Studies on the Subunit Structure of Rabbit Skeletal Muscle Phosphorylase Kinase†

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ABSTRACT: Rabbit skeletal muscle phosphorylase kinase was treated with sodium dodecyl sulfate and fractionated by gel filtration chromatography into two peaks. Peak I was shown to consist of previously identified subunits A and B having molecular weights of 118,000 and 108,000, respectively. Peak II was shown to consist of subunit C having a molecular weight of 41,000. Peak I showed slight heterogeneity on sedimentation equilibrium. The average molecular weight for this fraction was found to be 103,000 in 0.1% sodium dodecyl sulfate and 113,000 in buffer at pH 10.3. Peak II showed a molecular weight of 43,000 in 0.1% sodium dodecyl sulfate. The amino acid composition of peak I was shown to differ significantly from that of peak II. In the course of activation of phosphorylase kinase by [γ-^{32}P]ATP, subunit B was rapidly phosphorylated. Subunit A was also phosphorylated but at a slower rate following a short lag period. Subunit C did not take up phosphate during the activation reaction. At higher concentrations of ATP and Mg^{2+} (2.6/20 mM), nonspecific ^{32}P incorporation occurred without the addition of protein kinase. Little change in enzymic activity took place although considerable phosphorylation of subunits A and B occurred. During trypsin activation of phosphorylase kinase subunits A and B were rapidly degraded in contrast to subunit C which appeared to be more resistant to proteolysis.

The preceding paper (Hayakawa *et al.*, 1973) described a purification procedure for rabbit skeletal muscle phosphorylase kinase (ATP–phosphorylase phosphotransferase, EC 2.7.138) which yielded an essentially homogeneous enzyme. Physicochemical characterization of the kinase showed it to have a molecular weight of 1.33×10^6 and to be composed of three types of subunits with molecular weights of 118,000, 108,000, and 41,000 as revealed by disc gel electrophoresis in the presence of sodium dodecyl sulfate. These subunits, referred to as A, B, and C, respectively, were found to be present in amounts suggesting that the quaternary structure of the intact enzyme can be expressed by the formula, $A_4B_4C_8$.

In the present paper an effort has been made to characterize more fully the subunits of phosphorylase kinase. Enzyme dissociated by sodium dodecyl sulfate was divided into a heavy (mixture of subunits A and B) and a light fraction (subunit C) by gel filtration chromatography. Each of these fractions was characterized by sedimentation equilibrium and amino acid analysis. Activation of the kinase by enzymatic phosphorylation was studied as a function of phosphate uptake in the individual subunits. Preliminary observations were made as to the effect of tryptic activation on the subunit structure of phosphorylase kinase.

Experimental Section

Materials. The purification of nonactivated rabbit skeletal muscle phosphorylase kinase was carried out as described previously (De Lange *et al.*, 1968) with the additional steps given in the preceding paper (Hayakawa *et al.*, 1973). Crystalline rabbit skeletal muscle phosphorylase *b* was prepared as described previously (Fischer and Krebs, 1958). The catalytic subunit of rabbit skeletal muscle cyclic AMP[1]-dependent protein kinase was kindly supplied by Dr. C. O. Brostrom of this laboratory. [^{35}S]Sodium dodecyl sulfate was purchased from New England Nuclear Corp. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappel (1964) with minor modifications (Walsh *et al.*, 1971). Other materials were described in the preceding paper (Hayakawa *et al.*, 1973).

Activity Tests and Activation Reactions. Phosphorylase activity was determined in the direction of glycogen synthesis by the method of Cori *et al.* (1943). Phosphorylase kinase activity was determined by a slight modification (Brostrom *et al.*, 1971) of the method described earlier (Krebs *et al.*, 1964). Activation of phosphorylase kinase, as catalyzed by the catalytic subunit of the cyclic AMP-dependent protein kinase, was carried out at 30° in a 1.0-ml reaction mixture containing phosphorylase kinase, 1.4–1.8 mg/ml; [γ-^{32}P]ATP, 0.13 mM; magnesium acetate, 1 mM; β-mercaptoethanol, 20 mM; EDTA, 1.3 mM; sodium glycerol phosphate buffer (pH 6.8), 33 mM; and 2–4 μg of a purified catalytic sununit of rabbit muscle cyclic AMP-dependent protein kinase (Walsh *et al.*, 1971; Reimann *et al.*, 1971). Phosphorylation of phosphorylase kinase in the autocatalytic reaction (De Lange *et al.*, 1968; Krebs *et al.*, 1964) was carried out at 30° in a 1.0-ml reaction mixture containing phosphorylase kinase, 1.8 mg/ml; [γ-^{32}P]-ATP, 2.6 mM; magnesium acetate, 20 mM; β-mercaptoethanol, 20 mM; EDTA, 1.3 mM; and sodium glycerol phosphate buffer (pH 6.8), 33 mM. After incubation for a specified time in either type of activation reaction, aliquots were removed and diluted 20-fold with cold 10 mM sodium glycerol phosphate (pH 6.8) containing β-mercaptoethanol and kept at 0° until assays were carried out for phosphorylase kinase activity. For disc gel electrophoresis in the presence of sodium dodecyl sulfate (see

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[1] Abbreviation used: cyclic AMP, cyclic adenosine 3′,5′-monophosphate.

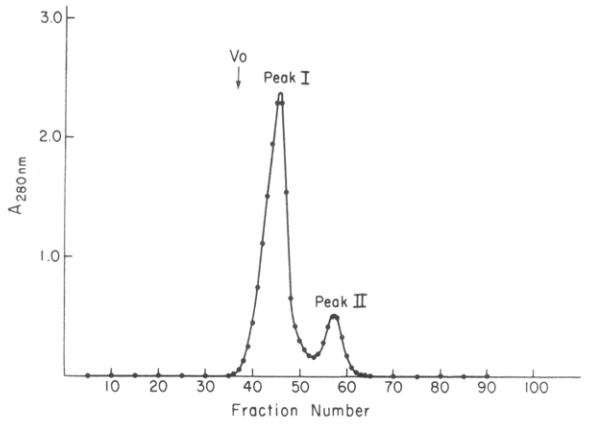

FIGURE 1: Fractionation of phosphorylase kinase subunits on a Sephadex G-200 column. Phosphorylase kinase was dissociated at 65° for 30 min in the presence of 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. After dialyzing against 50 mM ammonium bicarbonate buffer (pH 8.1) containing 0.1% sodium dodecyl sulfate, 18.7 mg of the enzyme was applied to a Sephadex G-200 column (2.5 × 86 cm) equilibrated with 50 mM ammonium bicarbonate buffer (pH 8.1) containing 0.1% sodium dodecyl sulfate. The flow rate was adjusted to 1 ml/25 min. Equal volume fractions (1.12 ml) were collected and absorbancies at 280 nm (A_{280nm}) were measured. The arrow indicates the void volume of the column.

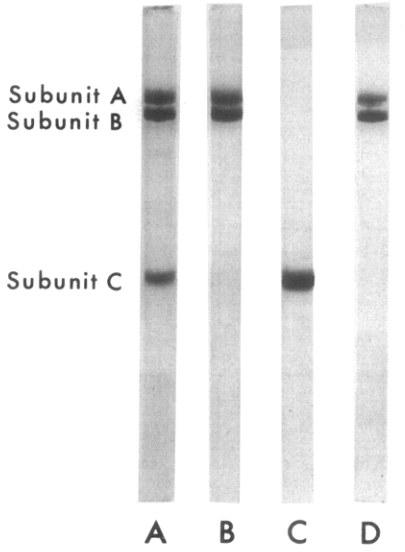

FIGURE 2: Disc gel electrophoresis patterns of phosphorylase kinase and its subunits in the presence of sodium dodecyl sulfate. (A) Native phosphorylase kinase; (B) peak I fraction (A and B subunits) from the Sephadex G-200 column; (C) peak II fraction (C subunit) from the Sephadex G-200 column; (D) peak I fraction retreated at 65° for 30 min in the presence of 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. Electrophoresis was performed using 7.5% acrylamide gel in Tris–glycine–borate buffer (pH 9.3) containing 0.1% sodium dodecyl sulfate, at 8 mA/gel for 2.5 hr. Proteins were stained with Coomassie Brilliant Blue and destained by diffusion in methanol–acetate solution as reported by Weber and Osborn (1969).

below) aliquots of the reaction mixture were mixed with $^1/_5$ volume of 6% sodium dodecyl sulfate and 6% β-mercaptoethanol and incubated for 30 min at 65° to dissociate the protein.

Ultracentrifugal Analyses. All sedimentation experiments were performed in a Beckman Model E ultracentrifuge equipped with an RTIC temperature control unit, Schlieren and Rayleigh interference optics. The molecular weights of the phosphorylase kinase subunits were determined by the sedimentation equilibrium method according to the meniscus depletion technique of Yphantis (1964) and the long-column meniscus depletion technique of Chervenka (1970). For the former equilibrium runs, a 12-mm six-channel Kel-F centerpiece was employed, and for the latter, a 12-mm double-sector capillary synthetic boundary cell was used. Molecular weights were determined as the average of those obtained from each of three black fringes. The partial specific volume ($\bar{v}$) was assumed to be 0.730 ml/g for both subunit fractions of phosphorylase kinase.

Disc Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate. Polyacrylamide gel electrophoresis was carried out in the presence of sodium dodecyl sulfate at alkaline pH (Hayakawa *et al.*, 1973). Proteins were first heated at 65° for 30 min in the presence of 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. Approximately 15-μg samples were then placed on the gels. After electrophoresis and staining with Coomassie Brilliant Blue (Weber and Osborn, 1969) densitometer tracings were made at 600 nm using a Gilford spectrophotometer equipped with a Model 2410 linear transport system in order to estimate the relative concentrations of individual protein bands. The gels were cut into 1-mm slices with a gel slicer, and each slice was then digested with 100 μl of 30% hydrogen peroxide at 85° for 1 hr. Radioactivity (^{32}P) was determined in a Packard Tricarb scintillation spectrophotometer, utilizing a scintillation fluid composed of 125 g of naphthalene, 7.5 g of 2,5-diphenyloxazole, and 375 mg of 1,4-bis[2-(5-phenyloxazolyl)benzene] dissolved in 1 l. of dioxane. Protein-bound phosphate was stable during the staining and destaining procedures as well as during the incubation at 65° in 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. The recovery of radioactivity after disc gel electrophoresis of sodium dodecyl sulfate was more than 90% of the ^{32}P originally bound to the enzyme as measured by the method reported previously (Walsh *et al.*, 1971).

Other Methods. Sodium dodecyl sulfate concentrations in the dialysate and in the protein solution were determined by radioactivity measurement of [^{35}S]sodium dodecyl sulfate or by extraction of an alkyl sulfate–Methylene Blue complex into chloroform and determining the optical density of the chloroform extract at 655 nm (Ray *et al.*, 1966; Reynolds and Tanford, 1970). Protein determinations were performed by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin ($E^{1\%}_{1\,cm}$ = 5.30 at 280 nm) as a standard (Sogami and Foster, 1962).

Results

Partial Separation of Phosphorylase Kinase Subunits by Gel Filtration. As reported in the previous paper (Hayakawa *et al.*, 1973) phosphorylase kinase can be dissociated into three different sized subunits, A, B, and C, as shown by disc gel electrophoresis in the presence of sodium dodecyl sulfate (also see Figure 2A). In an attempt to separate these subunits, phosphorylase kinase treated with sodium dodecyl sulfate was chromatographed on a Sephadex G-200 column equilibrated with 0.1% sodium dodecyl sulfate in 0.05 M ammonium bicarbonate. As shown in Figure 1, the elution profile showed one main component (peak I) and a minor component (peak II). On examination of these fractions by disc gel electrophoresis in the presence of sodium dodecyl sulfate, it was found that peak I consisted of subunits A and B (Figure 2B) and peak II contained subunit C (Figure 2C). In order to eliminate the

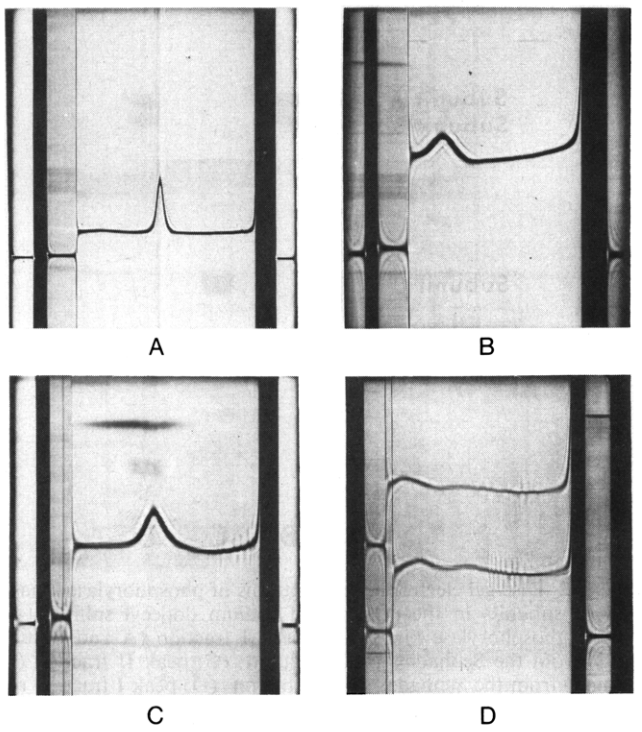

FIGURE 3: Sedimentation patterns obtained with phosphorylase kinase subunit fractions. (A) Peak I fraction from the Sephadex G-200 column in 1 mM sodium phosphate buffer (pH 9.0) containing 0.1 M NaCl and 0.1% sodium dodecyl sulfate after 77 min at 60,000 rpm; (B) peak II fraction from Sephadex G-200 column in 1 mM sodium phosphate buffer (pH 9.0) containing 0.1 M NaCl and 0.1% sodium dodecyl sulfate after 79 min at 48,000 rpm; (C) peak I fraction in 50 mM glycine–NaOH buffer (pH 10.3) containing 50 mM β-mercaptoethanol after 76 min at 60,000 rpm; (D) peak II fraction in 50 mM glycine–NaOH buffer (pH 11.0) (bottom) and in 50 mM NaOH (pH 12.0) containing 0.1 M NaCl (top) after 36 min at 60,000 rpm. Sedimentation proceeds from *right* to *left*; running temperature 20°; protein concentrations were 0.33, 0.045, 0.85, and 0.9 g/100 ml in A–D, respectively.

possibility that subunit C might be formed from either subunit A or subunit B, an aliquot of peak I was re-treated with 1% sodium dodecyl sulfate and 1% β-mercaptoethanol for 30 min and re-examined by disc gel electrophoresis (Figure 2D). No evidence was obtained, however, that peptide C might be formed by such treatment.

TABLE I: Molecular Weight of Peak I Fraction Determined by Meniscus Depletion Method at Different Protein Concentrations.[a]

Initial Protein Concn (g/100 ml)	Mol Wt
0.030	116,000
0.061	113,000
0.122	111,000

[a] Conditions: 50 mM glycine–NaOH buffer (pH 10.3) containing 50 mM β-mercaptoethanol, 20°, 42 hr at 18,000 rpm. The peak I fraction was obtained by gel filtration in the presence of sodium dodecyl sulfate which was removed by dialysis as explained in the text.

TABLE II: Sedimentation Coefficients and Molecular Weights of Phosphorylase Kinase Subunits in the Presence of Sodium Dodecyl Sulfate.[a]

	Method for Determination of Sodium Dodecyl Sulfate Concentration	g of Sodium Dodecyl Sulfate/g of Protein	$s_{20,w}$	Mol Wt
Peak I	[^{35}S]Sodium dodecyl sulfate	1.01	5.5	
	Colorimetry	1.26	5.2	103,000
Peak II	[^{35}S]Sodium dodecyl sulfate	1.91	3.6	
	Colorimetry	2.11	3.6	
		2.13	3.8	43,000

[a] Conditions: Both fractions were exhaustively dialyzed against 1 mM sodium phosphate buffer (pH 9.0) containing 0.1 M NaCl and 0.1% sodium dodecyl sulfate (Bakerman and Wasemiller, 1967).

Sedimentation Coefficients and Molecular Weights of the Fractionated Phosphorylase Kinase Subunits. The Schlieren patterns obtained in the ultracentrifuge for peaks I and II derived from phosphorylase kinase by gel filtration in the presence of sodium dodecyl sulfate are shown in Figure 3. Peak I, which is actually a mixture of subunits A and B as explained above, gave a nearly symmetrical single peak (Figure 3A) indicating that subunits A and B have the same sedimentation coefficient. Peak II (Figure 3B) also exhibited a single peak.[2] In order to remove sodium dodecyl sulfate, peak I was dialyzed for a number of days against 50 mM glycine–NaOH buffer (pH 10.3) containing 50 mM β-mercaptoethanol, until colorimetric analysis showed that the detergent had been essentially eliminated. The dialyzed solution showed a single symmetrical peak in the ultracentrifuge (Figure 3C), and from extrapolation to infinite dilution, an $s^0_{20,w}$ value of 4.4 was calculated. The weight average molecular weight of the peak I fraction determined by the meniscus depletion method (Table I) gave values in good agreement with what would be expected for a mixture of subunits A and B having molecular weights of 118,000 and 108,000, respectively (Hayakawa *et al.*, 1973). A plot of the logarithm of protein concentration *vs.* the square of the distance from the axis of rotation (not illustrated) showed slight curvature indicative of some heterogeneity. With the peak II fraction, efforts to remove sodium dodecyl sulfate while keeping the protein in solution were less successful. Even in a slightly more alkaline buffer, the sedimentation pattern of this material indicated the presence of aggregated forms (Figure 3D). Sedimentation velocities and molecular weights of peaks I and II were determined in the presence of 0.1% sodium dodecyl sulfate according to the method of Hersh and Schachman (1958). These results are summarized in Table II. Again, the results are in keeping with what was

[2] In the experiment of Figure 3A a 12-mm centerpiece was used whereas in the experiment of Figure 3B a 30-mm centerpiece was used.

TABLE III: Comparison of the Amino Acid Composition of Phosphorylase Kinase Subunits.[a]

Amino Acid	Mol of Amino Acid/1 × 10^5 g of Protein		
	Native Phosphorylase Kinase	Peak I Fraction	Peak II Fraction
Lys	42.9	42.1	48.1
His	18.1	18.3	19.4
Arg	40.5	39.8	48.3
Asp	68.7	66.9	65.5
Thr	35.7	35.8	35.2
Ser	44.5	49.8	40.2
Glu	89.3	87.7	85.8
Pro	36.4	37.3	37.4
Gly	45.4	47.3	44.0
Ala	42.5	42.8	43.7
Val	44.9	42.5	49.6
Met	20.5	19.9	19.3
Ile	37.8	36.9	33.8
Leu	80.5	82.9	71.7
Tyr	28.5	28.7	31.1
Phe	27.9	25.6	31.0

[a] All the samples were hydrolyzed for 24 hr.

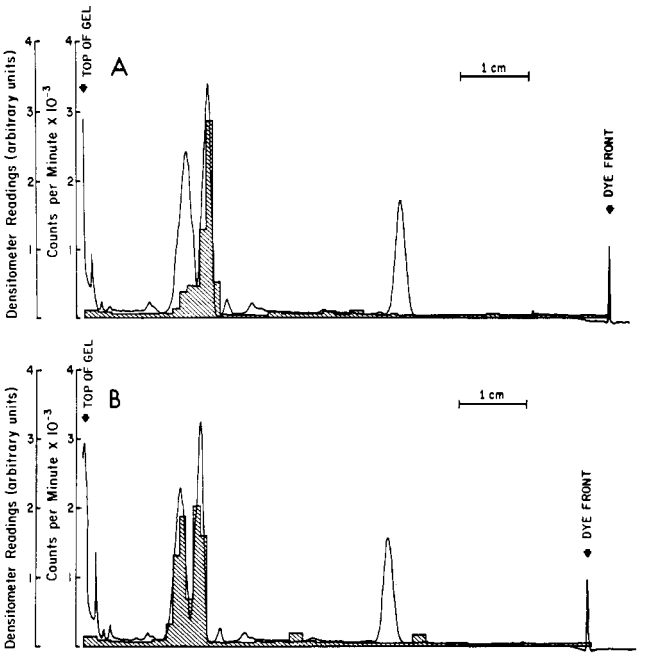

FIGURE 4: Activation and ^{32}P incorporation into the different subunits of phosphorylase kinase. Purified phosphorylase kinase (1.62 mg) was activated by incubation with 0.13 mM [γ-^{32}P]ATP and 1 mM magnesium acetate in the presence of 4 μg of a catalytic subunit of cyclic AMP-dependent protein kinase as described in the text. At intervals aliquots were removed and examined by disc gel electrophoresis in the presence of sodium dodecyl sulfate: A = activation for 2 min; B = activation for 10 min. Densitometer tracings of the gels were performed using a Gilford spectrophotometer equipped with a Model 2410 linear transport system at 600 nm after staining with Coomassie Brilliant Blue to indicate the positions and relative concentrations of individual protein bands. Radioactivity in each band was determined by transverse sectioning of the gel and counting individual 1-mm slices, as described in the Experimental Section.

obtained by disc gel electrophoresis in the presence of sodium dodecyl sulfate. The amount of bound sodium dodecyl sulfate in peak II seems unusually high in view of the observation of Reynolds and Tanford (1970) that constant binding ratios of either 0.4 g of sodium dodecyl sulfate/g of protein or 1.4 g/g are seen, *i.e.*, as a function of the equilibrium monomer concentration.

Comparison of the Partial Amino Acid Composition of Phosphorylase Kinase Subunits. Table III gives the partial amino acid composition of peak I (subunits A and B) and peak II (subunit C) obtained from phosphorylase kinase chromatographed by gel filtration in the presence of sodium dodecyl sulfate. It is clear that significant differences are present between these peaks particularly with respect to the content of lysine, arginine, serine, valine, leucine, and phenylalanine. Peak I was richer in serine and leucine whereas peak II was richer in lysine, arginine, valine, and phenylalanine.

Activation and Phosphorylation of Phosphorylase Kinase. In order to investigate the functional roles of the three different subunits of phosphorylase kinase, the enzyme was activated by [γ-^{32}P]ATP (Krebs *et al.*, 1964; De Lange *et al.*, 1968; Walsh *et al.*, 1971) and a study was made of the incorporation of phosphate into the individual subunits of the enzyme. The activation of phosphorylase kinase was catalyzed by the catalytic subunit of skeletal muscle cyclic AMP-dependent protein kinase which acts as a phosphorylase kinase kinase and has no requirement for cyclic AMP (Walsh *et al.*, 1971; Reimann *et al.*, 1971). The experiment was carried out by interrupting the activation reaction at different stages and running disc gel electrophoresis determinations in the presence of sodium dodecyl sulfate. The gels were then stained and sliced, and the radioactivity of the different bands was determined. It was noted that activation of the enzyme by ATP had no effect on the gel pattern obtained, *i.e.* in all cases the patterns were identical with that of Figure 2A. Figure 4 shows the result of an experiment in which incorporation into the different bands was determined after a brief period (Figure 4A) and a more prolonged period (Figure 4B) of activation. In the first instance (activation for 2 min) only subunit B was phosphorylated, but with a longer period of activation (10 min) subunit A was also phosphorylated. No phosphorylation of subunit C occurred at either time.

In a more complex experiment (Figure 5) the incorporation of phosphate into the different subunits of phosphorylase kinase during the time course of an activation reaction was correlated with the changes in enzyme activity that occurred at pH 6.8 and 8.2, *i.e.*, the two pH values at which phosphorylase kinase activity is commonly determined.[3] The experiment showed that there was a rapid incorporation of phosphate into subunit B. This was followed by the phosphorylation of subunit A after a brief lag period as would have been predicted from the experiments of Figure 4. The phosphorylation of subunit A started approximately at the time when subunit B was half-maximally phosphorylated. The increase in activity at pH 8.2 (top curve in Figure 5) followed a course more or less

[3] Nonactivated phosphorylase kinase has almost no activity at pH values of 6.8 or lower but manifests appreciable activity at pH 8.2. Activation of the enzyme, which occurs as a result of phosphorylation of serine residues in the enzyme, results in an increase in the activity at both pH values but particularly at pH 6.8 (Krebs *et al.*, 1964; De Lange *et al.*, 1968). Thus, an increase in the ratio of activity at pH 6.8 to activity at pH 8.2 has often been used as an index of enzyme activation.

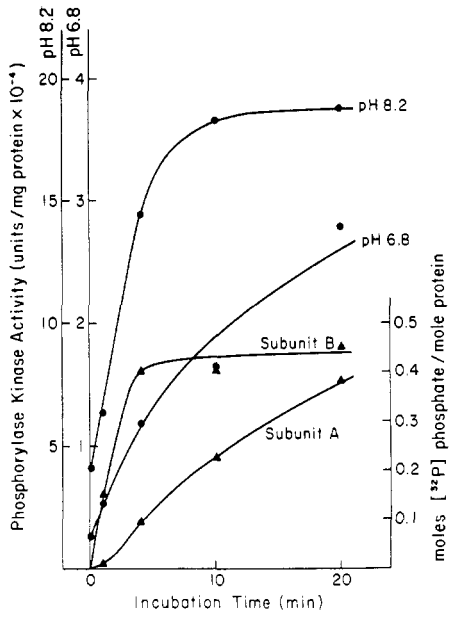

FIGURE 5: Time course of phosphorylase kinase activation and the incorporation of phosphate into individual subunits. Purified phosphorylase kinase (1.4 mg) was activated by incubation with [γ-^{32}P]-ATP and Mg^{2+} in the presence of 2 μg of a catalytic subunit of cyclic AMP-dependent protein kinase as in the experiment of Figure 4. At intervals aliquots were removed and assayed for phosphorylase kinase activity at pH 6.8 and 8.2 as indicated (circles). The samples were also examined by disc gel electrophoresis in the presence of sodium dodecyl sulfate for the incorporation of [^{32}P]-phosphate into subunits A or B (triangles).

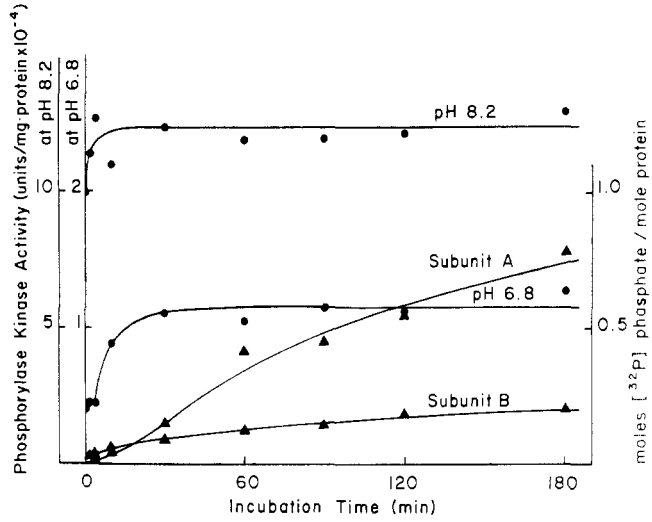

FIGURE 6: Time course of phosphorylase kinase activation and the incorporation of phosphate into individual subunits under conditions favoring self-phosphorylation. Purified phosphorylase kinase (1.8 mg) was incubated with 2.6 mM [γ-^{32}P]ATP and 20 mM magnesium acetate in the absence of added protein kinase catalytic subunit. At intervals aliquots were removed and assayed for phosphorylase kinase activity at pH 6.8 and 8.2 as indicated (circles) or examined by disc gel electrophoresis in the presence of sodium dodecyl sulfate for the incorporation of [^{32}P]phosphate into subunits A or B (triangles). Other conditions for the phosphorylation of phosphorylase kinase by itself are given in the Experimental Section.

parallel to the phosphorylation of subunit B. Activity as measured at pH 6.8 followed an independent course and was still increasing at 20 min as was phosphate incorporation into subunit A. In other experiments carried out for longer than 20 min (not illustrated) it was found that the activity at pH 6.8 usually reached a plateau or maximum within 25–30 min, but the incorporation of phosphate into subunit A often continued for as long as 60 min and the total amount incorporated exceeded 1 mol/mol of subunit. Even after prolonged periods of incubation under the conditions used in the experiments of Figure 5, subunit C was not phosphorylated. There was essentially no activation or phosphorylation of phosphorylase kinase at these ATP and Mg^{2+} concentrations unless the catalytic subunit of cyclic AMP-dependent protein kinase was present.

Phosphorylation of Phosphorylase Kinase without Added Protein Kinase. Earlier observations indicated that in addition to activation of phosphorylase kinase by protein kinase, activation could also occur by a self-phosphorylation of phosphorylase kinase (Walsh *et al.*, 1971). It was of interest to compare the pattern of phosphate incorporation into the phosphorylase kinase subunits with this type of activation with that which occurred when the enzyme was activated by the protein kinase. An experiment involving self-phosphorylation is shown in Figure 6. For this experiment the [^{32}P]ATP and magnesium acetate concentrations were increased 20-fold over that used in the experiments of Figures 4 and 5 inasmuch as the K_m for Mg^{2+}-ATP is higher for phosphorylase kinase than for the protein kinase. The initial 10–20-min time period showed a similar profile to the activation and phosphorylation of phosphorylase kinase in the presence of the catalytic subunit of the cyclic AMP-dependent protein kinase (Figure 5). However, it was observed that the incorporation of phosphate into subunit B was relatively low and also that phosphorylase kinase activity was increased less than threefold. The incorporation of phosphate into subunit A increased continuously even after 3 hr of incubation, but little change in activity occurred after 20–30 min of incubation. The incorporation of phosphate into the subunits, especially into subunit A, seems to have only a minor effect on the activity of phosphorylase kinase, suggesting that a nonspecific phosphorylation of phosphorylase kinase has occurred. Even under these conditions of high ATP and Mg^{2+}, no phosphorylation of subunit C could be detected.

Activation of Phosphorylase Kinase by Trypsin. Trypsin is known to cause a strong activation of purified muscle phosphorylase kinase, presumably as a result of limited proteolysis (Krebs *et al.*, 1964; Huston and Krebs, 1968). It was of interest, therefore, to see how this type of activation affected the subunit structure of the enzyme. Figure 7 shows the results of an experiment in which the time course of trypsin activation of the kinase was correlated with changes in the sodium dodecyl sulfate disc gel electrophoresis patterns of the enzyme. It is clear that subunits A and B appeared to be particularly susceptible to modification by trypsin. During trypsin activation a major new band corresponding to a molecular weight of 80,000 appeared and another band of 47,000 molecular weight also developed at the expense of subunits A and B. Subunit C appeared to be resistant to the action of trypsin. In another experiment with even more prolonged tryptic attack the bands corresponding to subunits A and B disappeared completely and the only major bands remaining were the band corresponding to peptide C and another band in the region corresponding to a protein with a molecular weight of 53,000.

Discussion

The molecular weight determinations by ultracentrifugal analysis of peaks I and II derived from phosphorylase kinase

by gel filtration chromatography were consistent with the known subunit composition of these fractions. Peak I, made up of subunits A and B, differing in molecular weight by only 10%, behaved almost as one component although slight heterogeneity was observed by sedimentation equilibrium. Peak II behaved as a single component exhibiting a molecular weight by sedimentation equilibrium in the presence of sodium dodecyl sulfate which agreed well with the value obtained by disc gel electrophoresis. The amino acid compositions of peaks I and II were sufficiently different to confirm the concept that phosphorylase kinase is made up of dissimilar subunits, a conclusion that was also supported by the phosphorylation data as well as those obtained using trypsin.

The study of subunit phosphorylation during activation of phosphorylase kinase by [^{32}P]ATP is difficult to interpret, but several points are clear. Unlike the phosphorylation of phosphorylase in which a single unique serine in the phosphorylase subunit is phosphorylated (Fischer *et al.*, 1959), the phosphorylation and activation of phosphorylase kinase are more complex. From results obtained several years ago in activation studies carried out at high magnesium and ATP concentrations (Riley *et al.*, 1968) it had already been concluded that the kinase possessed multiple phosphate-binding sites, and it was proposed that these could be categorized as consisting of a "specific activation site" plus a "nonspecific set of sites" unrelated to the activation process. The present study would tend to support the idea that if a specific activation site really exists, it is located in subunit B which is the more rapidly phosphorylated subunit. Most of the change in enzyme activity occurs during the phosphorylation of this subunit. It was noted, however, that an increase in phosphorylase kinase activity was still occurring *after* the reaction involving subunit B was finished. This would suggest that the phosphorylation of subunit A also plays a part in the activation process. Phosphorylation of subunit A that is unrelated to activation of the enzyme clearly does occur, however, under conditions of high ATP and magnesium concentrations as shown in Figure 6.

In the experiment of Figure 5 the phosphorylation of subunit B levels off at a point at which only 0.45 mol of [^{32}P]-phosphate has been introduced per mole of subunit. The reason for this is not clear, but it is possible that the kinase used was already partially saturated with nonradioactive phosphate. It is known (Mayer and Krebs, 1970) that nonactivated phosphorylase kinase contains some endogenous serine-bound phosphate as isolated. If this phosphate is bound at a site identical with that which is being phosphorylated by [γ-^{32}P]ATP in subunit B, it would, of course, lower the apparent stoichiometry of the reaction.

In view of the fact that activation of phosphorylase kinase (either as a result of phosphorylation or as a result of tryptic digestion) causes alterations in subunits A and B but does not affect subunit C, it is attractive to think that subunit C may be a catalytic subunit that is regulated by subunits A and B. There could be a similarity between phosphorylase kinase and the cyclic AMP-dependent protein kinases. It is known (Brostrom *et al.*, 1970; Gill and Garren, 1970; Tao *et al.*, 1970; Kumon *et al.*, 1970) that the latter consists of a regulatory subunit which binds to and inhibits a catalytic subunit. The protein kinase is activated by cyclic AMP which alters the conformation of the regulatory subunit so that it dissociates from the catalytic subunit. It is not known, however, whether the regulatory subunit of the cyclic AMP-dependent protein kinase need necessarily dissociate from the catalytic subunit for the enzyme to become active, *i.e.*, the conformational change in the regulatory sununit

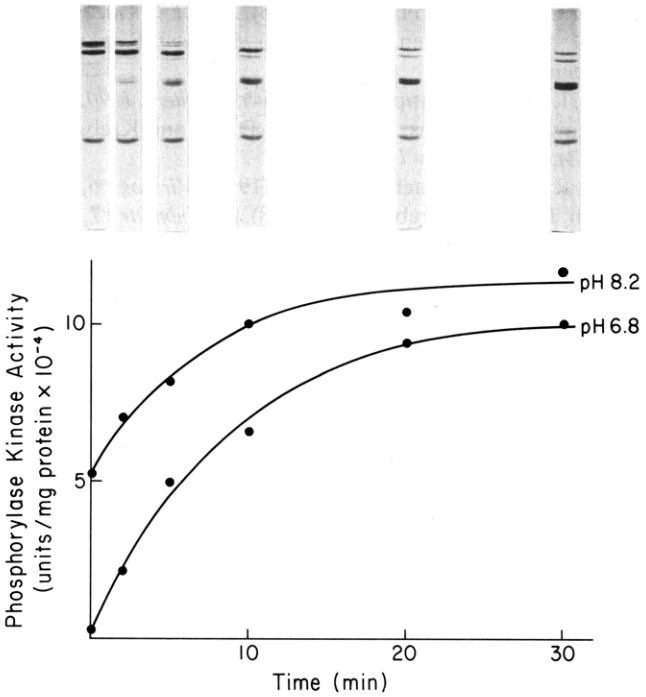

FIGURE 7: The time course of phosphorylase kinase activation by trypsin and the subunit patterns obtained by disc gel electrophoresis in the presence of sodium dodecyl sulfate. A reaction mixture containing 1.6 mg of phosphorylase kinase in 50 mM glycerol phosphate buffer (pH 6.8) containing 2 mM EDTA and 10% sucrose was incubated at 30° with 300 ng of trypsin in 0.5 ml total volume. Aliquots were removed and the reaction was stopped by addition of a sixfold excess (weight basis) of soybean trypsin inhibitor. Kinase activities were determined and the samples were examined by disc gel electrophoresis as described in the Experimental Section. The gel patterns obtained are superimposed on the activation time course at points corresponding to the times of sampling.

brought about by the finding of cyclic AMP might in itself relieve the inhibition brought about by subunit interaction. With phosphorylase kinase it is possible that phosphorylation or partial proteolytic attack of subunits A and/or B might alter their interaction with subunit C to allow the latter to become more active as a catalyst.

The next paper in this series will be concerned with a more extensive study of trypsin activation of phosphorylase kinase.

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Studies on the Subunit Structure of Trypsin-Activated Phosphorylase Kinase†

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ABSTRACT: Trypsin-activated phosphorylase kinase with a sedimentation constant of approximately 22 S can be dissociated to lower molecular weight species. In the presence of ATP at 5° a 13S component which has a molecular weight of approximately 350,000 is slowly formed. Rewarming the enzyme to 20° does not reverse the dissociation process. At lower enzyme concentrations, 9S and 6S species are formed as identified by sucrose gradient centrifugation. These subfragments of the enzyme are active in the assay, suggesting that the large molecular weight of phosphorylase kinase is not essential for catalysis of the phosphorylase *b* to *a* reaction. Analysis of the 6S fraction by gel electrophoresis in sodium dodecyl sulfate shows that subunits A and B which are present in native phosphorylase kinase are absent in this fraction. Subunit C, however, is still present. Cold denaturation of trypsin-activated kinase occurs and is accentuated in the presence of ATP. No effect of cold is seen with native nonactivated phosphorylase kinase or enzyme activated by phosphorylation. These forms of phosphorylase kinase are not dissociated by ATP.

In a previous report (Hayakawa *et al.*, 1973b), it was shown that phosphorylase kinase (ATP–phosphorylase phosphotransferase, EC 2.7.138) can be dissociated into three separable subunits by treatment with sodium dodecyl sulfate. Since no enzymic activity could be restored following this treatment, less stringent dissociating conditions were sought so that the relationships which these subunits have to the regulatory and catalytic function of phosphoylase kinase could be examined.

Nonactivated phosphorylase kinase, phosphorylase kinase that had been phosphorylated and activated by protein kinase, and trypsin-activated phosphorylase kinase were used in a comparative study. Phosphorylase kinase obtained by treatment with trypsin is catalytically active (Krebs *et al.*, 1964; Huston and Krebs, 1968) and sediments in the ultrcentrifuge with a sedimentation coefficient similar to that of nonactivated phosphorylase kinase (Krebs *et al.*, 1964) suggesting that only a limited attack of the enzyme by trypsin has occurred. The results reported herein show that trypsin-treated kinase dissociates upon dilution or by incubation with ATP to give active subfragments. The conditions found to dissociate trypsin-treated kinase to its subunits were ineffective with nonactivated and protein kinase-activated phosphorylase kinase.

Experimental Section

Materials. Phosphorylase *b*, nonactivated and protein kinase-activated phosphorylase kinase, and [γ-^{32}P]ATP were

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