment of Fig. 4, the phosphorylase kinase activation reaction displays a lag which is seen most clearly when the activation rate is low. This suggested that the reaction is autocatalytic and that the phosphorylase kinase, activated with respect to the phosphorylation of phosphorylase b, is also activated with respect to catalysis of its own activation reaction. Support for this concept was obtained by the experiment of Fig. 5, in which nonactivated kinase was used as a substrate for the activated enzyme. Curve A (Part A) shows the time course of kinase activation at a kinase concentration of 0.16 mg per ml. Curve D was an identical reaction mixture, to which an additional 0.16 mg of kinase in the activated form per ml was added at Arrow 1. Curve C was a control, giving the level of activity of the activated kinase introduced in D and also showing that the enzyme underwent no additional activation on incubation with ATP. Curve B was a control containing initially 0.16 mg of nonactivated kinase per ml to which an identical amount of nonactivated enzyme was added at Arrow 1. In Curve C an additional activation on incubation with ATP. Curve B was a control containing initially 0.16 mg of nonactivated kinase per ml to which an identical amount of nonactivated enzyme was added at Arrow 1. In Curve C an additional activation on incubation with ATP. Curve B was a control containing initially 0.16 mg of nonactivated kinase per ml to which an identical amount of nonactivated enzyme was added at Arrow 1.

Specificity of Phosphorylase Kinase—The experiment of Fig. 5 suggested that phosphorylase kinase catalyzes its own activation.
which is related to the phosphorylation of the enzyme as shown in Fig. 3. It was therefore of interest to determine how specific phosphorylase kinase is in relation to phosphorylation of proteins in general. Accordingly, experiments were carried out in which the kinase was incubated with a number of potential protein substrates in the presence of $\gamma$-ATP under conditions similar to those in activation reaction mixtures. The ratio of kinase to substrate ranged from 1:50 to 1:1 on a protein basis. With bovine serum albumin, chicken muscle lactic dehydrogenase, aldolase, phosphofructokinase, phosphoglucomutase, and glyceraldehyde-3-P dehydrogenase (the last four all from rabbit skeletal muscle) as substrates, no phosphorylation was seen. Casein, however, proved to be a substrate for phosphorylase kinase, as is shown in Table I. A relatively slow but definite phosphorylase kinase-catalyzed phosphorylation of this protein by $\gamma$-ATP took place, and the reaction was more rapid with activated kinase than with nonactivated enzyme. Activation of phosphorylase kinase in the absence of cyclic AMP was postulated to be an allosteric effector. Direct participation could result in dephosphorylation of cyclic AMP containing $350,000$ cpm. After 8 min at 30°, the kinase, which had been activated 3.5-fold, was precipitated with trichloracetic acid, washed, and counted. No tritium was present in the protein fraction. The supernatant fluid was analyzed for phosphorylase kinase activation by paper chromatography on Dowex 1 (formate) according to the procedure of Hurlbert et al. (28) followed by thin layer chromatography on cellulose plates.

All of the radioactivity was associated with the cyclic 3',5'-AMP fraction, which was recovered to the extent of $98\%$ as cyclic 3',5'-AMP containing 250,000 cpm. After 8 min at 30°, the kinase, which had been activated 3.5-fold, was precipitated with trichloracetic acid, washed, and counted. No tritium was present in the protein fraction. The supernatant fluid was analyzed for phosphorylase kinase activation by paper chromatography on Dowex 1 (formate) according to the procedure of Hurlbert et al. (28) followed by thin layer chromatography on cellulose plates. All of the radioactivity was associated with the cyclic 3',5'-AMP fraction, which was recovered to the extent of $98\%$ as compared to a control in which phosphorylase kinase was omitted from the activation reaction mixture. In another control with a complete reaction mixture containing $\gamma$-ATP it was determined that 10 mmoles of phosphate were transferred to the kinase under the conditions of this experiment. It could thus be calculated that total cyclic 3',5'-AMP destruction was at most 0.3% of the phosphate transferred from ATP to the enzyme.

The foregoing experiment indicated the lack of necessity for destruction of cyclic 3',5'-AMP in the activation of phosphorylase kinase. An alternative mechanism of action, still involving covalent changes in the cyclic nucleotide during the reaction, would be one in which cyclic 3',5'-AMP is regenerated. For example, the phosphate could be donated to a seryl residue

### Table I

<table>
<thead>
<tr>
<th>Experiment No. and proteins present during incubation</th>
<th>$^{32}$P bound to protein at 30 min</th>
<th>$^{32}$P bound to protein at 60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Nonactivated kinase</td>
<td>0.3</td>
<td>1.3</td>
</tr>
<tr>
<td>2. Activated kinase</td>
<td>2.0</td>
<td>1.8</td>
</tr>
<tr>
<td>3. Nonactivated kinase + casein</td>
<td>5.1</td>
<td>10.5</td>
</tr>
<tr>
<td>4. Activated kinase + casein</td>
<td>9.5</td>
<td>15.7</td>
</tr>
</tbody>
</table>

Several approaches were used in attempts to determine the mechanism through which cyclic 3',5'-AMP exerts its effect on phosphorylase kinase activation. These fell into two categories, the first of which involved possible mechanisms in which the nucleotide would participate directly as a coenzyme in a transphosphorylation reaction between ATP and the kinase, while in the second category cyclic 3',5'-AMP was postulated to be an allosteric effector. Direct participation could result in destruc-

![Fig. 6. Phosphorylase kinase activation rates with various cyclic 3',5'-AMP concentrations.](image-url)

Activation reaction mixtures at 30° contained 18 mM glycerol-P, 12 mM Tris, 0.3 mM EDTA buffer, pH 6.8, 3 mM cysteine, 12 mM magnesium acetate, 3.6 mM ATP, 0.3 mg of phosphorylase kinase per ml, and cyclic 3',5'-AMP as shown. Time of activation was 2 min; at the highest concentration of cyclic 3',5'-AMP used the extent of activation was 50% of the maximum achieved in 15 min. No activation occurred in the absence of cyclic 3',5'-AMP in this experiment. The inset shows a reciprocal plot of the data.

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of the enzyme and the cyclic nucleotide regenerated by phosphorylation of the adenylate-protein with ATP. Thus, in the presence of excess cyclic 3',5'-AMP, one might expect phosphate exchange into the cyclic nucleotide. To test this possibility, the enzyme was activated in the presence of γ-32P-ATP and an equimolar amount of cyclic 3',5'-AMP. The reaction was stopped and the reaction mixture was chromatographed on Dowex 1. No 32P was eluted in association with the cyclic 3',5'-AMP; all of the radioactivity was recovered as phosphoprotein, unreacted γ-32P-ATP, and a trace of inorganic phosphate.

In attempting to see whether the effect of cyclic 3',5'-AMP on phosphorylase kinase activation might be due to an allosteric type of interaction of the nucleotide with the kinase, the sensitivity of the kinase to denaturation by heat or urea was determined in the presence and in the absence of the compound. No protective effects were noted. Furthermore, incubation of phosphorylase kinase with iodoacetic acid under conditions leading to a 50% loss of activity was not influenced by cyclic 3',5'-AMP.

An attempt to demonstrate the direct binding of 3H-cyclic 3',5'-AMP was carried out by gel filtration of phosphorylase kinase on Sephadex G-50 in the presence of various concentrations of the tritiated nucleotide and other cofactors. Experiments of this type were complicated by the fact that one of the contaminants of phosphorylase kinase, phosphofructokinase, binds cyclic 3',5'-AMP tightly (37), and the presence of this enzyme to the extent of approximately 1% of the total protein had to be taken into account. At concentrations of cyclic 3',5'-AMP varying from 0.014 to 10 μM the maximum binding by the enzyme preparation was 1 mole/2.5 X 10⁵ g of protein. Calculations showed that this small amount of binding could in theory be due to the contaminating phosphofructokinase; however, inclusion of 1 mM ADP or ATP, which should have eliminated binding of cyclic 3',5'-AMP to phosphofructokinase (37), decreased the binding only slightly. The addition of Mg++ (6 mM) or Mg++ plus theophylline to inhibit diesterase activity did not increase the binding. In the presence of Mg++ and ATP, a mixture in which phosphorylase kinase phosphorylation was taking place, the extent of binding of cyclic 3',5'-AMP was the same as with ATP alone.

### Table II

*Effects of glycogen and cyclic 3',5'-AMP on phosphorylase kinase activation and phosphorylation rates*

<table>
<thead>
<tr>
<th>Tube No. and addition</th>
<th>Increase* in phosphorylase kinase activity at pH 6.8</th>
<th>Protein-bound 32P</th>
<th>Glycogen in precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>1,500 units/mg</td>
<td>0.27 mole/10⁵ g</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>2. Glycogen, 0.8%</td>
<td>4,000</td>
<td>0.66</td>
<td>1.5 mg</td>
</tr>
<tr>
<td>3. Cyclic 3',5'-AMP, 1 X 10⁻⁴ M</td>
<td>16,400</td>
<td>0.74</td>
<td>2.1 mg</td>
</tr>
<tr>
<td>4. Glycogen, 0.8%, + 1 X 10⁻⁴ M cyclic 3',5'-AMP</td>
<td>24,800</td>
<td>1.4</td>
<td>2.3 mg</td>
</tr>
</tbody>
</table>

* Phosphorylase kinase activity at pH 6.8 before activation was 2,300 units per mg.

### Table III

*Formation of glycogen-phosphorylase kinase complex*

ATP-free, activated phosphorylase kinase, 3.8 mg of protein per tube, was incubated for 30 min at 25° with various concentrations of glycogen in the presence of 10 mM magnesium acetate in 25 mM glycerol-P, 0.6 mM EDTA buffer, pH 6.8. The final volume was 2.0 ml. The flocculent precipitate which formed was collected by centrifugation for 15 min at 2000 × g and analyzed for glycogen (48) and protein. A slight precipitate was visible in the absence of glycogen.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Glycogen concentration</th>
<th>Protein in precipitate</th>
<th>Glycogen in precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>1.5</td>
<td>0.28</td>
</tr>
<tr>
<td>3</td>
<td>0.60</td>
<td>2.1</td>
<td>0.63</td>
</tr>
<tr>
<td>4</td>
<td>1.2</td>
<td>2.2</td>
<td>0.84</td>
</tr>
<tr>
<td>5</td>
<td>4.0</td>
<td>2.3⁴</td>
<td>0.93</td>
</tr>
</tbody>
</table>

* The phosphorylase kinase used in this experiment was the 40-precipitate fraction (12), which was only 90% pure, 2.3 mg of protein accounted for all the kinase present.

### Interaction of Phosphorylase Kinase with Glycogen—In a previous study (12) it was shown that glycogen increases the rate of the phosphorylase kinase activation reaction. Additional experiments were carried out to determine whether the increased rate of activation is also reflected as a similar effect on phosphorylation of the enzyme. The interrelationship between glycogen and cyclic 3',5'-AMP was also examined. Table II gives the results of an experiment in which glycogen and cyclic 3',5'-AMP were tested separately and together to determine their effects on activation and phosphorylation rates. The conditions of the experiment were similar to those of Fig. 3; the activation data are presented as the increase in activity of phosphorylase kinase at pH 6.8 over zero time levels based on a 5-min point. It can be seen that 0.8% glycogen increased the rate of activation about 2.5-fold and the rate of phosphorylation approximately 2-fold. Cyclic 3',5'-AMP by itself increased the activation rate more than 10-fold, but only tripled the rate of phosphorylation. Glycogen and cyclic 3',5'-AMP together were more effective than either alone. It should be noted that each substance was used at a concentration at which it was known to exert its maximal individual effect as determined in separate experiments.

In carrying out experiments of the type illustrated in Table II it was noticed that activation reaction mixtures containing glycogen became turbid within 10 to 15 min, with the eventual formation of a flocculent, white precipitate. The precipitate was readily soluble in buffer containing EDTA and was found to contain essentially all of the kinase activity and significant amounts of glycogen. When activation reactions were carried out with γ32P-ATP under these conditions, all of the protein-bound 32P was likewise present in the flocculent precipitate. Activated phosphorylase kinase, free of the activating components, was prepared and used to study this reaction, which was found to be dependent on the presence of Mg2⁺ ions as well as glycogen. Table III gives the results of an experiment in which the composition of the complex formed with increasing glycogen concentration was determined.² At the point of maximal pre-
Discussion

The additional purification steps described in this paper lead to a preparation of phosphorylase kinase that shows one major components on electrophoresis and in the ultracentrifuge. Assuming that this component does in fact represent the kinase, it can be calculated that this enzyme constitutes slightly more than \(1\%\) of the soluble proteins of rabbit skeletal muscle, since the specific activity of the enzyme in crude extracts is about 450 units per mg (12) and the purified preparation has an activity of 45,000 to 60,000 units per mg. Phosphorylase itself makes up 2\% of the soluble protein of rabbit muscle (47, 25). Thus on a weight basis the ratio of phosphorylase b to phosphorylase kinase would be around 2:1 under conditions in which all of the phosphorylase was present in the b form. On a molar basis the ratio would be higher, perhaps 10:1 or 15:1, since the molecular weight of phosphorylase kinase having an \(\epsilon_{280}\) value of 22.4 S is probably greater than \(1 \times 10^4\) and that of phosphorylase b is 185,000 (48).

It is hard to understand why there should be so much phosphorylase kinase in muscle when its only function is to regulate the activity of phosphorylase. On the other hand, considering the fact that in this particular enzyme-substrate relationship the substrate is a large protein molecule which diffuses relatively slowly when compared with low molecular weight substrates, it is perhaps not surprising to find that the kinase is quite abundant. In addition, in view of the tight binding of phosphorylase to glycogen which is known to occur in vitro (49, 46), it is probable that the diffusion of phosphorylase b is further restricted by virtue of such binding within the cell. In this connection it is of interest that phosphorylase kinase also binds to glycogen. It would seem probable that this property facilitates contact between phosphorylase b and the kinase and could provide a possible explanation for the regulatory role that glycogen itself has in metabolism (50).

One of the most interesting properties of phosphorylase kinase is the activation which occurs on preliminary incubation with Mg\(^{++}\)-ATP and the effect of cyclic 3',5'-AMP on this reaction. Activation is characterized by an increase in the ratio of activity at pH 6.8 to activity at pH 8.2, and is accompanied by phosphorylation of the enzyme. Cyclic 3',5'-AMP increases the rate of activation and also increases the rate of phosphorylation, although its effect on the latter process is not as great as the effect on activation. It is attractive to hypothesize that activation of the kinase occurs as a result of phosphorylation, but all of the evidence does not immediately support this idea. As indicated above, cyclic 3',5'-AMP stimulates activation more than it affects phosphorylation, and a similar phenomenon is seen with glycogen. Furthermore, there is a lack of correlation between kinase activation and phosphorylation during the time course of the activation reaction. Nevertheless, as will be shown in the following paper (43), the concept that phosphorylation causes activation receives support from the finding that activation can be reversed by treating the enzyme with a muscle phosphatase.

Phosphorylation and activation of phosphorylase kinase by ATP apparently take place by a catalytic mechanism and do not occur as a result of simple interaction between the enzyme and the nucleotide. Several observations indicate that the reaction is autocatalytic, i.e. that the enzyme catalyzes its own phosphorylation. The activation reaction shows a noticeable lag period, and the experiment of Fig. 5 indicates that phosphorylase kinase activated by preliminary incubation with Mg\(^{++}\)-ATP appears to be activated with respect to its own activation reaction. In addition, the ability of phosphorylase kinase to catalyze phosphorylation of casein shows that the enzyme is not absolutely specific for phosphorylase b, and its own phosphorylation could be interpreted as simply a further manifestation of something less than absolute specificity. Assuming for the moment that all of these protein phosphorylations are due to phosphorylase kinase, it is interesting to compare the relative activities of the kinase toward the various substrates. On the basis of a specific activity of 60,000 units per mg of nonactivated kinase at pH 8.2, or comparable activity at pH 6.8 after activation, it can be calculated that 4.8 moles of phosphate are transferred from ATP to phosphorylase b per min per mg of kinase. This is in contrast to a rate of only 0.5 to 3 mmoles per min per mg for the rate of phosphorylation of phosphorylase kinase at pH 6.8 (Fig. 3 and Table II), and a rate of 1.6 to 2.5 mmoles per min per mg of kinase for the phosphorylation of casein at pH 7.0 (Table I).

Several possible mechanisms for the action of cyclic 3',5'-AMP on phosphorylase kinase activation have been investigated. These experiments appear to eliminate a mechanism whereby the cyclic nucleotide participates directly in the reaction and is converted to some other nucleotide derivative during the course of the reaction. The formation of an adenylate-protein intermediate is also unlikely in view of the absence of an exchange of phosphate between cyclic 3',5'-AMP and ATP during the course of activation of the enzyme. This leaves an allosteric role for cyclic 3',5'-AMP as the most probable explanation of its action. Huijing and Larner (51) presented evidence that cyclic 3',5'-AMP affects glycogen synthetase kinase by an allosteric mechanism, and these same authors interpreted previously published work from this laboratory (12) as indicating an allosteric role for the nucleotide in phosphorylase kinase activation. The effect to which these workers called attention was an increased sensitivity in the phosphorylase kinase activation system to Mg\(^{++}\) ions in the presence of cyclic 3',5'-AMP (12, 51).

If cyclic 3',5'-AMP participates in the phosphorylase kinase activation reaction by an allosteric mechanism, then it should be possible to demonstrate binding of the nucleotide to whatever enzyme serves as the catalyst for this reaction. The present study, however, indicates that there is only slight binding of cyclic 3',5'-AMP to phosphorylase kinase. The extent of binding under the conditions used was so low that if it were due to phosphorylase kinase it would predict a very high dissociation
constant; evidence against this is the observation that the kinetic half-maximum concentration for activation of the enzyme is in the vicinity of 50 mM. A possibility not excluded, however, is that the binding of the cyclic nucleotide to phosphorylase kinase may require all reaction components and that the nucleotide will not bind to the activated protein. The small extent of binding of cyclic 3′,5′-AMP to phosphorylase kinase in the face of the strong effect of this nucleotide on activation, together with other observations relating to the kinetics of the activation reaction, suggests the possibility that the preparation may be contaminated with catalytic amounts of a phosphorylase kinase. The existence of such an enzyme, which might, for example, have an absolute requirement for cyclic 3′,5′-AMP, would not be in opposition to the autocatalytic mechanism for activation of phosphorylase kinase but could be viewed as supplementing this process. Experiments have shown that crude muscle extract contains factors affecting the activation reaction, and the existence of more than one protein phosphokinase in muscle has been suggested (22, 52, 53). Further work on this point is in progress.

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Robert J. DeLange, Robert G. Kemp, W. Dixon Riley, Ronald A. Cooper and Edwin G. Krebs


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