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Physicochemical Properties of Rabbit Skeletal Muscle Phosphorylase Kinase†

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ABSTRACT: Rabbit skeletal muscle phosphorylase kinase has been obtained in a homogeneous form having a sedimentation coefficient, $s^0_{20,w}$, of 26.1 S and a molecular weight of 1.33×10^6. The enzyme is subject to pressure denaturation which leads to the formation of polydisperse aggregated material which can be separated from the major fraction of the enzyme by gel filtration using Sepharose 4B. Three types of subunits are present in phosphorylase kinase as determined by disc gel electrophoresis in the presence of sodium dodecyl sulfate. These subunits (A, B, and C) have molecular weights of 118,000, 108,000, and 41,000, respectively. Molar amounts of each subunit are estimated to be 4.0, 4.7, and 8.6 for subunits A, B, and C, respectively, probably corresponding to a formula of $A_4B_4C_8$ for the enzyme. Titration of the free sulfhydryl groups of phosphorylase kinase with 5,5′-dithiobis(2-nitrobenzoic acid) shows the presence of one set of rapidly reacting groups and another set that reacts more slowly. The rapidly reacting sulfhydryl groups do not appear to be essential for enzyme activity. On prolonged exposure to 5,5′-dithiobis(2-nitrobenzoic acid), under conditions in which the slower reacting sets are titrated, the activity of the enzyme is lost.

Phosphorylase kinase (ATP–phosphorylase phosphotransferase, EC 2.7.138) which catalyzes the conversion of phosphorylase *b* to phosphorylase *a* has been studied extensively using the enzyme obtained from rabbit skeletal muscle. These studies have been concerned primarily with the activation of the kinase by ATP and adenosine 3′,5′-cyclic monophosphate (Krebs *et al.*, 1959; Krebs *et al.*, 1964; De Lange *et al.*, 1968; Riley *et al.*, 1968; Mayer and Krebs, 1970; Walsh *et al.*, 1971), but other properties of the enzyme have also been determined. The kinase is a large molecule as judged by its high sedimentation coefficient (Krebs *et al.*, 1964). The enzyme was found to interact with glycogen (De Lange *et al.*, 1968), and it was also determined that Ca^{2+} is essential for its activity. This latter property was originally discovered by Meyer *et al.* (1964) and was later put on a quantitative basis by Ozawa *et al.* (1967). Recently the binding of Ca^{2+} to the enzyme has been studied directly using ^{45}Ca (Brostrom *et al.*, 1971). The importance of Ca^{2+} in the regulation of phosphorylase activation in the glycogen–enzyme particle has been emphasized by Heilmeyer *et al.* (1970).

In the present work the procedure for purifying rabbit skeletal muscle phosphorylase kinase (Krebs *et al.*, 1964; De Lange *et al.*, 1968) was modified and extended so that homogeneous enzyme became available for physical characterization. This article reports the molecular weight of the kinase, the subunit and amino acid composition, and the reactivity of sulfhydryl groups of the enzyme.

Experimental Section

Materials. Early steps used in the purification of nonactivated rabbit skeletal muscle phosphorylase kinase, *i.e.* up to but not including the chromatographic step utilizing Sephadex G-200, were carried out as described previously (De Lange *et al.*, 1968). Additional purification of the enzyme is given in the Results section. Crystalline rabbit skeletal muscle phosphorylase *b* was prepared as described previously (Brostrom *et al.*, 1971). Sodium dodecyl sulfate, glutamic dehydrogenase, γ-globulin, and aldolase were purchased from Sigma Chemical Co. β-Mercaptoethanol was from Eastman Organic Chemicals. Crystalline bovine serum albumin was Pentex; DTNB[1] was from Calbiochem. Electrophoresis grade acryl-

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[1] Abbreviation used: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

amide, *N,N'*-methylenebisacrylamide, and ammonium persulfate were obtained from Bio-Rad. Sepharose 4B, Sephadex G-200, and Sephadex G-25 were from Pharmacia Fine Chemicals. The chloride form of Dowex 1-X8, 20–50 mesh, from Bio-Rad was used after conversion to the acetate form.

Enzyme Assay Procedures. Phosphorylase activity determinations were carried out in the direction of glycogen synthesis by the method of Cori *et al.* (1943). Phosphorylase kinase activities were determined by a slight modification (Brostrom *et al.*, 1971) of the method originally described (Krebs *et al.*, 1964).

Ultracentrifugal Analyses. All sedimentation experiments were performed in a Beckman Model E ultracentrifuge equipped with a RTIC temperature control unit, Schlieren and Rayleigh interference optics, and an ultraviolet system with a monochromator and photoelectric scanner. Photographic plates were measured with a Nikon profile projector, Model 6C. The molecular weight of phosphorylase kinase was determined by the sedimentation equilibrium method according to the meniscus depletion technique of Yphantis (1964). For the equilibrium runs, a 12-mm six-channel Kel-F centerpiece purchased from Beckman 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 calculated to be 0.730 ml/g from the amino acid composition.

Isoelectric Focusing. This was performed according to the method of Vesterberg and Svenson (1966). A sucrose density gradient containing 3% of pH 3–6 carrier ampholytes was prepared and used at 4° in a 110-ml LKB electrofocusing column. Purified phosphorylase kinase was passed through a Sephadex G-25 column, previously equilibrated with 50 mM sodium glycerol phosphate buffer (pH 7.0) containing 2 mM EDTA, to remove sucrose and reduce the ionic strength, and was then placed in the central fraction of the sucrose–ampholyte gradient. After electrofocusing at 400 V for 40 hr, approximately 50 2-ml fractions were collected from the column and analyzed for enzyme activity, protein concentration, and pH. Protein concentration was measured at 280 nm in the presence of 0.02 M sodium dodecyl sulfate.

Polyacrylamide Gel Electrophoresis. Ordinary acrylamide disc gel electrophoresis was performed in a 4% acrylamide and 0.11% *N,N'*-methylenebisacrylamide gel using a continuous buffer system containing 0.6 g of Tris and 2.88 g of glycine per 1000 ml (pH 8.3) (Ornstein, 1964; Davis, 1964). The gels were stained with Coomassie Brilliant Blue solution for about 1 hr and subsequently destained in acetic acid and methanol destaining solution (Weber and Osborn, 1969) by diffusion with continuous stirring. The acetate form of anion exchange resin was present to remove the dye. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out by the method of Weber and Osborn (1969). All protein samples were heated to 65° for 30 min in 10 mM sodium phosphate buffer (pH 7.0) containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol prior to placing them on the gels. With bovine serum albumin β-mercaptoethanol was omitted. For polyacrylamide gel electrophoresis in sodium dodecyl sulfate at alkaline pH, the gel buffer contained 100 g of Tris, 10 g of EDTA, 3.8 g of boric acid, and 1 g of sodium dodecyl sulfate per 500 ml (pH 9.3). The gel buffer, diluted 1:1 with water, was used as a running buffer. Ordinarily the high pH system gave better resolution of the various bands of phosphorylase kinase.

Analyses for Amino Acids and Free Sulfhydryl Groups. For the determination of the amino acid composition of phosphorylase kinase and its subunits, the proteins were dialyzed exhaustively against distilled water and lyophilized. Usually about 1.5-mg samples of the lyophilized material, which had been kept under vacuum over dry silica gel, were weighed out using a Cahn Gram Electrobalance. The protein was hydrolyzed under reduced pressure with 2 ml of 6 N HCl at 110° for 24–96 hr, and the hydrolysates were evaporated using a Craig rotary evaporator at a temperature of 40°. The residues, dissolved in 2 ml of 0.2 M sodium citrate buffer (pH 2.2), were analyzed on a Beckman Model 121 amino acid analyzer as described by Spackman *et al.* (1958) and modified by Spackman (1963). Cysteine was determined as cysteic acid after performic acid oxidation as described by Moore (1963), and also as cystine after air oxidation of cysteine at neutral pH as described by Moore and Stein (1963). The total number of SH groups in phosphorylase kinase was determined by reaction with DTNB after denaturing the protein in 6 M guanidine-HCl (Vanaman and Stark, 1970). Free sulfhydryl groups were determined by titration with DTNB using a Cary Model 16 spectrophotometer equipped with a Varian Model G-2000 recorder. Tryptophan was determined spectrophotometrically by the method reported by Goodwin and Morton (1946).

Other Methods. Protein determinations were performed by a biuret procedure (Layne, 1957) using crystalline bovine serum albumin as a standard.

Results

Modification of the Procedure for Purifying Phosphorylase Kinase. The purification of nonactivated phosphorylase kinase from rabbit skeletal muscle as described previously (De Lange *et al.*, 1968) yields a protein preparation that appears to be homogeneous by free boundary electrophoresis and sedimentation velocity ultracentrifugation utilizing Schlieren optics. In sedimentation equilibrium studies, however, the enzyme showed appreciable heterogeneity, so additional purification was undertaken. This was achieved by substituting Sepharose 4B for Sephadex G-200 in the final chromatography step of the procedure together with the inclusion of sucrose as a stabilization agent in the column equilibration buffer (Brostrom *et al.*, 1971). In a typical experiment, phosphorylase kinase prepared from 3.6 kg of skeletal muscle up to and including the ammonium sulfate fractionation stage by the method of De Lange *et al.* (1968) was applied to a column of Sepharose 4B. The elution profile is shown in Figure 1. Three protein peaks were obtained and two peaks (I and II) of enzyme activity. The protein peak which was eluted last and had no phosphorylase kinase was also separable using Sephadex G-200 in the procedure of De Lange *et al.* (1968), but separation of the kinase itself into two peaks was not achieved using this latter gel.

The properties of the two species of phosphorylase kinase separated using Sepharose 4B are summarized in Table I. Peak II was approximately twice as active as peak I at pH 8.2 but both fractions showed the same activity at pH 6.8.[2] Peak I was visibly turbid whereas peak II was clear, a difference that is manifested in the appreciable optical density shown by peak I at 310 mμ. Ultracentrifugal analysis of peak I using the

[2] Phosphorylase kinase activity determinations are often carried out at pH 6.8 and 8.2 in order to differentiate the nonactivated form of the enzyme from the activated form (Krebs *et al.*, 1964). Nonactivated phosphorylase kinase has a ratio of activity at pH 6.8 to activity at pH 8.2 between 0.01 and 0.05, whereas the activated form has a pH 6.8:8.2 activity ratio of about 0.6. Nonactivated phosphorylase kinase is approximately half as active as the activated form at pH 8.2.

TABLE I: Comparison of the Properties of the Two Fractions of Nonactivated Phosphorylase Kinase Isolated by Sepharose Chromatography.

	Fraction	
	I	II
Specific activity at pH 8.2 (units/mg of protein)	39,200	81,000
Specific activity at pH 6.8 (units/mg of protein)	2,120	2,120
Ratio $A_{310\ nm}/A_{280\ nm}$	0.43	<0.02

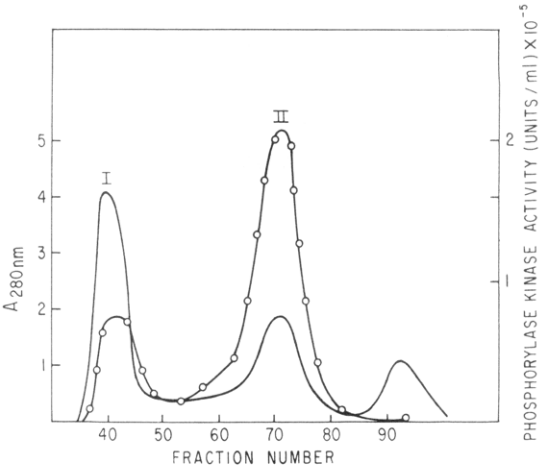

FIGURE 1: Chromatography of phosphorylase kinase on Sepharose 4B. Phosphorylase kinase from 3.6 kg of rabbit skeletal muscle was prepared by the method of De Lange *et al.* (1968) up to and including fractionation with ammonium sulfate. The precipitate obtained in this step was dissolved in 50 mM glycerol phosphate buffer, pH 6.8, containing 2 mM EDTA and 10% (w/v) sucrose. This solution, containing 1.3×10^7 units of phosphorylase kinase, was applied to a Sepharose 4B column (2.5 × 90 cm) equilibrated with the above buffer. Fractions of 4.5 ml were collected and assayed for phosphorylase kinase activity (○). Ultraviolet absorption was determined at 280 nm (——).

photoelectric scanner system (not illustrated) showed that it consisted of very large polydisperse material. Peak II, on the other hand, exhibited the properties of an essentially homogeneous enzyme. A study of its physical properties constitutes the major portion of this paper. It should be noted that the specific activity of nonactivated phosphorylase kinase prepared by this procedure, *i.e.*, peak II, is approximately 25% higher than that obtained in the earlier preparation (De Lange *et al.*, 1968).

The enzyme of peak I (Figure 1) is believed to represent high polymeric forms of native phosphorylase kinase which arise as artifacts during the isolation procedure due to the sensitivity of this enzyme to increased hydrostatic pressure. Earlier studies carried out by Mr. Richard Horvitz in this laboratory had shown that total recovery of phosphorylase kinase activity was very poor in sucrose density gradient centrifugation experiments and this was particularly true when the experiments were carried out at very high angular velocities. As illustrated in Figure 2, the sensitivity of the enzyme to pressure was confirmed by centrifuging phosphorylase kinase (peak II) at different speeds. At relatively low speed (20,000 rpm, 30,000*g*) the photoelectric scanning trace showed that phosphorylase kinase sedimented as a typical single component (Figure 2A). On the other hand, at relatively high speed (44,000 rpm, 150,000*g*) the sedimentation profile (Figure 2B) was characterized by marked sawtooth spikes which is indicative of convective disturbances which lead to extremely sharp concentration gradients (Schachman, 1959). One of the early steps of the phosphorylase kinase purification scheme involves sedimentation of the enzyme for 3 hr at 100,000*g*. When a 20% (w/v) sucrose solution is used as a cushion in the bottom of the centrifuge tube (Brostrom *et al.*, 1971) the amount of peak I material as seen in Figure 1 can be reduced and the yield of peak II enzyme is increased.

Acrylamide Gel Electrophoresis. On acrylamide gel electrophoresis, highly purified phosphorylase kinase[3] shows an essentially homogeneous band with only trace amounts of impurities (Figure 3).

Sedimentation Velocity and Equilibrium Studies on Nonactivated Phosphorylase Kinase. The Schlieren pattern obtained in a typical sedimentation velocity experiment using purified nonactivated phosphorylase kinase, *i.e.* peak II of Figure 1, is shown in Figure 4. A single symmetrical peak is seen. The concentration dependence of the sedimentation coefficient was determined by plotting $1/s_{20,w}$ against concentration (not illustrated). A linear regression analysis of these data gave the equation, concentration (g/100 ml) = $0.006026 \cdot (1/s_{20,w}) + 0.03827$ (± 0.00078). From the ordinate intercept a value for $s^0_{20,w}$ of 26.1 ± 0.5 (SD) was obtained. The regression coefficient was significantly different from zero ($P < 0.05$).

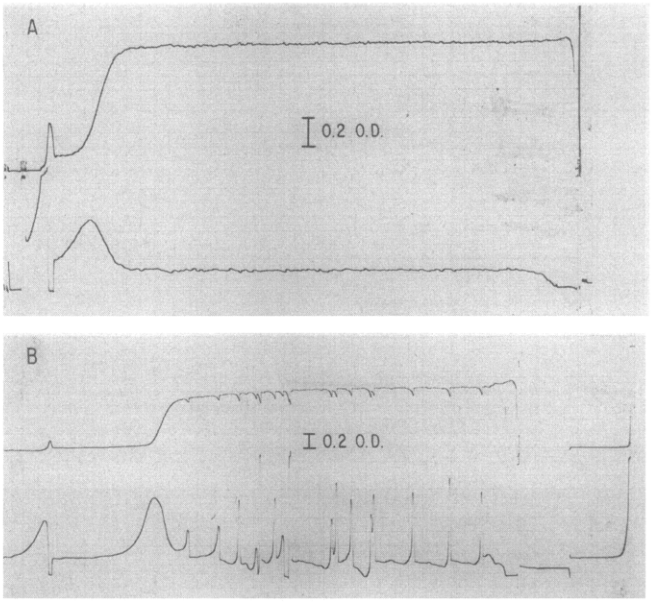

FIGURE 2: Effect of centrifugal speed on the dispersion of phosphorylase kinase using the photoelectric scanning system. The upper half of each scan shows the concentration tracing and the lower half shows its derivative at 280 nm: A, 0.1 g/100 ml, after 48 min, at 20,000 rpm or 30,000*g*; B, 0.14 g/100 ml, after 32 min, at 44,000 rpm or 150,000*g*. The solvent was 50 mM sodium glycerol phosphate buffer (pH 6.8) containing 2 mM EDTA and 10% (w/v) sucrose. Sedimentation is from *left* to *right*. OD refers to absorbancy units.

[3] Unless indicated otherwise, the phosphorylase kinase utilized in all studies reported in this paper was the peak II fraction from Sepharose 4B chromatography.

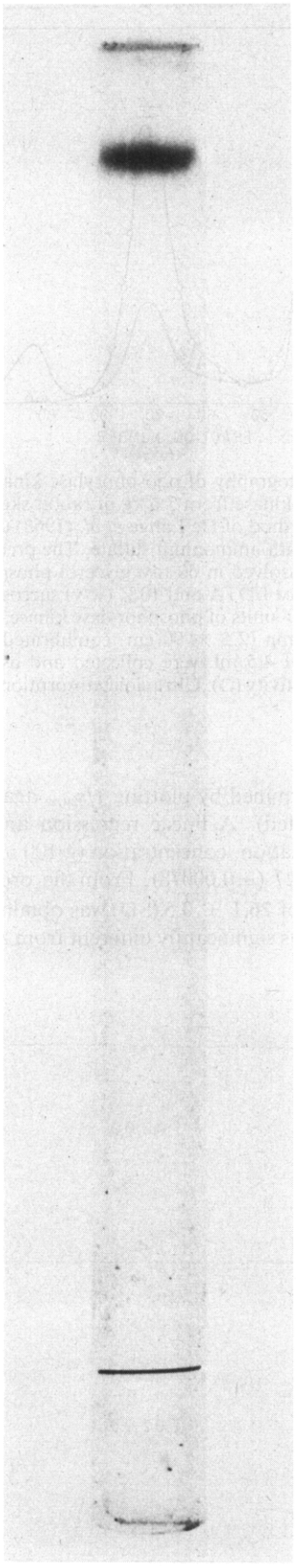

FIGURE 3: Acrylamide gel electrophoresis of phosphorylase kinase. 5 μg of purified enzyme was applied to the top of a gel in 50 mM sodium glycerol phosphate buffer (pH 6.8) containing 2 mM EDTA and 20% sucrose. Electrophoresis was for 45 min at 1.5 mA. The running gel was polymerized in an acrylic tube (4.5 × 85 mm) according to Bishop *et al.* (1967). The composition of the gel, the electrolyte solutions, and the staining and destaining procedures were as described in the Experimental Section.

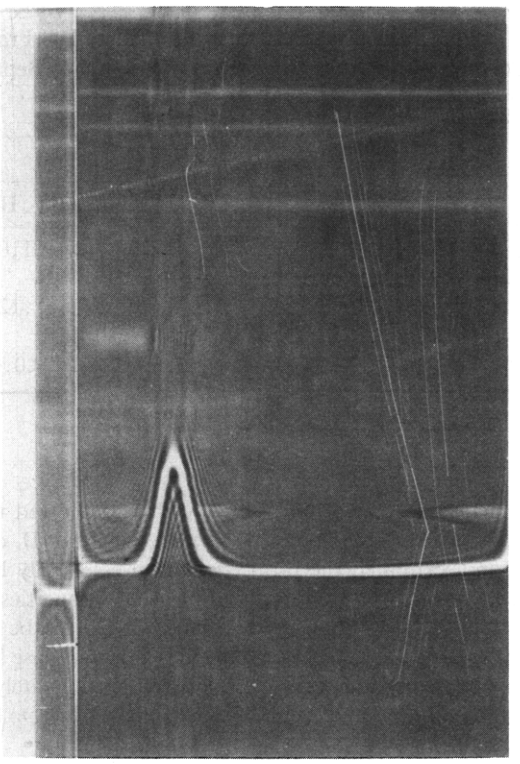

FIGURE 4: Sedimentation velocity Schlieren pattern obtained with purified phosphorylase kinase, 0.19 g/100 ml, in 50 mM sodium glycerol phosphate buffer (pH 7.0) containing 50 mM β-mercaptoethanol. Centrifugation carried out at 20,000 rpm for 112 min; bar angle, 60°. Sedimentation is from *left* to *right*.

TABLE II: Molecular Weights of Phosphorylase Kinase Determined by Mensicus Depletion Equilibrium Centrifugation at Various Protein Concentrations.[a]

Initial Protein Concn (g/100 ml)	Optical System	Mol Wt × 10^{-6}
0.1	Interference	1.22
0.05	Interference	1.28
0.03	Interference	1.29
0.027	Uv	1.28
0.015	Interference	1.33
0.013	Uv	1.28
0.0125	Interference	1.35
Extrapolated value to infinite dilution		1.33

[a] Experimental conditions: 50 mM sodium glycerol phosphate buffer (pH 7.0) containing 50 mM β-mercaptoethanol; 5°; 42–75 hr at 6400 rpm.

The weight average molecular weight of phosphorylase kinase was determined at several different initial protein concentrations using the sedimentation equilibrium method of Yphantis (1964). Only a slight degree of concentration dependence was found (Table II). On extrapolation to infinite dilution, a value of 1.33 × 10^6 was obtained. A plot of the logarithm of concentration *vs.* the square of the distance from the center of rotation was linear indicating a high degree of homogeneity of the kinase.

TABLE III: Amino Acid Composition of Phosphorylase Kinase.

Amino Acid	Av or Extrapolated Value[a] (g of Amino Acid Residue/100 g of Protein)	Mol/Mol of Enzyme (1.33 × 10^6 g)
Tryptophan	1.67	120
Lysine	5.73	595
Histidine	2.67	259
Ammonia[b]	1.11	923
Arginine	6.56	559
Aspartic acid	8.31	960
Threonine	3.89	512
Serine	4.76	728
Glutamic acid	13.09	1349
Proline	3.63	497
Glycine	2.72	634
Alanine	3.10	580
Half-cystine[c]	1.25	161
Valine	4.97	666
Methionine	2.44	247
Isoleucine	5.15	601
Leucine	10.28	1190
Tyrosine	4.97	406
Phenylalanine	4.31	390
Total	90.61	

[a] Tristram and Smith (1963). [b] Determined by subtracting the amount of ammonia derived from the decomposition of threonine and serine. [c] Determined as cysteic acid after performic acid oxidation.

From the $s^0_{20,w}$ and the molecular weight a frictional ratio (f/f_0) of 1.17 was calculated according to the formula given by Pedersen (1940).

Isoelectric Focusing. A sample of phosphorylase kinase was examined by isoelectric focusing. A single turbid band of protein corresponding to an isoelectric point of 5.77, together with several minor bands, was obtained (Figure 5). No enzymatic activity was detected in any of the fractions. An attempt to restore the activity by adding Ca^{2+} and boiled supernatant of phosphorylase kinase to the peak fraction was not successful.

Ultraviolet Absorption. The absorption spectrum of phosphorylase kinase in the ultraviolet range is shown in Figure 6. There is a maximum at 279 nm with small shoulders at 283 and 292 nm and a minimum at 251 nm. The $A_{280}:A_{260}$ ratio is 1.75. The $A^{1\%}_{280\,nm}$ was determined to be 11.8 using the biuret protein determination with bovine serum albumin as the standard.

Amino Acid Composition. Table III summarizes the results of amino acid analyses on phosphorylase kinase. The data represent the averages obtained using duplicate hydrolysates from two different preparations. In addition to the value for half-cystine shown in Table III, this amino acid was also determined after conversion of cysteine to cystine by air oxidation by the method of Moore and Stein (1963). This method yielded a value of 156 mol/mol of protein and is in good agreement with a value of 161 mol/mol of protein from performic acid oxidation.

From the amino acid composition, a partial specific volume ($\bar{v}$) of 0.730 ml/g was calculated assuming that amides are equally distributed in glutamic and aspartic acids.

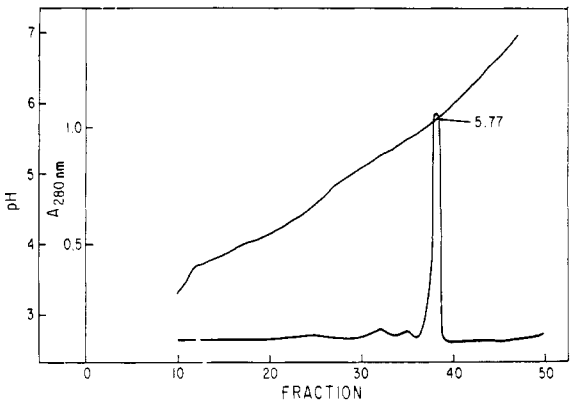

FIGURE 5: Isoelectric focusing of phosphorylase kinase. The enzyme (3 mg) was applied to the column and run as described in the Experimental Section. Two-milliliter fractions were collected and their optical density was determined.

Titration of Sulfhydryl Groups. Phosphorylase kinase contains one set of sulfhydryl groups which reacts rapidly with DTNB and another set which reacts slowly with this reagent. Figure 7 shows the time course of the reaction with varying ratios of reagent to enzyme, and Figure 7B shows a plot of the 10-min points against the ratio of reagent to enzyme. From the latter plot, it is seen that approximately 1.5 mol of rapidly reacting sulfhydryl is present per 10^5 g of phosphorylase kinase, *i.e.* 20 mol/mol of protein. Other sulfhydryl groups in the enzyme react more slowly and even after 3 hr the reaction is still proceeding (Figure 8). When phosphorylase kinase is denatured in the presence of 6 M guanidine hydrochloride before titration with DTNB, 11.9 mol of sulfhydryl group per 10^5 g of protein (159 mol/mol of protein) is titrated. This value is in good agreement with the half-cystine value

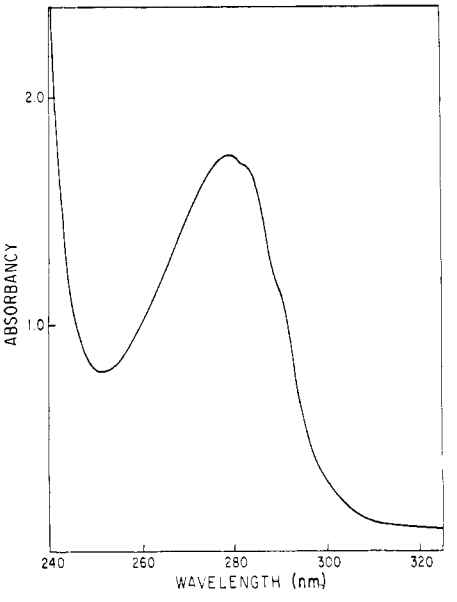

FIGURE 6: Absorption spectrum of phosphorylase kinase. A solution of 1.48 mg of protein/ml in 5 mM sodium glycerol phosphate buffer (pH 7.0) containing 0.2 mM EDTA was examined using a Cary-14 spectrophotometer.

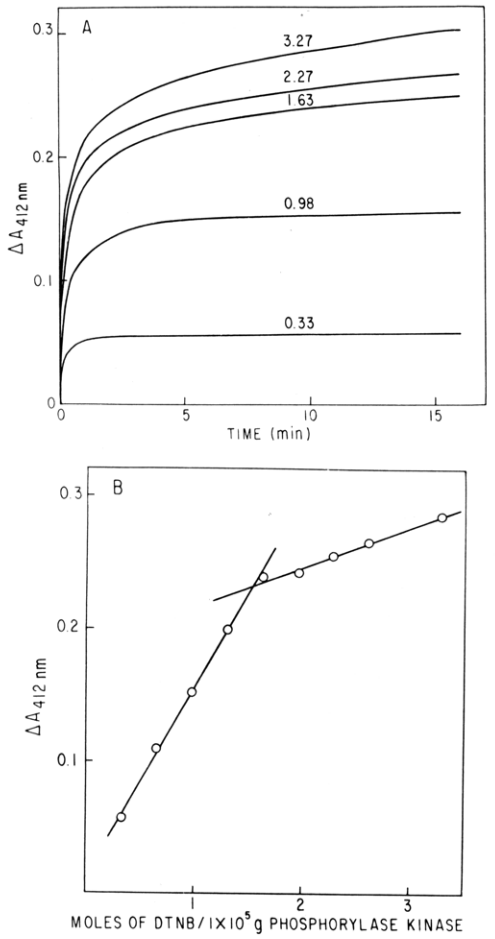

FIGURE 7: The reaction of phosphorylase kinase with DTNB. (A) The time course of the reaction with changing ratios of DTNB to phosphorylase kinase. Reaction mixtures contained 100 μl (306 μg of protein) of phosphorylase kinase in 50 mM sodium glycerol phosphate buffer (pH 6.8) containing 2 mM EDTA and 10% (w/v) sucrose and 600 μl of 35 mM sodium phosphate buffer (pH 7.5). The reactions were started by the addition of aliquots of DTNB solution. Changes of absorbance (ΔA) at 412 nm against each control without enzyme were measured at room temperature. The numbers on each curve indicate the moles of DTNB per 1×10^5 g of phosphorylase. (B) Change in $A_{412\ nm}$ after 10 min. Data from Figure 9A except for second, fourth, sixth, and eighth points. The time courses for these ratios of DTNB to phosphorylase kinase were not illustrated in Figure 9A.

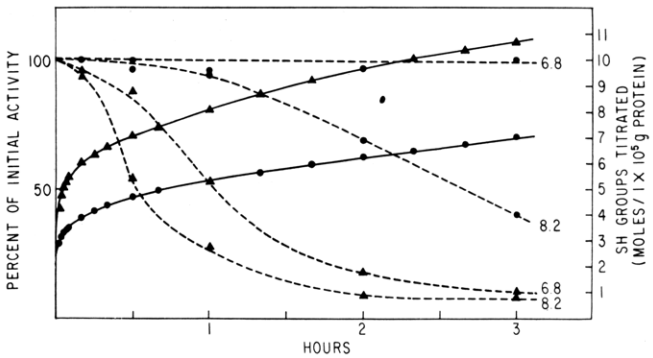

FIGURE 8: The effect of the reaction of phosphorylase kinase with DTNB on enzymatic activity. The solid lines show the time course of the reaction with DTNB and the dashed lines show enzymatic activity at ratios of 2.5 (●) and 25.0 (▲) mol of DTNB/mol of sulfhydryl group in the enzyme. Enzyme activities were determined at pH 6.8 and 8.2 as indicated in the figure. Activity tests were carried out for a 5-min period during which time reaction rates were linear. The initial phosphorylase kinase activities were 2600 units/mg at pH 6.8 and 84,500 units/mg at pH 8.2.

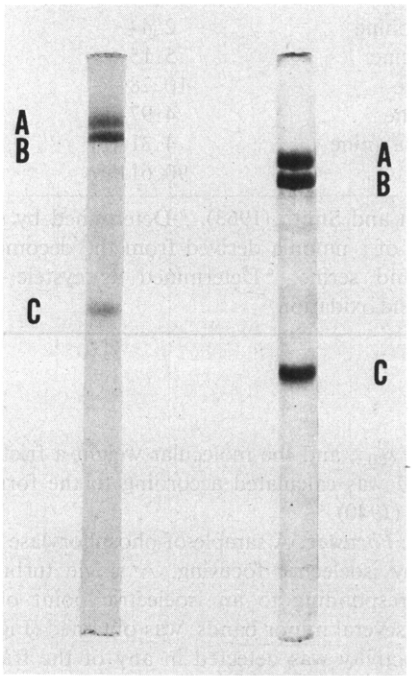

FIGURE 9: Disc gel electrophoresis of phosphorylase kinase in the presence of sodium dodecyl sulfate: left side, pH 7 buffer system; right side, pH 9.3 buffer system. See the Experimental Section for details concerning these buffer systems. The letters A, B, and C are used to designate the bands.

obtained from the amino acid analysis (Table III), suggesting that all the half-cystine residues exist in the sulfhydryl form.

The rapidly reacting sulfhydryl groups are not essential for phosphorylase kinase activity. This is shown in the experiments of Figure 8. When the enzyme was treated with DTNB, either at 2.5 or at 25 mol of reagent/mol of sulfhydryl group, there was essentially no loss of activity within the first 10 min. With prolonged treatment, however, activity was gradually lost. It is of interest that enzymatic activity as measured at pH 8.2 was lost more rapidly than activity at pH 6.8.

Dissociation of Phosphorylase Kinase by Sodium Dodecyl Sulfate. Phosphorylase kinase is readily dissociated by sodium dodecyl sulfate and shows three bands on disc gel electrophoresis in the presence of this substance (Figure 9A and B). The molecular weights corresponding to these bands or subunits are 118,000 ± 1400, 108,000 ± 1900, and 41,000 ± 1600, respectively, as estimated using different samples from different preparations of the enzyme and utilizing several standard proteins[4] as markers. After staining with Coomassie Brilliant Blue the relative densities of the bands were determined using a Gilford spectrophotometer equipped with a Model 2410 linear transport system and found to be 35.3 ± 2.5:38.0 = 2.0:26.6 ± 3.9, respectively, for eight different samples. Using these ratios and the molecular weights of each subunit, the number of subunits can be calculated to be 4.0, 4.7, and 8.6/mol (1.33×10^6 g) of original enzyme.

[4] The markers used as standards were the monomer and dimer of bovine serum albumin, phosphorylase *a*, the H and L chains of γ globulin, glutamic dehydrogenase, and muscle aldolase.

Discussion

The substitution of Sepharose 4B for Sephadex G-200 in the gel filtration step used in the purification of skeletal muscle phosphorylase kinase permits the isolation of this enzyme in an essentially homogeneous form having a higher specific activity than that achieved heretofore (De Lange *et al.*, 1968). The major advantage of the larger pore size of the gel is that it permits separation of an appreciable amount of polydisperse high molecular weight protein having relatively low phosphorylase kinase activity. This latter material is probably formed as a result of pressure denaturation during an earlier ultracentrifugation step used in the preparation.

Phosphorylase kinase has a molecular weight of 1.33×10^6 and appears to be made up of three kinds of subunits, A, B, and C, having molecular weights of 118,000, 108,000, and 41,000, respectively. From the scanning of stained disc gel electrophoresis patterns obtained in the presence of sodium dodecyl sulfate, it is estimated that the amounts of each subunit are such that a likely formula for the enzyme is $A_4B_4C_8$. It is recognized, of course, that this method for determining protein is subject to large errors so that this formula is viewed as tentative.

The amino acid composition of phosphorylase kinase is not unusual. Tryptophan is the least abundant amino acid. Determination of half-cystine residues by titration with DTNB agrees with the value determined as cysteic acid, so there is no evidence for any disulfide bridges in the enzyme. Some of the sulfhydryl groups of phosphorylase kinase, about 20 out of a total of around 160/mol of enzyme, react very rapidly with DTNB but another set reacts slowly. The rapid reacting sulfhydryl groups are not essential for activity.

Acknowledgments

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