Phosphorylation and Activation of Red Skeletal Muscle Phosphorylase Kinase Isozyme*

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Stanley W. Tam‡, Rajendra K. Sharma, and Jerry H. Wang
From the Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3

Red skeletal muscle phosphorylase kinase was purified from bovine muscle very near to homogeneity. The purified sample was still contaminated by a trace amount of white muscle isozyme. The purification procedure involved precipitation at pH 5.7, DEAE-cellulose chromatography, and calmodulin-Sepharose 4B column chromatography. The overall yield of the enzyme was approximately 30%. Analysis of the isozymes from bovine and rabbit skeletal muscles showed that all consisted of 4 types of subunits: α or α′, β, γ, and δ. The red muscle isozyme differed from white muscle isozyme only in having α′ instead of α subunit regardless of animal species. The regulatory properties of the bovine red muscle isozymes were found to be similar to the rabbit white muscle isozyme in its dependence on Ca⁡²⁺ for activity, exhibiting a low pH 6.8 to 8.2 activity ratio and being activated by the Ca²⁺-dependent protein kinase and autocatalytic reaction. Several of the regulatory properties between these two isozymes are different, however. While both isozymes contained calmodulin as a subunit, only the rabbit white muscle isozyme is further stimulated by exogenous calmodulin. In the protein kinase-catalyzed reaction, the extent of phosphorylation and activation of rabbit white muscle isozyme depends on the concentration of magnesium acetate. No such differences are observed for the bovine red muscle isozyme. The phosphorylation and activation of phosphorylase kinase in the autocatalytic reaction, both isozymes behave similarly with a rapid first phase of phosphate incorporation and enzyme activation followed by a second slower phase.

Glycogen phosphorylase kinase (ATP:phosphorylase-b phosphotransferase, EC 2.7.1.38) has been purified from rabbit skeletal muscle to a homogenous state (1, 2). The enzyme preparation, however, contains two isozymes which originate from the two types of muscle, red and white skeletal muscles (3). The predominant isozyme of the enzyme preparation is the white muscle isozyme, which is composed of 4 types of subunits called α, β, γ, and δ subunits in decreasing molecular weight (1, 2, 4). The δ subunit has recently been shown to be calmodulin (4). Rabbit muscle phosphorylase kinase contains a slight amount of the red muscle isozyme which differs from the predominant isozyme in having an α′ instead of an α subunit. The α′ subunit is slightly smaller than α subunit (3).

The molecular and regulatory properties of skeletal muscle phosphorylase kinase have been intensively characterized by using the enzyme preparations from rabbit muscle. The purified enzyme usually exists in a nonactivated state which is characterized by its very low activity at pH 6.8 and below, but showing considerable activity at higher pH values (5, 6). The enzyme can be converted to activated states either by limited proteolysis (7) or by an ATP-dependent phosphorylation reaction which is catalyzed by either cAMP or cGMP dependent protein kinases (8, 9), a Ca²⁺-protease-activatable protein kinase (10), or phosphorylase kinase itself (8, 11, 12, 23, 28). Among the various modes of the enzyme activation, the activation by the cAMP-dependent protein kinase-catalyzed and autocatalytic reactions have been the best characterized. In all cases, the activation of phosphorylase kinase involves multiple phosphorylations which occur on both α and β subunits.

For the cAMP-dependent protein kinase-catalyzed reaction, Cohen (1) reported that the enzyme activation correlated with the phosphorylation of a specific serine residue on the β subunit. However, Hayakawa et al. (2) did not observe the correlation of the enzyme activation with any specific phosphorylation of the subunits. Recently, it was found that the subunit phosphorylation of phosphorylase kinase in the cAMP-dependent protein kinase reaction depended on the concentration of Mg²⁺ (13). At a higher concentration of Mg²⁺, 10 mM in contrast to 1 mM, the enzyme was rapidly activated to about 1/2 to 1/3 of the final activation; this activation was then followed by a much slower phase of additional activation (13). The rapid phase of activation appeared to correlate with the specific β subunit phosphorylation; whereas the slow activation correlated with multiple phosphorylations of a subunit. For the autocatalytic reaction, it was noted that the process is greatly inhibited by inorganic and organic phosphates (11). The change of enzyme activity during the auto-phosphorylation reaction correlated with the phosphorylation of both α and β subunits (11, 12).

All the early studies on phosphorylase kinase focused on the rabbit muscle enzyme. Thus, the characterizations mainly apply to the white muscle isozyme which is present as the predominant form. Recently, Cooper et al. (14) have purified bovine cardiac phosphorylase kinase to close to homogeneity and characterized the enzyme in terms of its molecular and regulatory properties.

In this study, we report the purification of the red muscle phosphorylase kinase from bovine skeletal muscle and the characterization of the purified enzyme in terms of its subunit structure and activation by calmodulin. We also examined the

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† Predoctoral fellow of the Muscular Dystrophy Association of Canada.
regulation of the enzyme by phosphorylation reactions catalyzed by either the cAMP-dependent protein kinase or autocatalytic reaction. Subtle differences between the mechanisms of activation of the rabbit white muscle and bovine red muscle phosphorylase kinases are noted.

EXPERIMENTAL PROCEDURES1,2

RESULTS

Purification of Bovine Red Skeletal Muscle Phosphorylase Kinase—Procedures and results for the typical purification of bovine red muscle phosphorylase kinase are presented in the Miniprint. Analysis by SDS-gel electrophoresis revealed that the purified red muscle isozyme still contained trace amounts of white muscle isozyme (Fig. 1). The purified enzyme has a pH 6.8 to 8.2 activity ratio of 0.01 to 0.06 and a specific activity at pH 6.8 of about 2 units/mg of protein.

Comparison of Bovine and Rabbit Muscle Isozymes—The subunit structures of the bovine isozymes purified by using the above procedure were examined by SDS-gel electrophoresis. It revealed that both bovine isozymes contain three types of subunits: α, α ′, β, and γ (Fig. 1, lanes C and D). Other than the contaminating peptides found in the bovine white muscle isozyme preparations, the only difference in terms of electrophoretic mobility is that bovine red muscle isozyme contains the α ′ subunit while bovine white muscle isozyme contains the α subunit. Similarly, as shown previously (3), rabbit red muscle phosphorylase kinase differs from the white muscle isozyme only in containing α ′ instead of α subunit (Fig. 1, lanes E and F). When a mixture of rabbit and bovine white muscle isozyme was subjected to SDS-gel electrophoresis, the resulting electrophoresis pattern shows that subunits of the isozymes from the different species co-migrate.

Stoichiometry of Bovine Red Muscle Isozyme—It is known that phosphorylase kinase purified from rabbit back and leg muscle contained mostly white muscle isozyme (1, 2). Cohen (1) has shown that the molar ratio of subunit αβγ is 1:1:1. The stoichiometry of bovine red muscle isozyme purified according to the above procedure was determined. The enzyme was analyzed by means of SDS-gel electrophoresis according to the method described by Hayakawa et al. (23) at 4 mA/gel for 8 h. After staining and destaining, the gel was scanned at 620 nm (Fig. 2A). Since α ′ and β subunits were not well resolved, the peak area under (α ′ + β) and γ was determined and the mass ratio of (α ′ + β):γ was calculated to be 6.80:1. This mass ratio corresponds to a molar ratio of 1.14:1 for (α ′ + β):γ if molecular weights of the subunits α ′, β, and γ are taken as 133,000 (3), 12,800, and 45,000 (1), respectively. In another set of experiments, the gels were run for 22 h to provide a better resolution between α ′ and β with the loss of γ subunit (14). The molar ratio between α ′ and β calculated in a similar way was 1:1.18 (Fig. 2B). Based on these results, it may be suggested that bovine red muscle phosphorylase kinase consists of the same numbers of α ′, β, and γ subunits.

Purified rabbit skeletal muscle phosphorylase kinase contains 1 mol of δ subunit/mol of αβγ unit (29). This δ subunit has been shown to be identical with calmodulin (4, 30). The possible existence and the stoichiometry of δ subunit in the purified bovine red muscle isozyme are determined. Since calmodulin is poorly stained with Coomassie blue, we determined the amount of δ subunit in phosphorylase kinase by means of its ability to activate calmodulin-dependent phosphodiesterase as described under “Experimental Procedures.” The result indicated that bovine red muscle and white muscle isozyme contain 1.10 and 0.98 mol of δ subunit/mol of α (or α ′) βγ unit, respectively. In addition, rabbit red muscle and white muscle isozymes were found to contain 1.10 and 1.02 mol of δ subunit/mol of α (or α ′) βγ unit. Although the molecular weight of native red muscle phosphorylase kinase was not determined, the enzyme was found to elute from

![Fig. 1. Analysis of phosphorylase kinase isozymes from rabbit and bovine muscles by SDS-gel electrophoresis. A, a mixture of equal amount of red muscle isozyme from rabbit and rabbit skeletal muscle. B, a mixture of equal amount of white muscle isozyme from rabbit and bovine skeletal muscle. C, bovine red muscle isozyme. D, bovine white muscle isozyme. E, rabbit red muscle isozyme. F, rabbit white muscle isozyme. G, sample purified from DEAE-cellulose column chromatography.](image-url)
Muscle Phosphorylase Kinase—Various investigators (1, 2, 8) studied skeletal muscle which contains white muscle isozyme predominantly. In one previous study, it was observed that the activation of the phosphorylation and activation of the phosphorylase kinase appears to be 1.34 million (1). Based on the molecular weights of the isozyme may be calculated to be 1.29 million.

The rabbit white muscle isozyme is stimulated by calmodulin over the same pH range (Fig. 3B). It is also noted that the bovine red muscle isozyme is inert to exogenous calmodulin. Although all the isozymes contain calmodulin as a subunit, only the rabbit white muscle isozyme could be stimulated by exogenous calmodulin in the presence of Ca2+ (15). In the present study, the effects of exogenous calmodulin on bovine red muscle isozyme were further examined and compared (Fig. 3). The rabbit white muscle isozyme is stimulated by calmodulin in essentially the entire pH range tested: 6.8 to 8.2 (Fig. 3A). The extent of stimulation appears greatest at pH 7.5. On the other hand, bovine red muscle isozyme is inert to exogenous calmodulin over the same pH range (Fig. 3B). It is also noted (Fig. 3B) that the bovine red muscle phosphorylase kinase exhibits a pH activity profile which is characteristic of the nonactivated rabbit muscle enzyme. The enzyme is essentially inactive at neutral and acidic pH values but shows marked increase in activity as pH is raised above 7.0.

Comparison of Protein Kinase-catalyzed Phosphorylation and Activation of Bovine Red and Rabbit White Skeletal Muscle Phosphorylase Kinase—Various investigators (1, 2, 8) have carried out extensive studies of the phosphorylation and activation of muscle phosphorylase kinase catalyzed by cAMP-dependent protein kinase. These studies have been carried out mainly using the enzyme preparation from rabbit skeletal muscle which contains white muscle isozyme predominantly. In one previous study, it was observed that the correlation between the activation and phosphorylation of rabbit muscle phosphorylase kinase depended on the concentration of Mg2+ in the cAMP-dependent protein kinase-catalyzed reaction (13).

The bovine red muscle phosphorylase kinase appears similar to the rabbit muscle enzyme in being markedly activated in a cAMP-dependent protein kinase-catalyzed reaction, but different from the white muscle enzyme in showing little dependence on the concentration of Mg2+ in the protein kinase reaction. Fig. 4 shows that, in both 2 and 10 mm Mg2+, the phosphorylation and activation of the phosphorylase kinase isozyme proceeded in two phases: a rapid phase involving the incorporation of 1 to 2 phosphates/phosphorylase kinase monomer, then a gradual and continuous further phosphorylation. Quantitatively, however, both the enzyme activation and the protein phosphorylation were slightly slower at lower concentrations of Mg2+ (Fig. 4B versus 4A). The pH 6.8 to 8.2 enzyme activity ratio changed from 0.01 to 0.59 following phosphorylation of the red muscle isozyme.

Relationship between Subunit Phosphorylation and Enzyme Activation—To test the relationship between the enzyme activation and protein phosphorylation, bovine red skeletal muscle phosphorylase kinase was phosphorylated in the cAMP-dependent protein kinase-catalyzed reaction. The subunits of the phosphorylated enzyme were then separated and their radioactivity contents measured. Similar to that observed for the rabbit muscle phosphorylase kinase, only the α′ and β subunits of the bovine red muscle isozyme were phosphorylated. Fig. 5 shows the subunit phosphorylation patterns of the red muscle isozymes catalyzed by protein kinase at 2 (Fig. 5, A and C) or 10 mm Mg2+ (Fig. 5, B and D). It may be seen that essentially all the incorporated phosphate is associated with the α′ and β subunits (Fig. 5, A and B). The high radioactivity at the bottom of the gel was due to free

Regulation of Phosphorylase Kinase

Fig. 2. Densitometric tracing of the bovine red muscle isozyme after SDS-gel electrophoresis. A, the gel was run at 4 mA/gel for 8 h. B, the gel was run at 4 mA/gel for 22 h.

Fig. 3. The pH dependence of the activity of phosphorylase kinase. A, rabbit white muscle isozyme, B, bovine red muscle isozyme. The reactions were carried out at various pH values in 25 mm β-glycerophosphate, 25 mm Tris, 10 mm Mg2+, 15 mm β-mercaptoethanol, 4 mg/ml of phosphorylase b, 1 mm [γ-32P]ATP in the presence of 1.13 μg/ml of calmodulin and 0.1 mm Ca2+(○) and 0.1 mm Ca2+ alone (△).
affect a better separation of \( \alpha' \) and \( \beta \) subunits, the radioactivity associated with the \( \alpha' \) subunit could be seen to be much higher than those with the \( \beta \) subunit.

The time course of the subunit phosphorylation of the bovine red muscle phosphorylase kinase was examined and the results for the phosphorylation reaction at 10 mM Mg\(^{2+}\) is shown in (Fig. 6). The phosphorylation of \( \alpha' \) and \( \beta \) subunits followed different time courses. Subunit \( \beta \) was phosphorylated very rapidly to reach the incorporation of 0.8 mol of phosphate/subunit in about 5 min. Little additional phosphorylation of this subunit was observed over the next hour of incubation. In contrast, subunit \( \alpha' \) was phosphorylated rapidly to incorporate 1 mol of phosphate/subunit after 8 min and this phosphorylation was followed by further gradual phosphorylation over the next 70 min of the reaction.

When the time course of the enzyme activation and those of the subunit phosphorylation are compared, the initial rapid enzyme activation may be correlated with the initial rapid phosphorylation of either \( \alpha' \) or \( \beta \) subunits or it may be related to the incorporation of both phosphates. The slower additional enzyme activation afterwards appears to be correlated with the phosphorylation of the \( \alpha' \) subunit only.

The time courses of the enzyme activation and the phosphorylation in the protein kinase-catalyzed reaction in medium containing low concentration of Mg\(^{2+}\), 2 mM, were found to be virtually identical with those at high concentrations of Mg\(^{2+}\), 10 mM. It is noted that the enzyme activation, protein phosphorylation, as well as the subunits' phosphorylation of bovine red muscle phosphorylase kinase in media containing either 2 or 10 mM Mg\(^{2+}\) are similar to those of the rabbit white muscle phosphorylase kinase in media of high concentrations of Mg\(^{2+}\).

**Autophosphorylation and Activation of Bovine Red Mus-**

![Fig. 4. Phosphorylation of bovine red muscle isozyme by equal amount of catalytic subunit of the cAMP-dependent protein kinase at 2 mM Mg\(^{2+}\) (A) and 10 mM Mg\(^{2+}\) (B) as described in Fig. 5. Phosphorylase kinase (0.23 mg/ml) was phosphorylated in the reaction mixture either in the presence (○) or absence (●) of catalytic subunit. An aliquot of the reaction mixture was assayed for phosphorylase kinase activity at pH 6.8 after being phosphorylated in the presence (△) or absence (▲) of catalytic subunit.](image)

![Fig. 5. The incorporation of phosphate into different subunits of bovine red muscle phosphorylase kinase. Red muscle phosphorylase kinase (0.23 mg/ml) was phosphorylated by 0.3 units/ml of catalytic subunit under standard conditions in 2 mM Mg\(^{2+}\) (A and C) and 10 mM Mg\(^{2+}\) (B and D) for 80 min. After phosphorylation, an aliquot of the sample was subjected to SDS-gel electrophoresis as described under "Experimental Procedures" for 8 h (A and B) and 22 h (C and D). The gels were subsequently sliced into 1-mm segments and the radioactivity was counted.](image)

**Autophosphorylation and Activation of Bovine Red Muscle Phosphorylase Kinase**—The activation of bovine red muscle isozyme associated with autophosphorylation was examined in Tes buffer at pH 7.5. Fig. 7A shows that the phosphorylation reaction proceeded in two phases, a rapid incorporation of phosphates initially and a slower second phase. The enzyme activation appears to correlate with the phosphorylation reaction in having a rapid first phase and a slower second phase. The pH 6.8:8.2 activity ratio changed from 0.06 in the nonactivated state to 0.95 at the end of the phosphorylation reaction. In terms of subunit phosphorylation, it was observed that \( \beta \) subunit was phosphorylated to a maximum of 1 mol of phosphate/subunit with little or no further phosphorylation. In contrast, the phosphorylation of \( \alpha' \) subunit proceeded in two phases, a rapid first phase and a slower second phase. Hence, the initial rapid increase in enzyme activity is related to the phosphorylation of \( \alpha' \) and/or \( \beta \) subunit. The second phase of enzyme activation clearly correlates with the phosphorylation of \( \alpha' \) subunit only. A virtually identical result was observed with purified rabbit white muscle isozyme (data not shown). In addition, the autophosphorylation of rabbit white

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ATP\(^{12}\). When SDS-gels are run for longer times (22 h) to affect a better separation of \( \alpha' \) and \( \beta \) subunits, the radioactivity associated with the \( \alpha' \) subunit could be seen to be much higher than those with the \( \beta \) subunit.
FIG. 6. Time course of the subunit phosphorylation of bovine red muscle phosphorylase kinase. Red muscle phosphorylase kinase (0.23 mg/ml) was phosphorylated by 0.3 units/ml of catalytic subunit in standard reaction mixture in the presence of 10 mM Mg$^{2+}$. After phosphorylation, the phosphates incorporated into $\alpha'$ subunit (○) and $\beta$ subunit (□) were determined as described under "Experimental Procedures." An aliquot of the sample was also assayed for phosphorylase kinase activity (●) in standard reaction mixture as described.

FIG. 7. Phosphorylation of bovine red muscle phosphorylase kinase isozyme by the autophosphorylation reaction. A, autophosphorylation reaction was carried out in a standard reaction mixture containing 50 mM Tes, pH 7.5, 10 mM magnesium acetate, 10% sucrose, 2 mM [$\gamma$-32P]ATP, and 0.1 mg/ml of phosphorylase kinase and in the presence of 0.1 mM Ca$^{2+}$ (○) or 1 mM EGTA (●). An aliquot of the reaction mixture was also assayed for phosphorylase kinase activity at pH 8.0 under standard conditions as described under "Experimental Procedures." After the enzyme had been phosphorylated either in the presence of Ca$^{2+}$ (○) or EGTA (●). B, subunit phosphorylation pattern: $\alpha'$ subunit (□) and $\beta$ subunit (●).

muscle isozyme was inhibited by $\beta$-glycerophosphate buffer while the inhibitory effect was much less when bovine red muscle isozyme was used (data not shown). The significance of this observation is not clear at the present.

**DISCUSSION**

Most of the characterization of phosphorylase kinase has been carried out using purified rabbit skeletal muscle enzyme preparation which contains two isozymes. Jennissen and Heilmeyer (3) originally demonstrated the existence of the two isozymes of phosphorylase kinase and showed that they derived from the two muscle types, red and white muscles. The white muscle type of phosphorylase kinase was the predominant form in most of the preparations used. In a recent study (15), the two forms of phosphorylase kinase were fractionated by affinity chromatography on a calmodulin-Sepharose 4B column. Preliminary comparative studies indicated that the two rabbit muscle isozymes exhibited subtle differences in regulatory properties. Since rabbit skeletal muscle contains low amounts of the red muscle phosphorylase kinase, extensive comparative studies of the isozymes are difficult.

In the present study, a procedure has been developed for the purification of red skeletal muscle phosphorylase kinase from bovine leg muscle. Relatively large quantities of enriched enzyme could be obtained. The bovine red muscle phosphorylase kinase is similar to the rabbit red muscle isozyme in terms of their electrophoretic patterns, pH 6.8 to 8.2 activity ratio, as well as some of the regulatory properties examined. Thus, the comparative studies of the bovine red muscle and rabbit white muscle phosphorylase kinase would contrast the two isozyme types rather than the enzymes of different animal origins.

One difference in regulatory properties between rabbit red and white muscle phosphorylase kinase noted in the previous study (15) is that the white muscle enzyme, but not the red muscle enzyme is activatable at pH 7.0 by the addition of calmodulin to the enzyme reaction. The observation has been confirmed and extended in this study using bovine red skeletal muscle phosphorylase kinase. Over a wide range of pH, purified rabbit white muscle phosphorylase kinase was stimulated by calmodulin; whereas the bovine red muscle isozyme was not affected even though both forms of the enzyme contain about equal amounts of the $\delta$ subunits (calmodulin) and show Ca$^{2+}$-dependent activity.

The activation and phosphorylation of rabbit muscle phosphorylase kinase by the protein kinase and phosphorylase kinase itself have been characterized extensively using enzyme preparations from rabbits (1, 2, 8, 11, 12, 23, 28). The present study shows that the activation and phosphorylation of the bovine red muscle phosphorylase kinase by both mechanisms have similar general characteristics as those of the rabbit white muscle isozyme. The conversion of the enzyme from its nonactivated state to the activated form is accompanied by an increase in the pH 6.8 to 8.2 activity ratio from 0.01-0.06 to about 0.42-0.59 and 0.95 in the protein kinase-catalyzed reaction and autocatalytic reaction, respectively. The protein phosphorylation occurs exclusively on the two larger subunits, $\alpha'$ and $\beta$ units.

There are, however, subtle differences in the patterns of the enzyme activation and subunit phosphorylation of the two muscle isozymes of phosphorylase kinase. Unlike those of rabbit white muscle isozyme, the activation and phosphorylation of the bovine red muscle phosphorylase kinase are not significantly affected by the concentration of Mg$^{2+}$ in the protein kinase-catalyzed reaction. In either 2 mM or 10 mM Mg$^{2+}$, the time course of the activation of the red muscle isozyme consists of an initial rapid phase followed by a slow and gradual further increase in the enzyme activity. Analysis of the subunit phosphorylation indicates that both $\alpha'$ and $\beta$ subunits are phosphorylated to about one phosphate/subunit during the rapid activation phase. The slow activation phase is accompanied by the phosphorylation of the $\alpha'$ subunit. Thus, the activation of the bovine red muscle isozyme appears to be associated with the phosphorylation of both $\alpha'$ and $\beta$ subunits. In contrast, the activation of rabbit white muscle
phosphorylase kinase has been suggested to be associated with the phosphorylation at a specific site on the β subunit at low concentrations of Mg\(^{2+}\) (1) and with the phosphorylation of both α and β subunits at high concentrations of Mg\(^{2+}\) (15). The significance of the small difference in the activation and phosphorylation of the two muscle isozymes is not clear at present. In addition, the autophosphorylation of rabbit white muscle isozyme is greatly inhibited by β-glycerophosphate buffer. The degree of inhibition is much less when bovine red muscle isozyme is used. The significance of this is not known yet.

On the basis of SDS-gel electrophoretic analysis, cardiac and red muscle phosphorylase kinase have been suggested to be identical (27). Bovine cardiac phosphorylase kinase has been purified to close to homogeneity and shown to contain α' instead of α as its largest subunit (14). Recently the purified cardiac phosphorylase kinase has been characterized in detail terms of its activation and phosphorylation in the cAMP-dependent protein kinase-catalyzed reaction (14, 27). The regulatory properties of the cardiac enzyme appear to be significantly different from those of the red muscle isozyme observed in the present report. The cardiac enzyme has a relatively high pH 6.8 to 8.2 activation ratio, about 0.2 (14). The pH 6.8 activity of the enzyme is activated only 2- to 4-fold in the protein kinase-catalyzed reaction, and this activation is associated with a maximum of 1 to 2 mol of phosphate incorporated/mol of phosphorylase kinase (27). On the other hand, the red skeletal muscle phosphorylase kinase shows a low pH 6.8 to 8.2 activity ratio, 0.01-0.06. The enzyme activity at pH 6.8 is activated in the protein kinase reaction by more than 10-fold and the activation is associated with the incorporation of 12 to 16 mol of phosphates/mol of the enzyme. It should be noted that the difference between the observed regulatory properties of the two isozymes is not because the purified cardiac phosphorylase kinase is in a partially activated state. Treatment of the enzyme with phosphoprotein phosphatase did not change the properties of the enzyme (27). Thus, it seems possible that the cardiac and red muscle phosphorylase kinases are distinct isozymes. An alternative suggestion is that the difference in the regulatory properties of the two isozymes may be due to the different purification procedures used. Further studies are needed to clarify this point.

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Regulation of Phosphorylase Kinase

**Supplemental Material for**

**Phosphorylation and Activation of Red Skeletal Muscle**

**Phosphorylase Kinase Isotyping: Procedure of the Purification**

Dissley W. Tan, Rajinder P. Saggi, and Jerry H. Wang

**Experimental Procedures**

Materials

The n-methylated rabbit skeletal muscle phosphorylase kinase was prepared by the method of Cohen (11). The whole muscle was extracted from frozen rabbit skeletal muscle according to the method of Sjöström and Williams (12). The extract was fractionated with acetone to yeman 3% TCA. Partially purified catalytic subunits of the CaMP-dependent phosphorylase kinase were prepared by the method of Kissel and Halter (11). Calmodulin was prepared from bovine brains by the method of Ham and Weiss (13). A typical preparation from double distilled water was performed. The latter provided 8-mercaptoethanol and 10% sucrose. Analysis of the bovine phosphorylase kinase purified after SDS-allylamine columns contains significant amount of red muscle isoform (ref. 1, 12).

Culmination-Sepharose 4B affinity chromatography. After dialysis, the kinase was recovered in a elution buffer (50 mM Citrate, pH 5.0) and then applied to a 1 ml bed size of Culmination-Sepharose 4B in a high pressure chromatography system (14). The column was washed with the same buffer until no proteins could be detected in the eluate. Peaks were detected at 0.5 ml fractions. After dialysis against 20% 3-[(3-chloromethyl)-1H-imidazol-5-yl]propionic acid, 2 mM EDTA, and 10 mM dithiothreitol and its 1.0 M sucrose. Activity of bovine kinase purified after SDS-cyano-cellulose columns contained significant amount of red muscle isoform (ref. 1, 12).

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The n-methylated rabbit skeletal muscle phosphorylase kinase was prepared by the method of Cohen (11). The whole muscle was extracted from frozen rabbit skeletal muscle according to the method of Sjöström and Williams (12). The extract was fractionated with acetone to yeman 3% TCA. Partially purified catalytic subunits of the CaMP-dependent phosphorylase kinase were prepared by the method of Kissel and Halter (11). Calmodulin was prepared from bovine brains by the method of Ham and Weiss (13). A typical preparation from double distilled water was performed. The latter provided 8-mercaptoethanol and 10% sucrose. Analysis of the bovine phosphorylase kinase purified after SDS-allylamine columns contains significant amount of red muscle isoform (ref. 1, 12).

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