Factors Affecting the Activity of Muscle Phosphorylase b Kinase

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(Received for publication, February 13, 1959)

Resting muscle contains a higher proportion of phosphorylase b than of phosphorylase a as determined by analysis of extracts (1). Under the influence of epinephrine (2, 3) or with muscle contraction (4), the amount of phosphorylase a increases. Two enzymes which are involved in the interconversion of the two forms of phosphorylase have been described. One of these, phosphorylase b kinase, catalyzes the phosphorylation of phosphorylase b by adenosine triphosphate to give phosphorylase a (5-7); the other enzyme, phosphorylase phosphatase, catalyzes the hydrolytic cleavage of phosphate from phosphorylase a to give phosphorylase b (9). It appears reasonable to assume that the relative activities of these two enzymes would be important factors in determining the proportion of phosphorylase a and b in muscle (4, 11).

It has been found that phosphorylase b kinase exists in fresh rabbit muscle extracts in a form that is inactive at pH 7.0 or below. The enzyme becomes highly active in this region when incubated for a short period of time with Ca++ ions. As it has been suggested that the release of bound Ca++ may be a link between excitation of muscle and contraction (12, 13) or that Ca++ may be mobilized and undergo transfer from one site to another in connection with muscle contraction (14, 15), it is possible that the present effect is related to an important control process. Activation of phosphorylase b kinase would result in the production of phosphorylase a, which in turn catalyzes the first step in glycogenolysis.

The outstanding investigations of Sutherland et al. (16-18) have shown that the increased formation of active liver phosphorylase observed in the presence of epinephrine is mediated by adenosine 3',5'-phosphoric acid formed from adenosine triphosphate. They also have observed the formation of this substance in other tissues including skeletal muscle. No explanation has been given, however, as to how this compound acts in shifting the balance between the active and inactive forms of phosphorylase. In the present investigation it is shown that in muscle extract the effect of adenosine 3',5'-phosphoric acid is in the activation of phosphorylase b kinase. In the activation process, which is apparent when the kinase is assayed below its pH optimum, Mg++ ions and adenosine triphosphate are required for the cyclic nucleotide effect. A preliminary report of this work has been given (19).

METHODS

Materials—Crystalline rabbit muscle phosphorylase b was isolated as described previously (20). Phosphorylase a and P32-labeled phosphorylase a were made from phosphorylase b with ATP and phosphorylase b kinase (7). Samples of crystalline cyclic 3',5'-AMP2 were kindly furnished by Dr. David Lipkin and Dr. Earl W. Sutherland. The glycogen used in the assay of phosphorylase activities was obtained from Krishell Laboratories, Inc., and was freed from traces of AMP3 and other impurities by passage through Dowex 1-X10, 200 to 400 mesh, in the OH- form and Dowex 50-X4, 100 to 200 mesh, in the H+ form. The effectiveness of this treatment was ascertained by spectrophotometric analysis of hydrolyzed glycogen solutions before and after the procedure.

Muscle Extracts—White female rabbits were anesthetized deeply with sodium pentobarbital solution and the blood was drained from the jugular veins. The muscles from the lower extremities and back were removed immediately, packed in crushed ice, and then passed through an ordinary meat grinder in the cold room. Portions of 100 g of the ground muscle were mixed with 200 ml amounts of cold distilled water and homogenized for 1 minute in a Waring Blender. The homogenate was then centrifuged at 4,000 X g for 30 minutes and the supernatant solution was collected after filtering through glass wool to remove traces of lipid material. The extracts were kept at 0° and used within 3 hours. pH adjustments of the extract to neutrality were made by careful addition of 1 N NaOH.

Acid Precipitation—The pH of the extracts was lowered to 5.7 to 5.8 by the addition of 1 N acetic acid. The precipitate which formed was collected by centrifugation at 10,000 X g and partially dissolved in sufficient 0.08 m sodium glycerophosphate to give a final volume approximately 1/5 that of the extract used, and the pH was adjusted to 7.0 by careful addition of 1 N NaOH. This fraction was stored at -20°. On some occasions the solution was made 0.002 m with respect to EDTA before freezing and storage.

2 The abbreviations used are: cyclic 3',5'-AMP, adenosine 3',5'-phosphoric acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

3 In a previous publication (7) the activity of phosphorylase a, as measured in the absence of AMP, was erroneously found to be equal to the activity of this form of the enzyme measured in the presence of AMP. This was due to traces of AMP in the glycogen used in the reaction mixture for the activity test. The slight activity reported for charcoal-treated samples of crystalline phosphorylase b (20), presumably determining in the absence of AMP, was also due to this contaminant.

4 Most of the protein appears to go into solution although the final mixture is still turbid; it can be clarified by centrifugation at 25,000 X g without loss of phosphorylase b kinase.
Phosphorylase b Kinase Activity of Muscle Extracts—Phosphorylase b kinase of fresh rabbit muscle extracts is inactive when tested at pH 7 or below (Curve A of Fig. 1). Above this pH the enzyme is active, reaching its maximum around pH 8.5. This behavior is markedly different from that obtained with the preparations of purified phosphorylase b kinase described previously (6), which had their optimum in the same region but were more than half maximally active at pH 7. Since the kinase activity determinations are carried out at more than a 1000-fold dilution of the extract, it is unlikely that nonspecific effects due to salts or other components in the extract would affect the pH optimum curve appreciably and thus account for the observed difference.

Activation of Phosphorylase b Kinase by Ca++. When muscle extracts are incubated with added Ca++ ions for a short period of time, a striking activation of the kinase results. This activation is made manifest when the kinase activities are determined at pH values below the optimum range (Curve C of Fig. 1). The activation process is rapid with a maximum activity being reached after 3 to 5 minutes of incubation at 30°; the level of activity remains constant when the incubation times are prolonged to 45 minutes. No reversal of the activation is seen when a 2-fold molar excess of EDTA over the Ca++ ions is added after activation has occurred. This concentration of EDTA added before the addition of the Ca++ ions prevents the activation. Other metals studied including Zn++, Mg++, Cu++, Fe++, Mn++, and Ni++ have not been found to be effective in activating the kinase.

Fractionation of “pH 7-inactive” Kinase from Muscle Extract—When the pH of fresh muscle extract is lowered to 5.7 to 5.8 (see “Methods”), the precipitate which forms contains the phosphorylase b kinase. If this precipitate is dissolved in sodium glycerophosphate buffer, pH 7, and assayed the same day, it can be shown that the kinase is present in essentially the same “pH 7-inactive” form as in the original extract (Curve A of Fig. 2). Slight activation may have occurred, however, since there is now detectable activity at pH 7 and lower. On incubation with Ca++ the enzyme is again markedly activated (Curve B of Fig. 2); here there appears to be some increase in activity even at high pH values near the optimum which was not seen to any marked degree in the extracts. If the acid precipitate fraction is stored without EDTA, it is slowly transformed to the activated type of enzyme. Storage with EDTA holds the enzyme in its “pH 7-inactive” state. Once the acid precipitate fraction has been activated with Ca++, it is not converted back to the “pH 7-inactive” form by dialysis against repeated changes of neutral 2 X 10^{-4} M EDTA. The Ca++-activated and -dialyzed kinase does not lose activity when added back to the nonactivated acid precipitate fraction.

Dual Role of Ca++. In the experiments described thus far, the effect of Ca++ on the kinase has been studied by adding the metal to the muscle extracts, incubating for a short period, and then assaying at a high dilution of the treated extract. Under these conditions Ca++ is known to activate the enzyme, and the activated form is so different from the nonactivated form that the two can be readily separated. When muscle extracts are treated with Ca++, however, the enzyme is not converted back to the “pH 7-inactive” state. Once the acid precipitate fraction has been activated with Ca++, it is not converted back to the “pH 7-inactive” form by dialysis against repeated changes of neutral 2 X 10^{-4} M EDTA. The Ca++-activated and -dialyzed kinase does not lose activity when added back to the nonactivated acid precipitate fraction.

Phosphorylase b kinase which is inactive at pH 7.0 and below will be referred to as “pH 7-inactive” enzyme. A change in the kinase so that its activity is increased at pH 7 will be referred to as “activation.”

6 This fraction is stored in the frozen state in plastic tubes. After thawing and use on 2 or 3 successive days, the enzyme will have become activated.

Phosphorylase b Kinase Activities—Phosphorylase b kinase activities were determined at pH values indicated. The usual assay system for measurement of initial reaction rates (see “Methods”) was employed. A sodium glycerophosphate-glycine buffer instead of a sodium glycerophosphate-Tris buffer was used at pH 9.1. In Curve A the extract was diluted and assayed without treatment. In Curves B and C the extract was adjusted to pH 7.0 and incubated for 15 minutes at 30° with either 1 X 10^{-4} M cyclic 3’,5’.AMP or 3 X 10^{-3} M calcium acetate, respectively, before dilution and assay. A control experiment in which extract was adjusted to pH 7.0 and incubated without additions gave a curve identical to Curve A.

FIG. 1. Phosphorylase b kinase activity of muscle extracts; effects of Ca++ and cyclic 3’,5’.AMP. Phosphorylase b kinase activities were determined at a final dilution of 1 to 1,220 of muscle extract at the pH values indicated. The usual assay system for measurement of initial reaction rates (see “Methods”) was employed. A sodium glyceraophosphate-glycine buffer instead of a sodium glyceraophosphate-Tris buffer was used at pH 9.1. In Curve A the extract was diluted and assayed without treatment. In Curves B and C the extract was adjusted to pH 7.0 and incubated for 15 minutes at 30° with either 1 X 10^{-4} M cyclic 3’,5’.AMP or 3 X 10^{-3} M calcium acetate, respectively, before dilution and assay. A control experiment in which extract was adjusted to pH 7.0 and incubated without additions gave a curve identical to Curve A.

Phosphorylase b Kinase Activities—Phosphorylase b kinase activities were determined by a modification of the method described previously (6). Reaction mixtures are made up as follows: 0.1 ml of 0.25 M Tris 0.25 M sodium glyceraophosphate buffer, 0.1 ml of H2O or additions. 0.2 ml of phosphorylase b solution in 0.015 M neutral cysteine, 0.1 ml of kinase solution diluted in cold 0.015 M neutral cysteine, and 0.1 ml of 6 X 10^{-2} M Mg(AC)2.1.8 X 10^{-3} M ATP solution to start the reaction, which is carried out at 30°. The pH of the buffer and of the Mg-ATP solution are adjusted to give whatever final pH is desired. At 5 minutes an aliquot is removed and diluted in 0.04 M sodium glyceraophosphate-0.03 M cysteine buffer containing 0.001 M EDTA, pH 6.8, for assay of phosphorylase activity according to the method of Illingworth and Cori (21). A unit of phosphorylase b kinase activity is defined as 6 (i.e. that amount of enzyme that gives rise to the formation of 100 units of phosphorylase activity (measured without AMP) per ml of kinase reaction mixture in 5 minutes at 30°. The amount of phosphorylase b in the reaction mixture corresponds to about 10,000 units (6 mg) and assays are carried out at sufficient dilution of the kinase so that no more than 1/6 of the phosphorylase b will be converted within 5 minutes. In addition to the measurement of initial reaction rates, as described in the above assay system, the entire time course of the phosphorylase b to a reaction was followed with similar reaction mixtures. In this case aliquots were removed for phosphorylase assay at additional times beyond 5 minutes.

Phosphorylase Phosphatase Activities—Phosphorylase phosphatase activities were determined essentially as described by Keller and Cori (22) except that the phosphorylase a concentration was approximately thirty times greater than in their assay. This required an intermediate dilution before the assay for the phosphorylase.

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Fig. 2. Phosphorylase b kinase activity of acid precipitate fraction before and after activation with Ca++. Curve A: acid precipitate fraction control. Kinase activities were carried out at a 1 to 24,000 final dilution of the fraction at the pH values indicated. Curve B: the acid-precipitate fraction was incubated with \(5 \times 10^{-3}\) M Ca++ for 15 minutes at 30°C. Kinase activities were carried out as in A.

FIG. 3. Effect of Ca++ in the kinase test system with muscle extract as source of enzyme. Fresh muscle extract was assayed at a final dilution of 1 to 1,200. [Ca++] = \(1 \times 10^{-4}\) M in the assay reaction mixture where indicated. The pH of the assay is shown beneath the columns.

conditions Ca++ is present at very low concentrations (of the order of \(10^{-4}\) M) during the kinase reaction itself. When Ca++ is introduced directly into the kinase reaction mixture an interesting dual role for this metal appears (Fig. 3). Now Ca++ acts either as an activator or as an inhibitor, depending upon the pH. Below pH 8 the first of these effects prevails; above this point the enzyme is inhibited. The explanation for this complicated picture lies in the superposition of two separate and opposite actions of this metal. First, as shown above, Ca++ causes a change in the “pH 7-inactive” kinase present in muscle extracts, so that the enzyme becomes active at a lower pH:

**pH 7-inactive** phosphorylase b kinase Ca++

"activated" phosphorylase b kinase (1)

The second action is an inhibition of the phosphorylase b kinase reaction, which proceeds according to Equation 2:

2 Phosphorylase b + 4 ATP Mg++

phosphorylase a + 4 ADP (2)

This inhibition occurs over the entire pH range of activity of the kinase, but below pH 8.0 the effect of forming active kinase as in Equation 1 predominates.

Inhibition of Phosphorylase b Kinase Reaction by Ca++—With the use of an acid-precipitated fraction activated with \(5 \times 10^{-3}\) M Ca++ and then diluted so that the amount of this metal carried over into the assay reaction mixture would be of the order of \(10^{-4}\) M, the inhibitory properties of Ca++ in the phosphorylase b to a reaction itself were studied. Fig. 4 shows the effect of increasing concentrations of Ca++ on the kinase activity; in this experiment with \(1 \times 10^{-3}\) M Mg++ the reaction is inhibited 50% at \(2 \times 10^{-3}\) M Ca++.

Inhibition is noncompetitive with respect to ATP (Fig. 5), but is competitive with respect to Mg++ (Fig. 6). The value of \(K_a\) for Mg++ is \(1.9 \times 10^{-3}\) M and the value of \(K_i\) for Ca++ is \(3.0 \times 10^{-4}\) M under the conditions of the experiment illustrated in Fig. 6.

Effect of Ca++ on Phosphorylase b Kinase Reaction in Presence of EDTA—It appeared to be of interest to study the interrelationship that might exist between Mg++ and Ca++ in the phos-

7 The anomalous plot shows inhibition by high ATP concentrations which is especially marked in the presence of Ca++.
FIG. 5. Effect of varying ATP on the inhibition of phosphorylase b kinase by Ca++. Conditions as in experiment of Fig. 4. ATP concentration as shown. \( [\text{Ca}^{++}] = 1.60 \times 10^{-5} \text{m} \) in A.

Fig. 6. Effect of varying Mg++ on the inhibition of phosphorylase b kinase by Ca++. Conditions as in legend of Fig. 4 except that the source of kinase was a different activated and diluted acid precipitate fraction. \( [\text{Ca}^{++}] = 1.43 \times 10^{-6} \text{m} \) in A.

phorylase b kinase reaction in the presence of an agent that chelated these metals. In the experiment of Fig. 7 increasing amounts of EDTA are added to the assay reaction mixture in the presence (Curve A) and absence of Ca++. (Curve B). The source of kinase is again the activated and diluted acid precipitate fraction. It can be seen that without Ca++ the extent of inhibition increases with increasing EDTA until it becomes essentially complete at the point where the concentration of the chelating agent and Mg++ are equal (1.4 × 10^{-2} m). The reaction with Ca++ (Curve A) is inhibited relative to that without Ca++ (Curve B) at the lower EDTA concentrations, but the reverse is true at the higher EDTA levels. These results can be understood on the basis of the ability of Ca++ to displace the more loosely bound Mg++ from EDTA.

Whether this model system with EDTA is comparable to anything existing in muscle or in muscle extracts is not known. Muscle contains substances (phosphates, nucleotides, proteins, and so forth) which can serve as chelating agents for metals, and under certain circumstances the displacement of one metal by another could serve as a regulatory mechanism.

Effect of Cyclic 3',5'-AMP on Phosphorylase b Kinase Activity—When fresh muscle extracts are incubated with cyclic 3',5'-AMP, their phosphorylase b kinase activity is increased; this is especially noticeable when the kinase assays are carried out at pH values below the optimum for this enzyme (Curve B of Fig. 1). In this latter respect the activation of the kinase in the extract by the cyclic nucleotide resembles the action of Ca++ ions, except that the extent of activation is not as great as with the metal. In contrast to the effect of Ca++ ions, the activation of the kinase by cyclic 3',5'-AMP appears to be transient as is shown in the experiment of Table I.

The activation of phosphorylase b kinase by cyclic 3',5'-AMP requires the presence of a labile factor in the muscle extract. This is evident from the experiment of Table I, where it can be seen that readdition of cyclic 3',5'-AMP after incubation of the extract for 60 minutes fails to reactivate the kinase appreciably. Evidence that the factor lost from the extract during the incubation period might be ATP is seen by the effectiveness of this substance when it is added along with cyclic 3',5'-AMP; moreover, in separate experiments it was shown that sufficient ATPase activity exists in this type of extract to account readily for the destruction of endogenous ATP during an incubation period of 60 minutes at 30°C. The experiment of Table I shows that cyclic 3',5'-AMP is also lost from the extract during the incubation, since addition of ATP alone at 60 minutes has no effect. The requirement for ATP to enable cyclic 3',5'-AMP to act can also...
be shown when an initial dilution of fresh muscle extract is made before its incubation with activating components (Table II). Now, even though the extract contains endogenous ATP, the dilution lowers its concentration to a level where cyclic 3',5'-AMP alone is not effective.

Time Course of Phosphorylase b to a Reaction with "pH 7-inactive" Kinase—It can be seen in the experiment of Table II that incubation of diluted muscle extract with ATP and Mg++ ions caused some activation of phosphorylase b kinase in the absence of added cyclic 3',5'-AMP. This effect was more marked in the incubation carried out at pH 8.4 than in the experiment at pH 7.0. Since ATP and Mg++ ions are both components of the phosphorylase b to a reaction itself, this reaction was studied beyond the 5-minute period used in the measurement of initial reaction rates in the kinase assay. 8 An experiment at various pH's with dilute muscle extract as a source of "pH 7-inactive" kinase, is shown in Fig. 8. At the two lower pH values the reactions are clearly autocatalytic with zero initial reaction rates. With the particular extract used in the experiment of Fig. 8 some phosphorylase a formation was evident at 5 minutes at pH 6.8; this amounted to 6% of that found at pH 6.0. More commonly, as shown with the extracts used in Fig. 1 and Tables I and II, essentially no phosphorylase a was formed at pH 7.0 or lower in 5 minutes, the period of time employed in the usual kinase assay (see "Methods"). As the pH is increased the requirement for activation becomes less apparent, until at pH 8.2 the enzyme seems to be fully active initially.

In another experiment at pH 6.8 (Fig. 9), cyclic 3',5'-AMP is included in the phosphorylase b kinase reaction mixture (Curve B). Its presence does not abolish the lag period, but accelerates the activation process and results in the attainment of a rate which is equal to that shown in Curve C, where the extract has been activated by preincubation with Ca++ ions. In the control reaction (Curve A), without added cyclic 3',5'-AMP the rate is still increasing slowly at 20 minutes. Protein-free filtrates of reaction mixtures similar to that in Curve B at 15 minutes have not been found to contain anything that will abolish the lag phase of the kinase reaction. Heating, addition of perchloic acid, and addition of organic solvents have been used as methods for deproteinization.

The specificity of cyclic 3',5'-AMP for its role in the activation of phosphorylase b kinase has been tested with other nucleotides alone or in combination with ATP by preincubating these substances with "pH 7-inactive" kinase in the presence of Mg++. No other nucleotide was found to be effective. Those tested include: cyclic 2',3'-AMP, 2'-AMP, 3'-AMP, 5'-AMP, ADP, ATP, GMP, GDP, GTP, UMP, UDP, UTP, CMP, CDP, CTP, IMP, IDP, and ITP.

Localization of Effects of Cyclic 3',5'-AMP to Activation of Phosphorylase b Kinase—The problem in localizing an effect that shifts the balance between the phosphorylated and the nonphosphorylated forms of phosphorylase has been discussed by Rall and Sutherland (17). This is especially difficult when extracts or crude fractions containing both phosphorylase kinase and phosphorylase phosphatase are studied under conditions in which both reactions can occur at demonstrable rates. In the present work, however, phosphorylase kinase assays are carried out at dilutions 10-fold higher than the maximum dilution at which any phosphorylase phosphatase activity can be detected in the usual phosphorylase phosphatase activity test.

To eliminate completely the possibility that some special condition might exist in the kinase reaction that would permit phosphorylase phosphatase to act, a control experiment was set up in which a trace of P32-labeled phosphorylase a was included in the kinase reaction mixture. Reactions were carried out under these conditions with fresh muscle extract, extract activated by Ca++ ions, and extract activated by cyclic 3',5'-AMP (Table III). In every instance all the radioactive phosphorus could

<table>
<thead>
<tr>
<th>pH 7.0</th>
<th>pH 6.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>units/ml</td>
<td>units/ml</td>
</tr>
<tr>
<td>No additions</td>
<td>200</td>
</tr>
<tr>
<td>Incubation for 10 minutes after addition of cyclic 3',5'-AMP</td>
<td>3,100</td>
</tr>
<tr>
<td>Incubation continued for 30 minutes</td>
<td>0</td>
</tr>
<tr>
<td>Incubation continued for 60 minutes</td>
<td>0</td>
</tr>
<tr>
<td>Incubation continued for 71 minutes (cyclic 3',5'-AMP readded at 61 minutes)</td>
<td>400</td>
</tr>
<tr>
<td>Incubation continued for 71 minutes (cyclic 3',5'-AMP and ATP added at 61 minutes)</td>
<td>3,400</td>
</tr>
<tr>
<td>No additions. Extract incubated for 71 minutes</td>
<td>100</td>
</tr>
</tbody>
</table>

TABLE II

Activation of phosphorylase b kinase in diluted muscle extract at different pH values

Complete incubation mixtures for the activation of the kinase were made up with the following components: 0.2 ml of 0.125 M 1/100-0.125 M sodium glycerophosphate buffer, 0.1 ml of 5 x 10^{-4} M cyclic 3',5'-AMP, 0.1 ml of 5 x 10^{-2} M Mg(Ac)2, 1.5 x 10^{-2} M ATP, and 0.1 ml of fresh muscle extract diluted 1 to 30. Incubations were carried out for 15 minutes at 30°C and aliquots were removed for assay of phosphorylase b kinase at pH 6.8.

<table>
<thead>
<tr>
<th>pH of incubation mixture after activation</th>
<th>Cyclic 3',5'-AMP and Mg-ATP omitted</th>
<th>Mg-ATP omitted</th>
<th>Cyclic 3',5'-AMP omitted</th>
<th>Complete system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/ml*</td>
<td>units/ml*</td>
<td>units/ml*</td>
<td>units/ml*</td>
</tr>
<tr>
<td>7.0</td>
<td>100</td>
<td>200</td>
<td>1,200</td>
<td>3,700</td>
</tr>
<tr>
<td>8.4</td>
<td>0</td>
<td>0</td>
<td>2,100</td>
<td>4,100</td>
</tr>
</tbody>
</table>

* Calculated back to original muscle extract.
Absence of phosphorylase phosphatase activity during phosphorylase kinase reaction

The kinase reaction mixtures at pH 7.5 contained 0.1 mg of crystalline P-32-labeled phosphorylase a in addition to the regular components. At 5 minutes and at 60 minutes aliquots were removed and the proteins were precipitated with 5% trichloroacetic acid, washed, dissolved in formic acid, plated, and counted as described previously (7). Phosphorylase a determinations were also made on separate aliquots removed at 5 minutes. Theoretical c.p.m. on samples = 330, assuming no phosphatase action.

<table>
<thead>
<tr>
<th>Treatment of extract</th>
<th>Protein-bound radioactive phosphorus at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 minutes</td>
</tr>
<tr>
<td>None</td>
<td>340</td>
</tr>
<tr>
<td>Activation* with 5 X 10^-4 M calcium acetate</td>
<td>398</td>
</tr>
<tr>
<td>Activation* with 1 X 10^-4 M cyclic 3',5'-AMP</td>
<td>325</td>
</tr>
</tbody>
</table>

* The extract showed 13,000 kinase units per ml before activation and 29,000 units per ml after activation with either Ca++ or cyclic 3',5'-AMP.

DISCUSSION

The results reported in this paper are not readily explained on the basis of the information available. It appears certain that phosphorylase b kinase can be extracted from muscle in a form that is not fully active. Seemingly diverse methods are available for the activation of the enzyme. These include: (a) incubation with Ca++, which can be carried out in the crude extract or with a partially purified preparation of the “pH 7-inactive” kinase; and (b) incubation with ATP and Mg++ ions. The latter type of activation is accelerated by addition of cyclic 3',5'-AMP, a substance which has no effect by itself. The studies involving the time course of the phosphorylase b to a reaction at different pH values, with “pH 7-inactive” kinase, suggest that the kinase may be completely inactive initially and appears to be fully active at the higher pH values only because here the activation by ATP is essentially instantaneous. It has not been excluded that ATP may be giving rise to some cyclic 3',5'-AMP in addition to acting in conjunction with this substance in the activation process.

A highly speculative hypothesis to advance in relation to the observed phenomena is that phosphorylase b kinase itself exists in phosphorylated and dephosphorylated forms, which are active and inactive in a manner analogous to phosphorylases a and b. According to this hypothesis an enzyme system consisting of another kinase and a phosphatase might be involved in the activation and inactivation of phosphorylase b kinase. The effects of Ca++, pH, ATP, and cyclic 3',5'-AMP on the state of kinase activity could involve either the activating or inactivating enzymic reactions.

It is possible that phosphorylase b kinase is extracted from...
muscle in the form of an organized complex involving other proteins by which the kinase is inhibited. Activation by Ca++ might occur as the result of disruption of such a complex. The action of ATP and cyclic 3',5'-AMP could also involve some modification of this complex. It is of interest that the acid precipitate fraction of rabbit muscle, which can be isolated containing all the phosphorylase b kinase in its inactive form, also contains large amounts of phosphorylase b (9) and most of the phosphorylase phosphatase (22) of muscle. This fraction exhibits all of the behavior of the original extract in relation to the various types of activation seen (illustrated only with Ca++ in this paper). When the kinase of the fraction is activated by ATP and Mg++, with or without added cyclic 3',5'-AMP, all the endogenous phosphorylase b in the fraction is converted to phosphorylase a.

The phosphorylase b to a reaction appears to be sensitive to a variety of possible control mechanisms including variation of pH, changes in Mg++ or ATP concentrations (6), inhibition by Ca++, and perhaps most important, the activation and inactivation of phosphorylase b kinase reported here. Experiments are being undertaken to determine the state of activity of the kinase in relation to muscle contraction.

SUMMARY

1. Phosphorylase b kinase can be extracted from rabbit muscle in a form which shows no activity at pH 7.0 or lower.
2. The kinase which is inactive at pH 7.0 or below is activated by a short period of incubation with Ca++ ions. This type of activation has not been found to be reversible.
3. The inactive kinase is activated by preincubation with adenosine triphosphate in the presence of Mg++ ions. This activation is enhanced in the presence of added adenosine 3',5'-phosphoric acid.
4. In the phosphorylase b to a reaction itself Ca++ is a competitive inhibitor with respect to Mg++.

* Such a complex could not be large in size, since the “pH 7-inactive” kinase remains in the supernatant solution after centrifugation of muscle extracts at 100,000 × g.

** Unpublished results in this laboratory.

Acknowledgment—The authors wish to acknowledge the excellent technical assistance of Mrs. Marion E. Lord.

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