Isolation and Properties of the Catalytically Active \( \gamma \) Subunit of Phosphorylase \( b \) Kinase*

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Nonactivated phosphorylase kinase was dissociated to catalytically active lower molecular weight species by incubation with either ATP or LiBr at 0°C. Sucrose density gradient analysis showed that incubation with 100 mm ATP produced 14 S and 7.5 S active forms and incubation with 1 mm LiBr produced a 5 S active species. Large increases in the pH 6.8/8.2 activity ratio and loss of inhibition by EGTA were characteristics of the dissociated forms. After native phosphorylase kinase was dissociated optimally by incubation with 1.8 mm LiBr, a catalytically active subunit was purified to near homogeneity by gel filtration and blue dextran-Sepharose affinity chromatography. This catalytically active subunit of phosphorylase kinase was identified as the \( \gamma \) subunit (MW = 45,800) by using sodium dodecyl sulfate disc gel electrophoresis. In addition, by using radioactively labeled activated phosphorylase kinase, the distribution of the other subunits was determined while isolating the catalytic subunit. Some properties of the purified catalytically active \( \gamma \) subunit were examined. The apparent molecular weight as determined by gel filtration is 86,000, indicating that the active species is a dimer (\( \gamma_2 \)). The pH 6.8/8.2 activity is 0.67 to 1.0. The enzyme is also active when assayed in the presence of EGTA, indicating a loss of the absolute requirement of \( Ca^{2+} \) for activity. Like the holoenzyme, the purified \( \gamma \) subunit did not phosphorylate casein and histone. The kinetic parameters for the purified \( \gamma \) subunit were determined at pH 6.8 and 8.2 for MgATP and phosphorylase \( b \).

Phosphorylase kinase (ATP-phosphorylase phosphotransferase, EC 2.7.1.38) is a key enzyme involved in the regulation of glycogenolysis of rabbit skeletal muscle. Two distinct mechanisms for the regulation of enzyme activity have been reported to be of physiological significance. One involves the regulation of phosphorylase kinase activity in response to intracellular \( Ca^{2+} \) levels. The enzyme has been shown to have an absolute requirement of \( Ca^{2+} \) for activity (1, 2), suggesting a link between glycogenolysis and muscle contraction (1–3). Phosphorylase kinase is isolated from resting muscle in a form expressing little activity at physiological pH. The ratio of the activity determined at pH 6.8 compared with that at pH 8.2 (pH 6.8/8.2 ratio) is less than 0.05. The ratio can be increased to 0.38 through phosphorylation by cAMP-dependent protein kinase (5–8). This second mechanism of regulation has been shown to occur in vivo. More extensive activation of phosphorylase kinase can occur during phosphorylation by cAMP\(^{3}\)-dependent protein kinase in the presence of high Mg\(^{2+}\) concentrations (9). Nonactivated phosphorylase kinase can also be activated through phosphorylation by cGMP-dependent protein kinase (10), \( Ca^{2+}\)-protease-activated protein kinase (11), and phosphorylase kinase itself (5). Other mechanisms of activation can occur and reports of activation by a \( Ca^{2+}\)-dependent protease (12) and trypsin (5) have appeared.

The holoenzyme has a molecular weight reported as 1.28 \( \times \) 10\(^6\) (6) or 1.33 \( \times \) 10\(^6\) (7) and comprises three different subunits (6, 7, 13) with a stoichiometry of \( \alpha, \beta, \gamma \) (6) or \( \alpha, \beta, \gamma_{\gamma} \) (7). The molecular weights of the subunits have been reported as: \( \alpha = 145,000 \) (6), 136,000 (14), 118,000 (7); \( \beta = 128,000 \) (6), 120,000 (14), 108,000 (7); and \( \gamma = 45,000 \) (6), 42,000 (14), 41,000 (7). The exact functions of the subunits of this structurally complicated molecule have not been established. Very recently, Cohen et al. (15) have suggested that a low molecular weight, faintly staining band in SDS-gels of phosphorylase kinase from rabbit skeletal muscle is actually a fourth subunit designated as \( \delta \). They reported it to have a molecular weight of 17,000 and to be identical with the \( \alpha \) subunit. Cohen et al. (15) and Cheung (17) reported to be identical with the \( \alpha \) subunit along with proteolytic degradative products of the other subunit(s). These experiments showed that the large molecular structure was not essential for kinase activity, but failed to determine which subunit was involved in the catalytic reaction. Studies of phosphorylase kinase isolated from the Pacific dogfish, *Aquilus acanthias*, suggest that the \( \gamma \) subunit is catalytically active (20). The purpose of this work is to dissociate and purify the catalytic subunit of rabbit muscle phosphorylase kinase. LiBr effectively dissociated the enzyme. Nearly pure preparations of the catalytically active \( \gamma \) subunit were obtained by using molecular sieve and affinity chromatography in the purification scheme. The molecular weight, substrate specificity, \( Ca^{2+}\) requirement, kinetic parameters, and activity-pH profile of the purified catalytically active \( \gamma \) subunit were determined.

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*The abbreviations used are: cAMP, adenosine 3':5'-monophosphate; cGMP, guanosine 3':5'-monophosphate; SDS, sodium dodecyl sulfate; ATP, adenosine triphosphate; EGTA, ethylene glycol bis\( (\beta\)-aminoethylther) \( N,N'\) -tetracetic acid; MES, 2-(N-morpholino)ethanesulfonic acid; Tetradecapeptide, Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin.

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Materials and Methods

Phosphorylase b kinase was prepared from rabbit muscle by the method of DeLange et al. (21) as modified by Hayakawa et al. (13), and unless otherwise stated, the phosphorylase kinase so obtained was used in this report. Further purification of phosphorylase kinase by DEAE-cellulose chromatography was performed as described by Cohen (6). Activated phosphorylase kinase was prepared as described by Tabatabai and Graves (22). Phosphorylase b was prepared as described by Fischer and Krebs (23) except that 30 mM 2-mercapto-ethanol was substituted for cysteine. Residual AMP was removed by treatment with acid-washed Norite A. Phosphorylase was assayed in the direction of glycogen synthesis according to the method of Illingworth and Cori (24). Protein concentrations of the purified enzymes were determined spectrophotometrically by using absorbance indices of 12.0 (33) or 13.0 (25) for 1% protein solutions of phosphorylase kinase and phosphorylase, respectively. Protein determinations during experiments utilized the Coomassie Brilliant Blue G-250 microprotein assay as described by Bradford (26). Often the samples contained ATP, which increased the background slightly, but the standard curves were linear. Phosphorylase kinase was used as the standard for the assay. Other protein determinations were performed by the method described by Lowry et al. (27). Both of these protein determination methods gave results that agreed very closely when phosphorylase kinase was used as standard. This, together with the fact that Coomassie blue stained the a, b, and c subunits almost stoichiometrically (see "Results"), justifies the accuracy of all protein determinations found in this report.

Phosphorylase kinase was assayed as described (28) except that 0.1 mM CaCl₂ was included in the reaction mixture unless noted otherwise. Phosphorylase kinase activity was determined in some experiments by following the incorporation of "³²P from [γ-³²P]ATP into phosphorylase by using a filter paper assay (29). For the sucrose density gradient centrifugation experiments, glycogen phosphorylase b and hemoglobin were used as markers to estimate the S values of the fractions.

Blue dextran-Sepharose was prepared essentially by the method of Ryan and Vestling (30) except that 0.125 M Na₂HPO₄, pH 11, was included in the cyanoform bridge activation reaction mixture to aid in controlling the pH. The Blue dextran-Sepharose was stored in 0.1 M Na₂EDTA, pH 4.5, to aid in maintaining gel stability (31), and also in 3 mM sodium azide at 4°C.

Polyacrylamide gel electrophoresis in the presence of SDS was done by using the alkaline buffer system described by Hayakawa et al. (7). Densitometer tracings were made with a Honeywell Electronik 194 recorder added in series with a Zeiss PM II spectrophotometer and a System I computing integrator (Spectra-Physics). The gels were scanned at 550 nm.

Rabbit muscle lactate dehydrogenase was obtained from Cal-Biochem., pig heart malate dehydrogenase from Sigma Chemical Co. Pig heart aspartate aminotransferase was a gift from D. E. Metzler's research group; hemoglobin from M. A. Rouvgie, both at Iowa State University. Lactate dehydrogenase and malate dehydrogenase were assayed as described in the Worthington Enzyme Manual (32) and aspartate aminotransferase by the method of Furnish et al. (33). [γ-³²P]ATP was prepared by the method of Glynn and Chappell (34). All other materials used were commercially available.

Results

Evidence for Dissociation of Phosphorylase Kinase by ATP and LiBr—Preliminary studies were done by using various concentrations of ATP as a potential dissociating agent. Nonactivated phosphorylase kinase was incubated with ATP at 0°C under a variety of conditions. Sucrose density gradient centrifugation of nonactivated phosphorylase kinase that had been incubated with 100 mM ATP for 33 h at 0°C produced two activity peaks located near the middle of the gradient, Fractions 5 and 10 (Fig. 1). The sedimentation coefficients of the peaks found at Fractions 5 and 10 were approximately 7 S, respectively, indicating that incubation of nonactivated phosphorylase kinase with 100 mM ATP will dissociate the 23 S native enzyme (6) to active low molecular weight species.

The effects of pH and EGTA on the activity of the dissociated phosphorylase kinase species were determined. The pH 6.8/8.2 activity ratio was 0.44 and 0.32 for Fractions 5 and 10, respectively. Activation by autophosphorylation during the incubation period was not likely because no Mg²⁺ was included in the incubation mixture or the gradient. Furthermore, Carlson et al. (35) had shown that there was no evidence to link autophosphorylation with dissociation. Inclusion of 0.83 mM EGTA in the assay reaction mixture caused no inhibition of activity, and the reaction progress curves were linear. These characteristics of ATP-dissociated phosphorylase kinase were used as indicators of dissociation in later experiments. No effect of pH was seen when the pH of the incubation mixture, or gradient, or both, were pH 6.8 or pH 8.6. When ATP was used as the dissociating agent, no condition was found that produced active fragments with an S value less than approximately 7.5 S, indicating that full dissociation of the holoenzyme to individual subunit forms probably had not occurred.

Two properties, characteristic of ATP-dissociated phosphorylase kinase, have also been observed for nonactivated phosphorylase kinase after being incubated with NaNO₃, LiBr, or RbBr (36). That report showed that incubating nonactivated phosphorylase kinase with nearly molar concentrations of these salts at 30°C would stimulate the pH 6.8 activity and abolish the lag characteristic of the nonactivated phosphorylase kinase reaction. The effects of incubating nonactivated phosphorylase kinase with 0.83 M LiBr, NaNO₃, and RbBr at 0°C on its pH 6.8, pH 8.2 activities, and pH 8.2 activity in the presence of EGTA were re-examined. After 4 h of incubation with LiBr, the pH 6.8/8.2 activity ratio had increased, and inhibition by EGTA was lost. NaNO₃ was less effective than LiBr in promoting the changes characteristic of ATP-dissociated phosphorylase kinase. RbBr caused little change in activity over that seen when phosphorylase kinase was incubated in the absence of concentrated salt (results not shown).

The effect of incubating nonactivated phosphorylase kinase with LiBr on its subunit structure is also shown in Fig. 1. Centrifugation of nonactivated phosphorylase kinase that had been incubated with 1 M LiBr for 9 h produced one catalytically active peak (Fraction 3) with a sedimentation coefficient of approximately 5 S, an increased pH 6.8/8.2 activity ratio,
and a loss of the absolute Ca\(^{2+}\) requirement at pH 8.2. However, it was found that the presence of Ca\(^{2+}\) in the incubation mixture stabilized the enzyme activity compared to when the incubation was performed in its absence. Fractions containing mixture stabilized the enzyme activity compared to when the 5 S phosphorylase kinase species were analyzed by using disc gel electrophoresis in the presence of SDS. All three subunits of phosphorylase kinase were found in these fractions indicating that, although LiBr is an effective dissociating agent, preparative sucrose density gradient centrifugation after LiBr treatment would not be a suitable method for purification of the catalytic subunit from other phosphorylase kinase subunits.

The effect of incubating nonactivated phosphorylase kinase with various concentrations of LiBr on enzyme activity at pH 6.8, pH 8.2, and pH 8.2 in the presence of 0.1 mM EGTA was examined. During the initial ½ h, incubation with 1.8 M LiBr resulted in a large loss of pH 8.2 activity which was mostly regained after 4 to 6 h (Fig. 2). The pH 6.8 activity steadily increased, and the inhibition by EGTA was decreased. Incubation with 3.6 M LiBr caused rapid loss of activity, and 2.7 M LiBr caused a significant loss that was not reversed during the incubation period. LiBr (0.9 M) also caused a loss of pH 8.2 activity that was not regained, and only small increases in the pH 6.8 activity and the pH 8.2 activity in the presence of EGTA were noted. Also included in the figure is a control incubation mixture having no LiBr added, which shows that nonactivated phosphorylase kinase was stable during the incubation period. Experimentation to support a satisfactory explanation for the fluctuations in the pH 8.2 activities has not been done, but these fluctuations were seen consistently when phosphorylase kinase was incubated under conditions that caused dissociation, including incubation with 100 mM ATP.

Preparation of the Catalytically Active γ Subunit of Phosphorylase Kinase—Nearly pure preparations of the γ subunit required a three-step isolation scheme. The native phosphorylase kinase was dissociated by incubation at 0°C with 1.8 M LiBr for 12 to 18 h, sometimes up to 25 h, partially purified by Sephadex G-150 chromatography, and further purified and concentrated by blue dextran-Sepharose affinity chromatography.

Fig. 3 shows the activity and elution profiles of LiBr-treated phosphorylase kinase, chromatographed on Sephadex G-150. Sixty-six per cent of the applied activity was recovered in all fractions, and two-thirds of the recovered activity was found in Fractions 23 through 32. Substantial purification of the γ subunit in fractions eluting after the major protein peak was seen when these fractions were analyzed by using SDS-gel electrophoresis (results not shown). Associated with the purification of the γ subunit is a concomitant increase of about 5-fold in specific activity. The α subunit eluted mainly in or near the void volume. Very little β subunit was found in any of the fractions. Presumably it precipitated in the gel matrix, as shown in the later section.

Although molecular sieve chromatography of the LiBr-treated phosphorylase kinase results in a substantial purification of the γ subunit, the protein solutions are extremely dilute, and an additional step is necessary for concentrating the enzyme. Blue dextran-Sepharose affinity chromatography (37) is chosen because it can both further purify and concentrate the catalytic subunit. In the experiment shown in Fig. 4a, fractions with specific activity greater than 300,000 Cori units/mg obtained from the Sephadex column (depicted in Fig. 3) were applied to a blue dextran-Sepharose column which was eluted with a linear ATP gradient. The effectiveness of using these two columns in series to purify the catalytic subunit can be seen in Fig. 4, a and b. The specific activity had increased from 89,000 Cori units/mg for the phosphorylase kinase incubated with LiBr to over 500,000 Cori units/mg for the peak fractions. The purification of the γ subunit from

![Fig. 2. Effect of incubation with LiBr on the activities of phosphorylase kinase. Phosphorylase kinase (0.86 mg/ml) was incubated at 0°C with 3.6% sucrose, 18 mM β-glycerophosphate, 0.73 mM EDTA, 0.73 mM CaCl\(_2\), 91 mM MES, pH 7.0, and varying concentrations of LiBr. No LiBr added (○); 0.9 M LiBr (□); 1.8 M LiBr (△); 2.7 M LiBr (●); 3.6 M LiBr (■). At various times aliquots of the incubation mixture were diluted in 40 mM β-glycerophosphate, 30 mM 2-mercaptoethanol, pH 6.8, and assayed for activity at pH 6.8, pH 8.2, and pH 8.2 in the presence of 0.1 mM EGTA.](http://www.jbc.org/)

![Fig. 3. Sephadex G-150 chromatography of LiBr-dissociated phosphorylase kinase. Nonactivated phosphorylase kinase (0.9 mg/ml) was incubated with 3.6% sucrose, 18 mM β-glycerophosphate, 0.73 mM EDTA, 0.73 mM CaCl\(_2\), 91 mM MES, pH 7.0, and 1.8 M LiBr for 18 h at 0°C. 2.7 ml of the incubation mixture was applied to a Sephadex G-150 column (132 x 1.7 cm) equilibrated with 100 mM MES, 0.5 mM CaCl\(_2\), 30 mM 2-mercaptoethanol, pH 7.0. 1.7-m1 fractions were collected. Kinase activity at pH 8.2 (○), protein concentration (△), and calculated specific activity (●) were plotted.](http://www.jbc.org/)
the other subunits is shown in the densitometric tracing of the peak fractions (Fig. 4b). Quantitation of the gel densities showed that there was over 30 times more γ subunit than any other protein on the gel for the peak fractions on a molar basis. These data strongly suggest that the catalytic subunit of phosphorylase kinase is the γ subunit.

Additional experiments were done to determine the yield and purity of the γ subunit that can be obtained by using the purification scheme on an 8-fold larger scale. The procedures followed were nearly the same as those described in the legend to Figs. 3 and 4, and identical with the procedures used for the purification of the catalytic subunit of ³²P-labeled phosphorylase kinase, which is described later under Results. Only the protein elution profile was monitored until the protein in or near the void volume had been eluted. The elutant from the Sephadex column was then applied directly to the blue dextran-Sepharose column without collecting fractions for analysis. This circumvented the probable loss of activity due to the catalytic subunit being in a dilute solution. From 18.8 mg of native phosphorylase kinase (2.6 mg of undissociated γ subunit), 0.9 mg of the catalytic subunit with a specific activity greater than 600,000 was recovered from the affinity column. The molar ratios of contaminating proteins to the catalytic subunit are shown in Table I. Fractions eluting from 46 mM ATP to 24.5 mM ATP, were pooled inclusively and represent the final product for future studies. The values for molecular weights of the phosphorylase kinase subunits and the subunit stoichiometry for the holoenzyme used in the calculations were α = 143,000, β = 133,000, γ = 45,800 determined in this laboratory. The subunit stoichiometry for native phosphorylase kinase was determined to be: α₁₁₀γ, β₁₁₆γ, γ₁₁₆ which agreed closely with the values published (6). The use of two different chromatographic techniques to purify the catalytic subunit of phosphorylase kinase dissociated by incubation with LiBr produces nearly pure preparations of the γ-subunit with recovery of approximately 10 to 30% of the activity and of the protein, depending on preparations. There was no β subunit present in the preparation of the catalytic subunit and only a small amount of α subunit was found as contaminant. The molar ratio of the γ subunit to the α subunit in the product is 50:1 (Table I). SDS-gel electrophoresis of the catalytically active subunit is shown in Fig. 3a. It was found that it had a $M_r = 45,800$ and that it comigrated with the γ subunit band of the holoenzyme when the two proteins were electrophoresed together (Fig. 5b). In addition, this subunit migrated as a single band in 7% polyacrylamide gel electrophoresis at 4°C (Fig. 5d) and was still active.

Contaminations of the catalytically active subunit due to α and δ subunits and Protein X could be reduced further when phosphorylase kinase purified after DEAE-cellulose chromatography was used as the starting material. However, the yield was decreased appreciably because this form of holoenzyme was less stable during the LiBr treatment (results not shown). Consequently, this method of preparation was abandoned.

During the purification of the catalytic subunit, most of the

![Fig. 4. Affinity chromatography of partly purified catalytic subunit and densitometric tracings of fractions. a. Fractions 23 through 32 (less 29 and 30) eluted from the Sephadex column depicted in Fig. 3 were applied to a 2-ml blue dextran-Sepharose column. The column was equilibrated with 100 mM MES, pH 7.0, 0.5 mM CaCl₂, 30 mM 2-mercaptoethanol, and the enzyme was eluted with a 10-ml linear ATP gradient in the equilibration buffer. The enzyme activity (C), protein concentration (D), and calculated specific activity (E) of the eluted fractions are plotted against the ATP concentrations eluting the fractions. b. Densitometric tracings of SDS-polyacrylamide gels showing purification of the γ subunit on blue dextran-Sepharose column as shown in a. Tracing (i) is of a gel native phosphorylase kinase; (ii) to (v) are of the fractions obtained from 17 to 25 mM ATP, 33 to 41 mM ATP, 49 to 57 mM ATP, 63 to 72 mM ATP, respectively, as depicted in a.]

**Table I**

| Fraction no. | α     | β     | X   | γ   |
|-------------|-------|-------|-----|-----|
| 34 mM ATP   | 0.01  | 0.00  | 0.27| 1.0 |
| 53 mM ATP   | 0.02  | 0.00  | 0.09| 1.0 |
| 60 mM ATP   | 0.02  | 0.00  | 0.03| 1.0 |
| 76 mM ATP   | 0.02  | 0.00  | 0.02| 1.0 |
| 81 mM ATP   | 0.04  | 0.00  | 0.03| 1.0 |
| 92 mM ATP   | 0.10  | 0.02  | 0.06| 1.0 |
| Pooled      | 0.02  | 0.06  | 0.04| 1.0 |

* The molecular weight of Protein X is 190,000.
of radioactivity associated with the peak protein fraction is likely to be caused by a subunit and Protein X contamination of the γ subunit because the γ subunit band recovered from an SDS-gel did not contain any 32P radioactivity (result not shown). Therefore, the use of radioactively labeled phosphorylase kinase clearly shows that the catalytic subunit is being separated from the α subunit, β subunit, and Protein X contaminants.

**Evidence against Limited Proteolysis of Phosphorylase Kinase during the Preparation**—The possibility of limited proteolysis of phosphorylase kinase occurring during the preparation of the catalytic subunit was considered, but no evidence was found to suggest that it had occurred. SDS-poly-
acrylamide gels of native phosphorylase kinase and phosphorylase kinase that had been incubated with 1.8 m LiBr for 3 days showed no difference in subunit structure or appearance of new protein bands, which is characteristic of proteolysis (8, 7, 19). Unidentified protein bands were not found in the densitometric tracings of SDS-gels for fractions eluting from Sephadex G-150 column. A characteristic of limited trypsin attack of activated phosphorylase kinase covalently labeled with $^{32}$P is the rapid release of radioactive labeled peptides (38). No evidence was found for the presence of $^{35}$P-labeled peptides when labeled phosphorylase kinase was used for the preparation of the catalytic subunit.

It is known that the activation of most of the Ca$^{2+}$-dependent proteases requires millimolar concentrations of Ca$^{2+}$, but an increase in the CaCl$_2$ concentration to 2 mM in the column buffers had no effect on both the activity and protein elution profiles as shown in Figs. 3 and 4a. Inclusion of three different protease inhibitors, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 4 $\mu$g/ml of 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK), and 4 $\mu$g/ml of leupeptin, in the incubation mixture did not affect the profiles shown in Fig. 2 indicating that the activation of pH 6.8 activity and the loss of sensitivity to EGTA were not a result of proteolysis. Furthermore, the preparation of $\gamma$ subunit was not affected by the presence of PMSF.

Properties of the Purified Catalytically Active $\gamma$ Subunit—The chromatographic homogeneity and apparent molecular weight of the $\gamma$ subunit were determined by gel filtration as shown in Fig. 7. The enzyme chromatographs essentially as one species with an approximate molecular weight of 86,000 indicating that the $\gamma$ subunit is a dimer.

The response of the purified $\gamma$ subunit as well as nonactivated phosphorylase kinase that had been incubated with 1.8 M LiBr to Ca$^{2+}$, EGTA, and pH are in marked contrast to that found for nonactivated phosphorylase kinase. Fig. 8 shows the activity progress curves for the purified $\gamma$ subunit at pH 6.8 and pH 8.2. The typical lag associated with the native enzyme is absent and the progress curves are completely linear at both pH 6.8 and pH 8.2. When 0.1 mM CaCl$_2$ or 0.1 mM CaCl$_2$ plus 0.1 mM EGTA was included in the reaction mixture, the rate of the reaction was the same as when neither was added. When the activity progress curve was followed with 0.1 mM EGTA in the reaction mixture (Fig. 8), a biphasic response was observed. The initial rate appeared to be the same as that seen when EGTA was omitted. The rate rapidly decreased and, after about 2 min, was stabilized at a rate approximately 30 to 40% of the initial rate. The reaction progress curve was linear, however, after the initial loss of activity for at least 6 min of reaction time.

The activity-pH profile curve of the $\gamma$ subunit is shown in Fig. 9. Unlike nonactivated phosphorylase kinase, which has almost no activity at pH 6.8, the $\gamma$ subunit has nearly full activity at neutral pH, and the pH 6.8/8.2 activity ratio is 0.70 to 1.0, depending on preparations. The pH optimum for the purified $\gamma$ subunit is the same as reported for other forms of phosphorylase kinase (20). The substrate specificity of the purified $\gamma$ subunit was also nearly the same as those reported for the holoenzyme (21). Of the substrates tested, only phosphorylase $b$ and the synthetic tetraheptapeptide representing the NH$_2$-terminal region of phosphorylase $b$ were good substrates. Other proteins tested as possible substrates were histone and casein. When the autophosphorylation of nonactivated phosphorylase kinase in the presence of purified $\gamma$
shown in Fig. 10, the presence of the purified phosphorylation was detected over a 30-min period.

subunit was compared with the autocatalytic reaction, as shown in Fig. 10, the presence of the purified γ subunit in the nonactivated phosphorylase kinase autophosphorylation reaction mixture at pH 8.2 increased the rate significantly as compared to that observed for the autocatalytic reaction. However, when the reaction was done at pH 8.2 in the presence of EGTA or at pH 6.8 in the presence of Ca" or EGTA, no additional phosphorylation over that seen for the autocatalytic reaction was detected. The purified γ subunit would not phosphorylate itself. The enzyme (0.13 mg/ml) was incubated with 5 mM MgATP, 1.8 mM [γ-32P]ATP, 50 mM MES, 15 mM 2-mercaptoethanol, 50 mM CaCl2, and 25% glycerol. No phosphorylation was detected over a 30-min period.

The apparent Kav values for phosphorylase b and MgATP and apparent Vmax values were determined for the purified γ subunit at pH 6.8 and 8.2. The assay conditions were those normally used for the phosphorylase kinase reaction except that free MgATP was 6 mM in all reaction mixtures. Phosphorylase was 100 μM when determining the KM,ATP, and MgATP was 3 mM when determining the Kphosphorylase b. At pH 8.2 the KM,ATP was 0.86 mM with a Vmax of 60 μmol/min/mg, and the Kphosphorylase b was 84 μM with a Vmax of 52 μmol/min/mg. At pH 6.8, the KM,ATP was 1.0 mM with a Vmax of 24 μmol/min/mg and a Kphosphorylase b of 200 μM with a Vmax of approximately 100 μmol/min/mg.

DISCUSSION

Phosphorylase kinase from rabbit skeletal muscle is composed of multiple subunits. This report suggests that one of these chains, γ, is a catalytically active subunit of phosphorylase kinase. Purification of a catalytic subunit of the holoenzyme has enabled a new approach to be taken in the study of its mechanisms of action and modes of regulation.

The procedures used in the purification of a catalytic subunit result in nearly pure preparations of the γ subunit. There is no β subunit and only small amounts of Protein X, and α and δ subunits which contaminate the enzyme preparation could largely be eliminated when highly purified phosphorylase kinase is used as the starting material. The predicted specific activity of pure catalytic subunit would be approximately 680,000 Cori units/mg because the holoenzyme and the LiBr-dissociated enzyme have specific activities of approximately 95,000 Cori units/mg and the γ subunit constitutes 0.14 of the total protein. The predicted value was approached by the peak fractions eluted from blue dextran-Sepharose column, with specific activities greater than 650,000 Cori units/mg being recorded.

A comparison of the purified dimeric catalytically active γ subunit with the native holoenzyme revealed two major differences in their catalytic properties. Whereas nonactivated phosphorylase kinase is essentially inactive when assayed at pH 6.8, the purified γ subunit is highly active at this pH. The purified γ subunit has also lost the absolute requirement of Ca" for activity characteristic of the native holoenzyme.

Nonactivated phosphorylase kinase has been shown to be activated by phosphorylation of the β subunit (6) or α and β subunits (9) by cAMP-dependent protein kinase, by phosphorylation of the α and β subunits during the autocatalytic reaction (7), and by limited proteolytic degradation of the α and β subunits (6, 7, 19). Activation of the enzyme is also the result of dissociation of the holoenzyme by LiBr. The pH 6.8/8.2 activity ratio of nonactivated phosphorylase kinase can be increased from less than 0.05 to 0.38 by protein kinase phosphorylation, to 0.5 by autophosphorylation (5), to 0.7 and greater by trypsinization (5, 6), and to 0.7 by dissociation. Earlier kinetic studies suggested that the increase in the pH 6.8 activity resulting from phosphorylation of the native phosphorylase kinase is caused by a decrease in the Km for phosphorylase b. The kinetic data in this report also show that the increased pH 6.8 activity of the purified γ subunit is a result of the lowering of the Km for phosphorylase b. A more extensive study of the kinetic parameters for the purified γ subunit should be completed to determine whether the Vmax is also affected by pH. These results imply that the pH 6.8 activity of nonactivated phosphorylase kinase is strongly inhibited by a regulatory subunit(s) that modulates the pH 6.8 activity by affecting the Km for phosphorylase b. The inhibition is partly relieved by protein kinase phosphorylations, more so by autophosphorylation, and trypsinization or dissociation which allow full pH 6.8 activity to be expressed.

The absolute requirement of Ca" for activity to be expressed by all forms of phosphorylase kinase previously described is fundamental to in vivo regulation of glycogenolysis. The purified γ subunit does not have an absolute Ca" requirement for activity, but inclusion of EGTA in the assay mixture did cause a time-dependent loss of activity to approximately 40% of the initial velocity at pH 8.2. The most likely explanation for the effect of EGTA on activity is that the purified catalytic subunit is stabilized by Ca" and its removal causes a time-dependent transition to a less active conformation or state of association or dissociation. The loss of activity is not likely the result of denaturation because the activity progress curve was linear after the initial loss of activity, nor the result of an EGTA-protein interaction because inclusion of equal molar amounts of EGTA and Ca" did not affect activity. The loss of the Ca" requirement for activity by the purified γ subunit suggests a mechanism of regulation for the native enzyme in response to Ca". In the absence of Ca", the other subunit(s) completely inhibit enzyme activity. The inhibition is relieved by the binding of Ca" by a regulatory subunit, possibly the δ subunit. The regulation of enzyme activity by Ca" and small metabolites has been established for several other enzyme systems. The skeletal muscle contractile process is regulated by the binding of Ca" to the troponin regulatory protein complex. The regulation of phosphorylase kinase in
response to Ca\(^{2+}\) may be analogous to the regulation of cAMP-dependent protein kinase in response to cAMP. Phosphorylase kinase may be similar, in that binding of Ca\(^{2+}\) promotes a conformational change that allows for expression of activity (without dissociation occurring).

The report by Fischer et al. (39) comparing the dogfish and rabbit muscle phosphorylase kinase showed that some similarities exist between the two enzymes, including the molecular weights of the holoenzyme and its subunits, and substrate specificity. The amino acid composition of \(\gamma\) subunit of dogfish phosphorylase kinase is nearly identical with actin except that no 3-methylhistidine is present. The \(\beta\) subunit of dogfish phosphorylase kinase was reported to be the catalytic subunit. This report, however, provides strong evidence that the \(\gamma\) subunit of rabbit muscle phosphorylase kinase is the catalytic subunit.

The purified catalytically active \(\gamma\) subunit of phosphorylase kinase has a specific activity near that reported for nonactivated kinase results in a conformational change that allows for expression of activity. Purification of the catalytic subunit. Possible explanations are that the subunit of rabbit muscle phosphorylase kinase is the catalytic subunit. The latter explanation is consistent with the results of Dickneite et al. (40) who showed that 1 mol of ATP analogue could be covalently labeled into both the \(\alpha\) and \(\beta\) subunits of phosphorylase kinase. However, when they correlated the degree of modification of each subunit with the residual activity, they found that when 1 mol of adenosine-5'-chloromethanepyrophosphonate was found/mol of the \(\gamma\) subunit, the enzyme still retained 90% of its original activity. Horl and Heilmeyer (43) recently reported that phosphorylase kinase found in the sarcoplasmic reticular membranes from rabbit muscle did not contain a protein corresponding to the molecular weight of the \(\gamma\) subunit, but proteins with molecular weights corresponding to the \(\alpha\) and \(\beta\) subunits were present. According to Fischer et al. (44) by using phosphorylase kinase, \(\alpha\)P-labeled at the \(\beta\) subunit, isolated a polypeptide fragment \(M_r = 33,000\) after chymotryptic digestion which is active and retains radioactivity. They claimed that the \(\beta\) subunit is the catalytic subunit. Another possible explanation is that the pH 8.2 activity of the \(\gamma\) subunit can be stimulated by other subunit(s).

We have provided evidence that an active species can be obtained from the holoenzyme in the absence of proteinase which contains no phosphate, cannot autophosphorylate itself, has a \(M_r\) of 45,800, and co-migrates with the \(\gamma\) subunit. Because of these results we suggest that the \(\gamma\) subunit of phosphorylase kinase is catalytically active.

**REFERENCES**

1. Meyer, W. L., Fischer, E. H., and Krebs, E. G. (1964) Biochemistry 3, 1033-1039
2. Ozawa, E., Hosoi, K., and Ebashi, S. (1967) J. Biochem. (Tokyo) 61, 531-533
3. Ozawa, E., and Ebashi, S. (1967) J. Biochem. (Tokyo) 62, 285-286
4. Krebs, E. G., Graves, D. J., and Fischer, E. H. (1959) J. Biol. Chem. 234, 2667-2673
5. Krebs, E. G., Love, D. S., Bravtovld, G. E., Trayser, K. A., Meyer, W. L., and Fischer, E. H. (1964) Biochemistry 3, 1022-1033
6. Cohen, P. (1973) Eur. J. Biochem. 34, 1-14
7. Hayakawa, T., Perkins, J. F., and Krebs, E. G. (1973) Biochemistry 12, 574-580
8. Yeman, S. J., and Cohen, P. (1975) Eur. J. Biochem. 51, 93-104
9. Singh, T. J., and Wang, J. H. (1977) J. Biol. Chem. 252, 625-632
10. Lincoln, T. M., and Corbin, J. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3239
11. Kishimoto, A., Takai, Y., and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7449
12. Huston, R. B., and Krebs, E. G. (1968) Biochemistry 7, 2116-2122
13. Hayakawa, T., Perkins, J. P., Walsh, D. A., and Krebs, E. G. (1973) Biochemistry 12, 567-573
14. Jennissen, H. P., and Heilmeyer, L. M. G. (1977) FEBS Lett. 42, 77
15. Cohen, P., Burchell, A., Foulkess, J. G., Cohen, T. W., Vanaman, T. C., and Nairn, A. C. (1978) FEBS Lett. 92, 287
16. Kaltiuchi, S., Yamazaki, R., and Nakajima, H. (1970) Proc. Jpn. Acad. 46, 589
17. Cheung, W. Y. (1970) Biochem. Biophys. Res. Commun. 38, 533
18. Cheung, W. Y. (1971) J. Biol. Chem. 246, 2859
19. Graves, D. J., Hayakawa, T., Horvitz, R. A., Beckman, E., and Krebs, E. G. (1973) Biochemistry 12, 580-585
20. Fischer, E. H., Blum, H. E., Byers, B., Heinmann, C., Kerrick, G. W., Lehky, P., Malencik, D. A., and Pocinwog, S. (1970) in Metabolic Interconversion of Enzymes, Arad, I. (Shaltiel, S., ed) pp. 1-8, Springer-Verlag, New York
21. Delange, R. J., Kemp, R. G., Riley, W. D., Cooper, D. A., and Krebs, E. G. (1968) J. Biol. Chem. 243, 2200-2206
22. Tabatabai, L. B., and Graves, D. J. (1978) J. Biol. Chem. 253, 2196-2202
23. Fischer, E. H., and Krebs, E. G. (1958) J. Biol. Chem. 231, 65-71
24. Ilengworth, B., and Cori, G. T. (1953) Biochem. Prep. 3, 1-9
25. Kastenschmidt, L. L., Kastenschmidt, J., and Helmreich, E. (1968) Biochemistry 7, 3590-3605
26. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
28. Brostrom, C. O., Hunkeler, F. L., and Krebs, E. G. (1971) J. Biol. Chem. 246, 1961-1967
29. Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1971) J. Biol. Chem. 246, 1966-1955
30. Ryan, L. D., and Vestling, C. S. (1974) Arch. Biochem. Biophys. 160, 279-284
31. Vaquelin, G., Lacombe, M.-L., Hanoune, J., and Strosberg, A. D. (1975) Biochem. Biophys. Res. Commun. 64, 1076-1082
32. Decker, L. A., ed, Worthington Enzyme Manual (1977) pp. 19-26, Worthington Biochemical Corp., Freehold, N. J.
33. Purbsch, F. S., Fonda, M. L., and Metzler, D. E. (1969) Biochemistry 8, 5169-5180
34. Glynn, I. M., and Chappell, J. B. (1964) Biochem. J. 99, 147-149
35. Carlson, G. M., and Graves, D. J. (1976) J. Biol. Chem. 251, 7460-7466
36. Carlson, G. M., and Graves, D. J. (1976) Biochemistry 15, 4476-4481
37. Stellwagen, E. (1977) Actes. Chem. Res. 10, 92-98
38. Cohen, O., Watson, D. C., and Dixon, G. H. (1975) Eur. J. Biochem. 51, 79-92
39. Brostrom, M. A., Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1970) Adv. Enzyme Regul. 8, 191-203
40. Dickpeiten, G., Jennissen, H. P., and Heilmeyer, L. M. G. (1978) FEBS Lett. 87, 297
41. Gulyaeva, N. B., Guskova, R. A., Barenova, L. A., Gulyaev, N. N., Valfsson, P. L., and Severin, E. S. (1977) Dokl. Akad. Nauk. SSSR 235, 696
42. Gulyaeva, N. V., Valtsson, P. L., and Severin, E. S. (1978) Biochim. Biophys. Acta 543, 373-381
43. Horl, W. H., and Heilmeyer, L. M. G. (1978) Biochemistry 17, 766
44. Fischer, E. H., Alaba, J. O., Brautigan, D. L., Kerrick, W. G. L., Malencik, D. A., Moechler, H. J., Picton, C., and Pocinwog, S. (1978) in Versatility of Proteins, Proceedings of the International Symposium on Proteins, March 6 to 8, 1978, Tuzupe, (Li, C. ed) p. 133, Academic Press

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J R Skuster, K F Chan and D J Graves

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