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Structural and Catalytic Properties of Lobster Muscle Glycogen Phosphorylase*

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SUMMARY

Purification of lobster phosphorylase was achieved by a mild method which involves ammonium sulfate and diethylaminoethyl cellulose column chromatography. Both lobster phosphorylase *b* and *a* were crystallized by dialysis against 0.01 M EDTA, pH 6.8, which was 30% saturated in ammonium sulfate. Structural and catalytic studies on crystalline lobster phosphorylase are reported. Striking differences from rabbit muscle phosphorylase were found in the amino acid composition, association-dissociation behavior, catalytic efficiency at cold temperature, cold inactivation, energy of activation, and kinetic constants including the allosteric constant. The molecular weight and the extinction coefficient for lobster phosphorylase *b* are also reported in this study and a model is presented to account for the allosteric properties of this enzyme.

Glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase EC 2.4.1.1) catalyzes the first reaction in the utilization of glycogen

$$\underset{(n \text{ glucosyl units})}{\text{Glycogen}} + \text{inorganic phosphate} \rightleftharpoons \text{glucose 1-phosphate} + \underset{(n-1 \text{ glucosyl units})}{\text{glycogen}}$$

Although the equilibrium of the reaction favors glycogen synthesis, this enzyme is believed to function *in vivo* only in the degradation of glycogen. Muscle glycogen phosphorylase has been isolated from various sources (1–10). Among these rabbit muscle phosphorylase has been the most thoroughly investigated.

Two comprehensive studies have previously been published on purified muscle glycogen phosphorylase of poikilothermic animals (9, 11). Frog muscle glycogen phosphorylase was purified and found to have an amino acid composition, molecular weight, and subunit structure similar to that reported for rabbit muscle glycogen phosphorylase. Lobster muscle glycogen phosphorylase, however, was reported to be different from rabbit phosphorylase in that a third form of phosphorylase called *c* was reported in crude extracts of lobster muscle (10, 11) and that purified phosphorylase *a* and *b* were found to have the same sedimentation constant (11).

A comparative study between lobster and rabbit muscle glycogen phosphorylase was undertaken with emphasis on the kinetics and subunit structure of these two enzymes, as these properties are important in the regulation of enzymic activity. In order to carry out these studies with lobster phosphorylase, this enzyme was prepared under mild conditions with the use of diethylaminoethyl cellulose column chromatography, and was crystallized with ammonium sulfate. The two-state exclusive binding model of Monod, Wyman, and Changeux (12) as utilized by Buc (13) was used in the evaluation of the kinetic data in the direction of glycogen degradation. Deviation from the model of Monod *et al.* of full enzyme activity in the absence of the allosteric effector AMP is discussed and is explained by a modified concerted transition model.

EXPERIMENTAL PROCEDURE

Materials

Live lobsters (*Homarus americanus*, 2.5 to 4 pounds each) were obtained from Hines and Smart, Boston, and were stored in a tank of aerated seawater for at least 1 day. Seawater salt mixture was obtained from Rila Marine Mix Company, Teaneck, New Jersey, and was dissolved in distilled water to a specific gravity of 1.025 at 8 ± 2°. Lobster phosphorylase *a* was obtained either by column chromatography on DEAE-cellulose as discussed in the text or from purified lobster phosphorylase *b* with the use of rabbit phosphorylase *b* kinase, Mg^{++}, and ATP according to the procedure used for the conversion of rabbit phosphorylase *b* to *a* (14). Frozen rabbit muscle was obtained from Pel-Freeze Biologicals, Inc., Rogers, Arkansas. Rabbit phosphorylase *b* was prepared according to the method of Fischer and Krebs (15). Glucose 6-phosphate dehydrogenase, phosphoglucomutase, TPN, and sodium glycerophosphate were obtained from Calbiochem. AMP and ATP were obtained from P-L Biochemicals. Previously swollen microgranular DEAE-cellulose (DE-52) was purchased from Reeve Angel Company, New York.

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Chromatographic columns and gradient flask assembly were obtained from Buchler Instruments. Potassium glucose-1-P, cysteine-HCl, 2-mercaptoethanol, dithiothreitol, and shellfish glycogen were obtained from Sigma. The latter was treated with Norit A and passed through a Dowex 1 column to remove any contaminating AMP. Tris from Fisher was twice crystallized before use. Electrophoresis gels and protein stains were obtained from Canalco Industries. All other chemicals used were of reagent grade. Deionized water was used throughout this work.

Methods

Determination of Extinction Coefficient—Lobster phosphorylase *b* was crystallized three times with ammonium sulfate as will be described later and dialyzed at 2–4° for 40 hours against four buffer changes (2 liters each) of 0.04 M Tris-0.01 M EDTA, pH 6.9. The extinction coefficient was determined by measuring the absorbance of five duplicate samples at 280 mμ. Using the same dialyzed enzyme solution, five other aliquots were measured and pipetted into weighed bottles and then dried for 6 hours in an oven the temperature of which was gradually raised to 70°. They were then placed under vacuum and dried to a constant weight by varying the temperature from 70–85° over a 3-day period. Aliquots of buffer were dried along with the enzyme samples in order to correct for the weight due to buffer.

Protein Concentration—The biuret method (16) or that of Lowry *et al.* (17) was used to determine protein concentration of crude enzyme preparations. Whenever bicarbonate or sulfate ions were present, the former method was not used. The concentration of purified enzyme solutions was determined from measurement of absorbance at 280 mμ assuming the extinction, $E^{1\%}_{280} = 13.5$, for lobster phosphorylase *b* solution in 0.04 M Tris-0.01 M EDTA, pH 6.9, determined as indicated above.

Disc Gel Electrophoresis—Polyacrylamide disc gel electrophoresis was run according to the procedure of Ornstein and Davis (18) except that 25% sucrose was used in place of the large pore gel. In order to obtain sharp bands and detect small concentrations of foreign protein (or proteins), varied amounts of enzyme (20 to 250 μg) were used. In addition to the buffer system described by Ornstein and Davis (18), the buffer system described by Davis *et al.* (8) was used. The electrophoresis run was carried out at 2–4° at 3 ma per tube. Some of the gel tubes in the run were removed when the tracking dye reached the bottom of the gel while others were left for several more hours. This was done to ensure that all existing protein bands remained on the gel and to obtain a good separation of other protein bands if more than one was present.

Activity was detected in the gel by incubation in hydrolyzed starch substrate containing calcium as described by Davis *et al.* (8) except that activity was terminated when a white band of calcium phosphate appeared in the gel. Test for protein was conducted by staining with Amido black. Destaining of the gel was accomplished by stirring it overnight in a 10% acetic acid solution.

Ultracentrifugation—Experiments involving sedimentation velocity by using Schlieren optics and sedimentation equilibrium with the use of Rayleigh interference optics were carried out with a Spinco model E analytical ultracentrifuge. The sedimentation velocity runs were carried out at 60,000 rpm with the use of a wedge cell and a normal cell to allow two samples to be run simultaneously. Sedimentation equilibrium measurements were performed either by the slow method at 4,800 rpm with the use of double sector cells or by the fast method at 12,000 rpm with Yphantis cells (19). The equilibrium runs were carried out and analyzed as described by Richards and Schachman (20) and by Van Holde (21).

Amino Acid Analysis—The enzyme solution used for determination of the extinction coefficient was also used for amino acid analysis. Equal aliquots were pipetted into 50-ml, 6-cm long neck flasks and lyophilized. Hydrolysis in constant boiling 5.7 N HCl of the lyophilized enzyme solutions was performed in the vacuum-sealed flasks at 110° for 18, 22, 49, and 70 hours. Extrapolation of the data to zero time was made for all amino acids. Half-cystine was determined by using the performic acid oxidation method and the corrections described by Hirs (22). Hydrogen bromide was also used according to Hirs for the destruction of excess oxidant. Tryptophan was determined spectrophotometrically by the method of Goodwin and Morton (23) and the use of the equation developed by Beaven and Holiday (24) for a protein sample with a known concentration.

Activity Measurements—A 0.2-ml portion of an appropriately diluted enzyme solution was mixed at zero time with 0.2 ml of substrate. The reaction was carried out at 30° and allowed to proceed for 5 min, after which the glycogen synthesis reaction was stopped by adding 8.2 ml of 0.305% ammonium molybdate in 0.061 N H_2SO_4. Phosphate released was measured by the method of Fiske and SubbaRow (25). Specific activity was expressed as micromoles of P_i released per min per mg or as Cori units per mg (26). The substrates used for the assay of the three lobster phosphorylase forms were as follows: for phosphorylase b_{II} (see under "Lobster Phosphorylase b_{II}" for definition), 0.032 M glucose-1-P, 4% glycogen, and 4×10^{-2} M AMP; for phosphorylase *b*, same as above except the AMP concentration was 4×10^{-3} M; for phosphorylase *a*, same as above except that no AMP was used. When the specific activity of lobster phosphorylase *b* was expressed in Cori units per mg, the substrate consisted of 0.032 M glucose-1-P, 2% glycogen, and 2×10^{-3} M AMP, pH 6.8, as used for the assay of rabbit phosphorylase *b*.

Kinetics in Direction of Glycogen Degradation—In kinetic studies with variable P_i and AMP at fixed glycogen concentration, the phosphorylase reaction was measured at 28° in a coupled assay enzyme system that utilized phosphoglucomutase and glucose-6-P dehydrogenase in the presence of TPN (27). Phosphorylase concentration in the reaction mixture was 10 μg per ml with a specific activity of 800 Cori units per mg. A large excess of the auxiliary enzymes glucose-6-P dehydrogenase and phosphoglucomutase was used, *viz.* 30 and 60 μg per ml, respectively. The initial rates proved to be linear by finding proportionally decreasing rates when one-half, one-quarter or one-eighth the amount of phosphorylase was added to aliquots of the reaction mixture containing high concentrations of P_i and AMP. The reaction mixture (total 2.85 ml, pH 6.8) contained 0.2 ml of 0.03 M EDTA, 0.1 ml of 0.03 M mercaptoethanol, 0.2 ml of 0.3 M Tris-0.15 M $MgCl_2$, 1.0 ml of 0.0018 M TPN, trace of glucose 1,6-diphosphate (5×10^{-8} M final concentration), 0.2 ml of 4.5% glycogen, and 0.15 ml of AMP and P_i of known concentration. The phosphorolysis reaction was initiated by adding to the above reaction mixture at 28°, 0.15 ml of the enzymes which had been freshly dissolved in an ice-cold buffer, pH 6.8, which consisted of 0.01 M Tris, 0.001 M EDTA, 0.001 M mercaptoethanol, and 1% bovine serum albumin, maintained at 0°.

Velocities used in the plots were obtained from the slopes and were expressed in OD units per sec.

Kinetics in Direction of Glycogen Synthesis—Kinetics in the direction of glycogen synthesis were carried out by adding a known amount of enzyme solution to a substrate at pH 6.8 containing 3% and 2×10^{-3} M final concentrations of glycogen and AMP, respectively. Glucose-1-P was varied between 0.016 and 0.004 M. Another substrate used in this type of study contained 4% glycogen and 4×10^{-3} M AMP with glucose-1-P varying between 0.048 and 0.004 M. When glycogen was the variable substrate, it was varied between 3.2 and 0.4% while the concentrations of AMP and glucose-1-P were fixed at 4×10^{-3} M and 0.048 M, respectively. The buffer used contained 0.04 M Tris-0.01 M EDTA, pH 6.8, to which 0.04 M mercaptoethanol was added when rabbit phosphorylase was studied. Initial rates at any given glucose-1-P or glycogen concentration were determined from a plot of the micromoles of P_i released against time for each set of points of the varied glucose-1-P or the varied glycogen. Velocities were expressed in micromoles of P_i released per min per mg.

RESULTS

Purification Procedure

Preparation of Crude Extracts—Cowgill (11) reported earlier a purification of lobster phosphorylase. As this procedure involved heat steps, and we observed in our preliminary work that heating altered the response of the enzyme to allosteric effectors, this method of purification was abandoned. The present purification procedure was adopted in order to minimize structural alterations of the enzyme and to obtain an enzyme with high specific activity.

The lobsters were killed by immersing each one for about 1 min in a bath containing either liquid nitrogen-isopentane or Dry Ice-ethanol. The tail muscle, which was unfrozen, was excised and rinsed with cold distilled water after the exoskeleton and all other visible extraneous matter had been removed. The muscle was ground in a meat grinder three or four times while still firm. Two times the weight (by volume) of deionized distilled water at room temperature was added to the ground muscle and extraction was performed by stirring at room temperature for 20 to 30 min depending on the amount of lobster muscle used. All following steps were then carried out at 2–4°. The ground muscle-water slurry was centrifuged at $7000 \times g$ for ½ hour and the supernatant was gently filtered through glass wool. Aliquots of 0.2 M EDTA and 0.01 M dithiothreitol, pH 7.0, were added to the extract to result in a final concentration of 0.01 M EDTA and 1×10^{-3} M dithiothreitol.

Ammonium Sulfate Fractionation—To each 100 ml of extract, 34.4 ml of saturated neutralized ammonium sulfate were slowly added with continuous stirring resulting in 25% saturation. The preparation was then stored overnight at 0–1° and then centrifuged as above at $7000 \times g$ for 1 hour. The supernatant was filtered through glass wool and additional saturated neutralized ammonium sulfate was added to 50% saturation. After storage overnight in the cold room, a heavy precipitate settled to the bottom. The supernatant was decanted and the remaining material was centrifuged for 1 hour at $7000 \times g$. The precipitate was resuspended in a minimum volume of 0.01 M neutral EDTA and dialyzed against the same solution for 1 to 2 days to convert all the phosphorylase *a* present to the *b* form. This step was later found to be unnecessary since phosphorylase *a* could be well separated from phosphorylase *b* by DEAE-cellulose chromatography. Phosphorylase *a* emerged in a sharp peak 140 tubes (12 ml each) after phosphorylase *b* from the DE-52 column described in Fig. 1. However, since the yield of phosphorylase *b* was lower in this case, the EDTA dialysis step was done routinely.

Ultracentrifugation—The enzyme was centrifuged at 78,480 $\times g$ for 1¾ hours. The pellet was discarded and the clear, light yellow supernatant was dialyzed against a dilute buffer (0.002

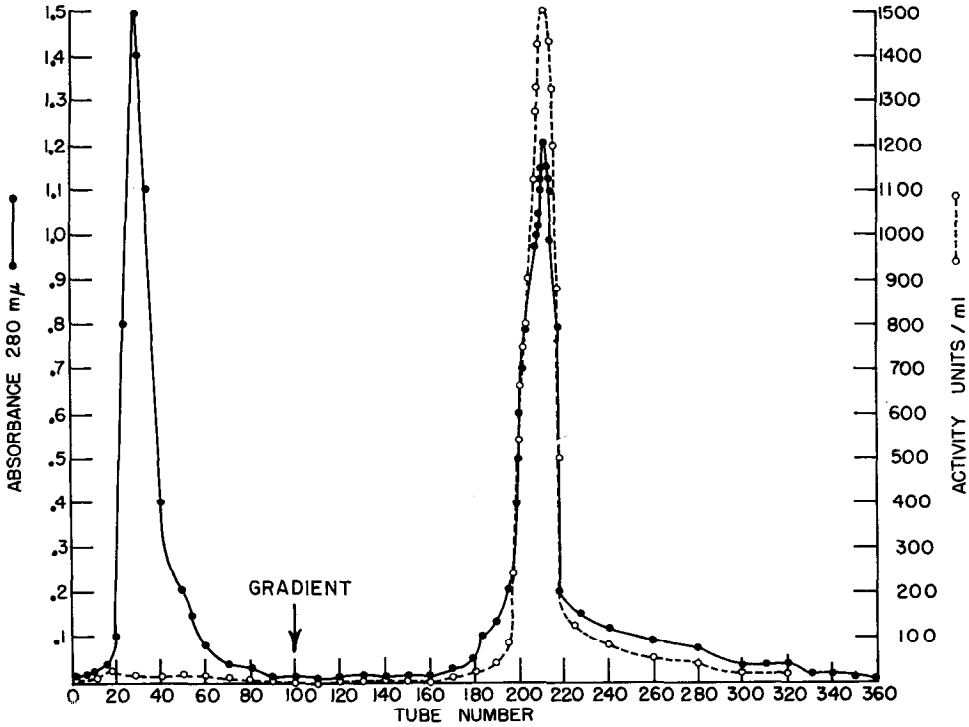

FIG. 1. Elution pattern of lobster muscle phosphorylase *b* from a DEAE-cellulose (DE-52) column (12 ml were collected in each tube). Initial buffer of 0.002 M Tris-0.001 M EDTA, pH 7.5, and gradient buffer of 0.04 M Tris-0.005 M EDTA, pH 7.5, were used. Details are in the text. The enzyme preparation is described in Table I.

M Tris-0.001 M EDTA, pH 7.5) overnight with several buffer changes.

Repetition of Ammonium Sulfate Fractionation—The above fractionation steps (25 and 50% ammonium sulfate saturation) were repeated, except that centrifugation after each ammonium sulfate treatment was done after only ½ hour of storage in the cold instead of overnight. Following this step, the enzyme was dialyzed against the dilute buffer as above, without repetition of ultracentrifugation. Although only a small increase in specific activity was achieved from this step, a sharper elution profile was obtained, and the DEAE-cellulose-purified enzyme responded very well to crystallization by ammonium sulfate.

Chromatography on DEAE-cellulose—Microgranular DEAE-cellulose (DE-52) was washed with 0.5 N NaOH and 0.5 N HCl, and equilibrated with the 0.002 M Tris-0.001 M EDTA buffer, pH 7.5. After deaeration, the DE-52 was packed under 5 pounds of nitrogen pressure into a water-jacketed column, 4 × 75 cm, at 1–2°. Then 2 to 3 g of protein from the above step were added to the column, and an initial elution with 0.002 M Tris-0.001 M EDTA, pH 7.5, under 3 pounds of nitrogen pressure was carried out to remove contaminating protein (or proteins). A concave gradient was then started by using 0.04 M Tris-0.005 M EDTA, pH 7.5, and a dropwise control from an upper 500-ml gradient flask to a lower 1000-ml mixing flask. Absorbance and activity measurements were taken for every five tubes and for each tube of the active fraction (or fractions). The elution profile is shown in Fig. 1. Only tubes with nearly constant specific activity were collected and were concentrated by dialysis against neutralized saturated ammonium sulfate. The precipitate was collected by centrifugation, resuspended in a minimum volume of 0.04 M Tris-0.01 M EDTA, pH 6.9, and dialyzed against the same overnight. The enzyme was then crystallized or stored frozen in this buffer.

When phosphorylase *b* and b_{II} were both present, b_{II} came off the column first. The formation of b_{II}, its pH profile, and its stability will be discussed later.

Crystallization—Lobster phosphorylase *b* was crystallized by dialyzing an enzyme solution (15 to 30 mg per ml) at 2–4° against a solution of 0.01 M EDTA, pH 6.8, which was 30% saturated in ammonium sulfate at 24°. The crystals, which formed in 6 to 12 hours, were collected by centrifugation and redissolved in a minimum volume of 0.04 M Tris-0.01 M EDTA, pH 6.8. A second and third crystallization were achieved by dialyzing the enzyme against 25% ammonium sulfate containing 0.01 M EDTA, pH 6.8. Lobster phosphorylase *a*, after its conversion from phosphorylase *b*, was similarly crystallized. Fig. 2 shows crystals of both phosphorylase *b* and *a* after the third crystallization and it appears that the crystals of both enzymes are similar in shape under the phase microscope. The addition of AMP and Mg^{++} used in crystallization of rabbit phosphorylase *b* (28) and other muscle phosphorylases (1, 3) failed to bring about crystallization of lobster phosphorylase *b*. A summary of the purification procedure for a typical preparation is shown in Table I.

Storage and Stability

Lobster phosphorylase *b* or *a* could be stored unfrozen at 1–2° in 0.04 M Tris-0.01 M EDTA, pH 7.5 or pH 6.9, for several weeks with no loss of specific activity. Both enzymes were also found to be stable if frozen in the above buffer for at least 6 months. Sulfhydryl compounds such as mercaptoethanol and cysteine were not included in the buffers used in the study of lobster phosphorylase because they were found to have no activation or stabilization effect on this enzyme.

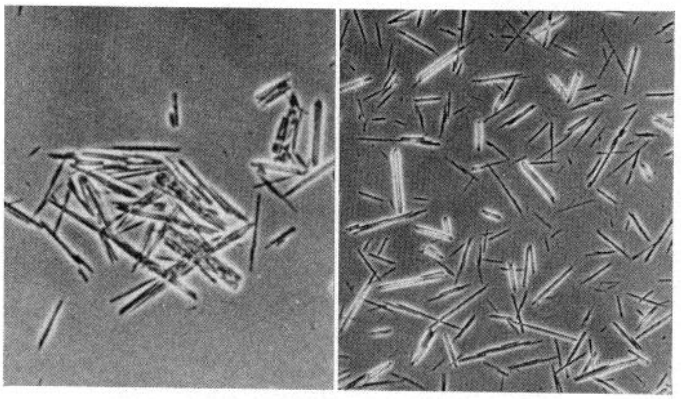

FIG. 2. Photographs of crystals of lobster phosphorylase *a* and *b*. Pictures were taken with a 35-mm film with a 200-fold magnification with the use of a phase contrast microscope. Photograph on the *left* represents crystals of phosphorylase *b*. Photograph on the *right* represents crystals of phosphorylase *a*. Conditions for crystallization are described under "Methods."

TABLE I

Purification profile of lobster muscle glycogen phosphorylase[a]

Fraction	Volume	Concentration	Total protein	Specific activity	Total activity	Yield
	ml	*mg/ml*	*mg*	*units/mg*[b]	*units*	%
Crude extract	3,000	14	42,000	3.6	151,200	100
25% ammonium sulfate	4,000	8	32,000	4.4	140,800	93
50% ammonium sulfate	225	25	5,625	13.2	74,250	50
Ultracentrifugation at 78,480 × *g* for 1½ hours	180	18	3,240	20.4	66,096	43
DEAE-cellulose column chromatography[c]	8	60	480	62.4	29,952	21
Crystallization with 30% ammonium sulfate	13	26	338	67.9	22,950	16
Crystallization with 25% ammonium sulfate	12	23	276	69.0[d]	19,044	13

[a] The data for purification of lobster muscle glycogen phosphorylase starting with 1100 g of muscle from 14 lobsters are given.

[b] Units of activity are expressed as micromoles of P_i released per min at 30° (see "Methods").

[c] This step represents DEAE-cellulose chromatography, concentration by dialysis against neutralized saturated ammonium sulfate, and dialysis against 0.04 M Tris-0.01 M EDTA, pH 6.8.

[d] This crystalline enzyme had a specific activity of 1100 Cori units per mg. Percentage ammonium sulfate in this table refers to percentage saturation at 24°.

Homogeneity

The enzyme was subjected to electrophoresis as described under "Methods." Purified lobster phosphorylase *b* or *a* gave one active protein band as shown in Fig. 3. The enzyme was also shown to be homogeneous in the ultracentrifuge. Fig. 4 illustrates a sedimentation velocity pattern of purified lobster phosphorylase *b* and *a*. A plot of log *C versus* r^2 shown in Fig. 5*A* from a slow sedimentation equilibrium run yields a straight line as expected for a homogeneous protein sample (20).

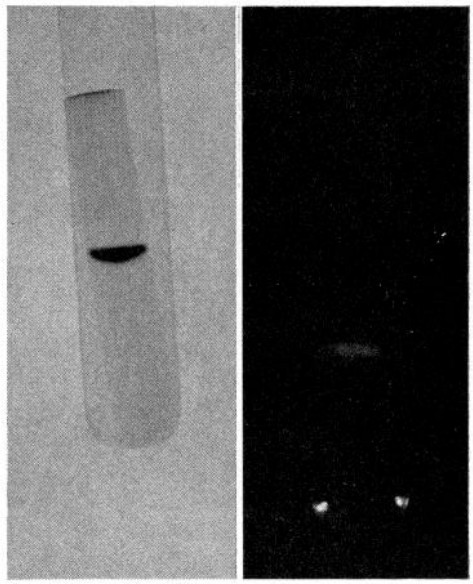

FIG. 3. Polyacrylamide gel electrophoresis photographs of lobster phosphorylase *b* (75 μg of purified enzyme were subjected to electrophoresis at pH 8.5 for 5 hours at 2 ma per tube). Photograph on the *left* represents protein test. Photograph on the *right* represents activity test. For details on gel and sample preparation, buffer used, and protein activity tests, see "Methods."

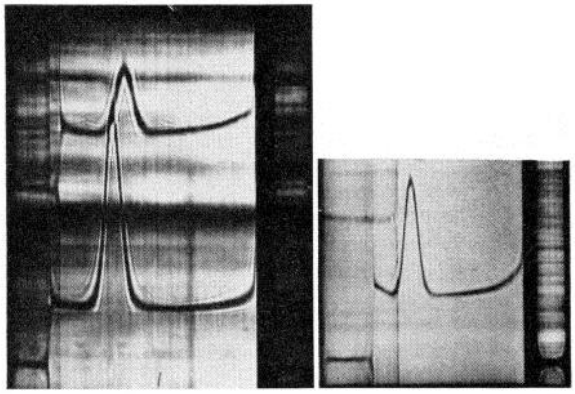

FIG. 4. Sedimentation velocity patterns of lobster phosphorylase *b* and *a*. Photograph on the *left* shows a sedimentation velocity pattern of phosphorylase *b* in 0.04 M glycerophosphate-0.01 M EDTA, pH 6.8. Enzyme concentration is 4 mg per ml in the *upper curve* and 11 mg per ml in the *lower curve*. Photograph on the *right* shows a sedimentation velocity pattern of phosphorylase *a* (7 mg per ml) in 0.14 M glycerophosphate-0.02 M EDTA, pH 6.8. $s_{20,w}$ of 8.5 S was found for both phosphorylase *b* and *a* at 5°. Pictures were taken approximately 50 min after speed at 60,000 rpm was reached. Direction of sedimentation is from *left* to *right*.

Molecular Weight

The data from the slow speed sedimentation equilibrium of lobster phosphorylase *b* (Fig. 5*A*) were analyzed according to Van Holde (21). With 0.75 ml per g as the partial specific volume ($\bar{v}$) (29) or 0.74 ml per g (30), molecular weights of 170 $\times 10^3$ and 164 $\times 10^3 \pm 3 \times 10^3$ g per mole were calculated. High speed sedimentation equilibrium experiments (Fig. 5*B*) also gave similar values (180 $\times 10^3$ g per mole with 0.75 for $\bar{v}$) when the slope of log *C versus* r^2 was calculated from points

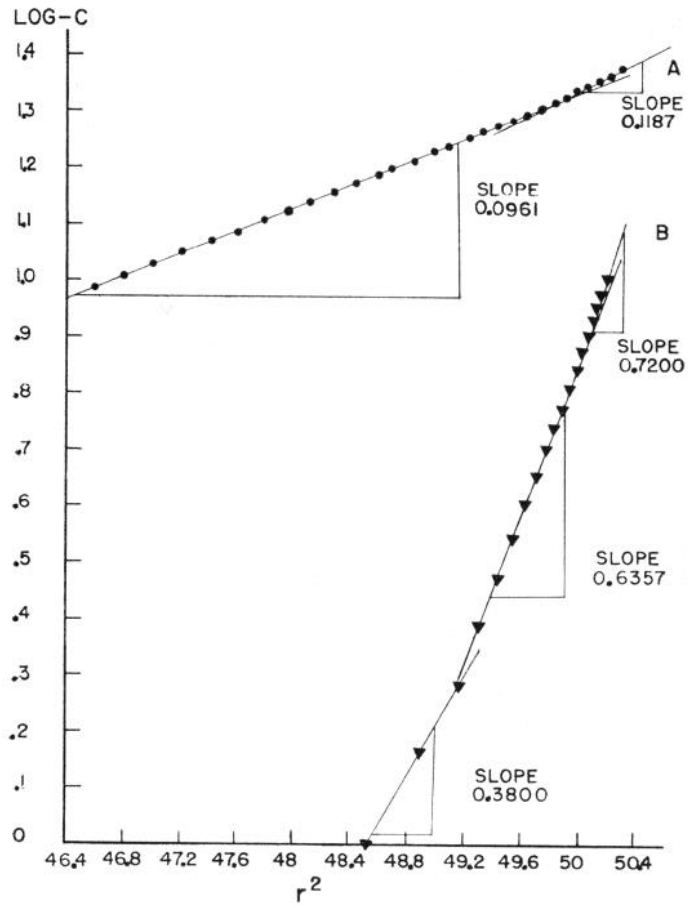

FIG. 5. High (*B*) and low (*A*) sedimentation equilibrium experiments of purified lobster phosphorylase *b*. Enzyme concentration was 2.2 and 0.52 mg per ml for the low (4,800 rpm) and the high (12,000 rpm) speed runs, respectively. The experiments were conducted for 22 and 30 hours at 20°, respectively, with the use of a Spinco model E analytical ultracentrifuge with an ANJ rotor. Protein gradients were determined by Rayleigh interference optics. Fringe displacements were read every half-fringe with a Nikon 6C microcomparator.

representing the center of the cell. Estimation of molecular weight for the protein solution near the meniscus and near the bottom of the cell gave values of 108 $\times 10^3$ and 204 $\times 10^3$ g per mole, respectively, as shown in Fig. 5*B*. This is consistent with the theory discussed by Yphantis (19) for a protein which may dissociate at very low concentrations approaching zero (near the meniscus of the Yphantis ultracentrifuge cell) and which tends to aggregate at very high concentrations (near the bottom of the cell).

Amino Acid Composition

The amino acid composition of lobster phosphorylase *b* calculated on the basis of a molecular weight of 170,000 is shown in Table II and is compared to results obtained with rabbit muscle phosphorylase (31).

Study of Enzyme Sedimentation Velocity and Subunit Structure

Ultracentrifugal studies of phosphorylase *b* with various buffers (glycerophosphate-cysteine, glycerophosphate-mercaptoethanol,

glycerophosphate-EDTA, Tris-cysteine, Tris-mercaptoethanol, Tris-dithiothreitol, Tris-EDTA) at pH values from 6.8 to 7.5 and at protein concentrations from 3.5 to 11 mg per ml gave one symmetrical homogeneous peak in the ultracentrifuge (Fig. 4) with an average $s_{20,w}$ value of 8.45 S (standard deviation of 0.24, 38 determinations).

Since rabbit muscle phosphorylase *b* and *a* exist as dimeric and tetrameric forms depending upon enzyme concentration, salts, and temperature (28, 32, 33), the sedimentation behavior of lobster phosphorylase *b* and *a* was studied under a variety of conditions. The sedimentation constants of lobster phosphorylase *b* and *a* in the presence of buffer were compared to those in AMP and Mg^{++} at 4°. A sedimentation constant of 8.4 ± 0.2 S was found for these four determinations. Other conditions, which are known to promote the association of rabbit phosphorylase *b* at cold temperature, such as high enzyme concentrations in the presence of AMP and sodium fluoride (34), and AMP and mercaptoethanol (35) did not affect the sedimentation constant ($s_{20,w}$ = 8.5 S) of either form of lobster phosphorylase. Thus, it appears that lobster phosphorylase, in contrast to rabbit phosphorylase, cannot be induced to form a tetramer.

The high speed sedimentation equilibrium experiments (Fig. 5*B*) indicated a tendency of lobster phosphorylase *b* to dissociate to a monomeric form at low enzyme concentrations. Experiments were also carried out to determine whether lobster phosphorylase *b* could dissociate at higher enzyme concentrations in the presence of salt solutions. Attempts to detect dissociation of lobster phosphorylase *b* in the ultracentrifuge with salts failed when 0.5 M, 1.0 M, 2.0 M, and 2.5 M KCl or when 0.5 M and 1.0 M NaCl were used. In the presence of 0.5 M ammonium sulfate or 0.3 M inorganic phosphate at pH 7.5 and 20°, the enzyme sedimentation constant was decreased by about 1 Svedberg unit. Phosphate concentrations from 0.05 to 0.2 M at pH 7.5 and 20° failed to bring about significant change in the sedimentation constant.

Alteration of the sedimentation constant of lobster phosphorylase *b* was effected when the enzyme was treated with 0.5 M phosphate as shown in Fig. 6. A sedimentation constant of 5.5 ± 0.3 S (average of nine runs) was obtained when the enzyme treated with 0.5 M phosphate was run in the ultracentrifuge at 3–5° or at 20–22° at the pH values of 6.5, 6.8, or 7.5. When 0.01 M AMP was added to an enzyme solution which had been treated with 0.5 M phosphate (pH 7.5 at 20°), two peaks were formed. As shown in Fig. 6, a fast sedimenting peak with an $s_{20,w}$ value corresponding to 8.5 ± 0.2 S and a slower peak with an $s_{20,w}$ of 5.5 ± 0.3 S (average of six runs) are apparent. Similar experiments carried out at 20° and pH 6.5 gave only one peak (Fig. 6) with an $s_{20,w}$ value of 5.3 ± 0.2 S (average of three runs). At cold temperature, the enzyme treated with AMP and phosphate gave only one peak whether at pH 7.5 (Fig. 6) or at pH 6.5 (Fig. 6), with an $s_{20,w}$ value of 5.4 ± 0.2 S (average of six determinations). Thus, AMP seems to promote association of the 0.5 M phosphate-treated enzyme only when the pH is 7.5 at 20° but fails to do so at cold temperature or acidic pH.

The sedimentation constant of lobster phosphorylase *b* with phosphate in these studies was found to be independent of enzyme concentration (4.5 to 11 mg per ml). The $s_{20,w}$ value of 5.5 S observed here in 0.5 M phosphate corresponds to the sedimentation constant found for a monomer of rabbit phosphorylase (36) and agrees closely with the equation of Kirshner and Tanford (37) in which the sedimentation constant varies as a function of $M^{2/3}$ where M is the molecular weight. At lower concentrations of phosphate (0.2 to 0.3 M) the enzyme sedimented as a single peak with a lower sedimentation constant, suggesting a rapid equilibrium between an associated and a dissociated pro-

TABLE II

Amino acid composition of lobster and rabbit muscle phosphorylase b[a]

Amino acid	Lobster	Rabbit
	residues/mole	
Lysine	91	87
Histidine	42	39
Arginine	94	118
Aspartic acid	170	182
Threonine	73	64
Serine	68	55
Glutamic acid	168	190
Proline	57	80
Glycine	88	92
Alanine	105	121
Half-cystine	20	16
Valine	85	113
Methionine	37	40
Isoleucine	112	90
Leucine	133	150
Tyrosine	63	68
Phenylalanine	58	71
Tryptophan	23	24

[a] Based on molecular weight of 170,000 for lobster phosphorylase *b* and 185,000 for rabbit phosphorylase *b* (31).

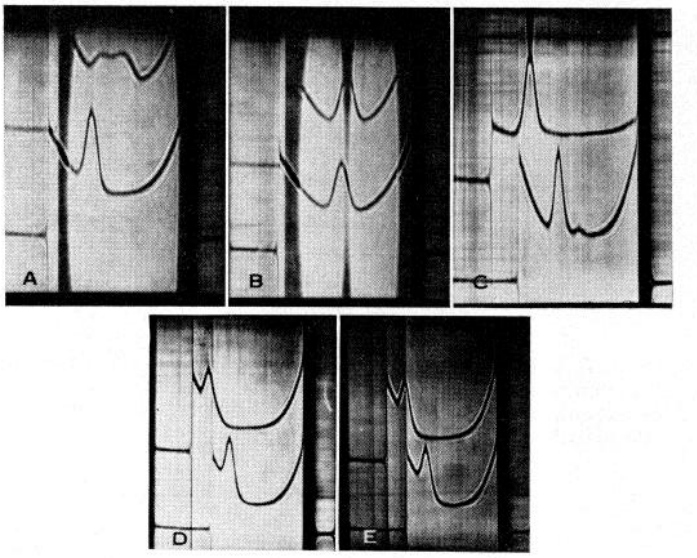

FIG. 6. Sedimentation velocity pattern of lobster and rabbit phosphorylase *b* in 0.5 M phosphate. *A: upper curve,* lobster phosphorylase *b* (6 mg per ml) in 0.02 M Tris, 0.005 M EDTA, 0.5 M P_i, and 0.01 M AMP, pH 7.5, at 20°; *lower curve,* same as in the *upper curve* except in the absence of AMP. *B:* same as the curves in *A* except that the temperature was 5°. *C:* rabbit phosphorylase *b* (6 mg per ml); *upper curve* in 0.05 M Tris-0.01 M EDTA, pH 7.5, and *lower curve* conditions as in the *lower curve* in *A*. *D:* lobster phosphorylase *b* (7 mg per ml), same as the curves in *A* except pH is 6.5. *E:* lobster phosphorylase *b* (7 mg per ml), same as the curves in *A* except temperature is 3°, pH 6 5. Pictures were taken 40 to 70 min after speed at 60,000 rpm was reached. Direction of sedimentation is from *left* to *right.*

tein as was previously observed with hemoglobin in salt solutions (37, 38).

In contrast to lobster phosphorylase *b*, rabbit phosphorylase *b* treated with 0.5 M P_i, pH 7.5, at 4° (Fig. 6) gave two peaks, one with an $s_{20,w}$ value of 8.6 ± 0.2 S and a faster moving peak with an $s_{20,w}$ of 13.1 ± 0.3 S which closely correspond to a dimer and tetramer, respectively. Warming this rabbit enzyme to 20° decreased the tetramer peak such that its sedimentation constant could not be measured with certainty. Lobster phosphorylase *a* (not shown) treated with 0.5 M phosphate, pH 7.5, at 20°, gave one peak with a sedimentation constant of 8.8 ± 0.3 S which indicated that lobster phosphorylase *a* was unaffected by the phosphate treatment in contrast to lobster phosphorylase *b* which dissociates.

When the phosphate-treated lobster phosphorylase *b* was dialyzed against 0.04 M Tris-0.01 M EDTA, pH 6.9, it was found to be fully active and sedimented as a homogeneous peak in the ultracentrifuge with a sedimentation constant of 8.3 S, corresponding to a dimeric form of phosphorylase *b*. This indicates that the dissociation by 0.5 M P_i to a monomer is reversible and has no adverse effect on enzymic activity. Furthermore, it was found that the enzyme treated with phosphate could be crystallized with ammonium sulfate at 30% saturation after the phosphate had been removed by dialysis.

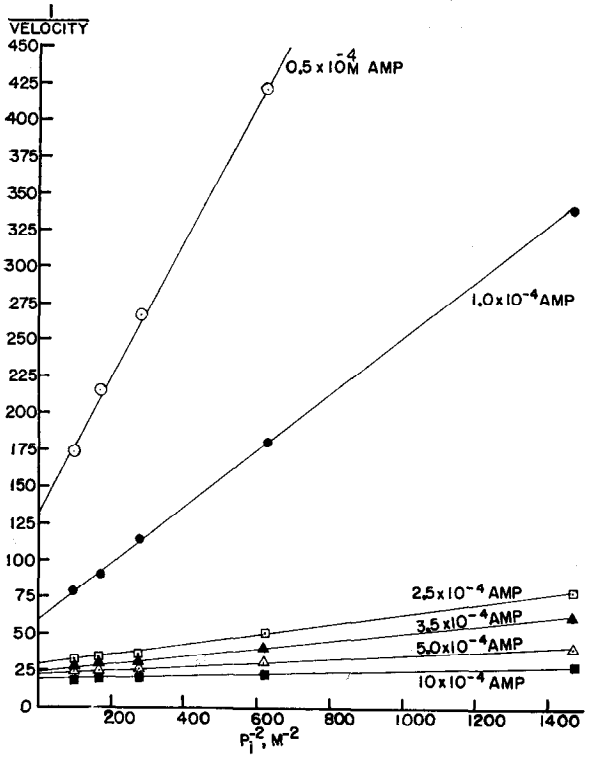

FIG. 7. Reciprocal plot of P_i at six levels of AMP. The velocity in OD units per min was obtained as described under "Methods" for the coupled assay system. For experimental conditions, see "Methods."

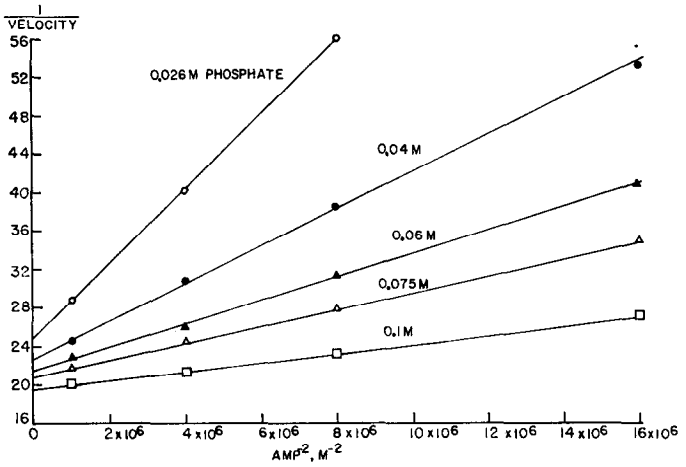

FIG. 8. Reciprocal plot of AMP at five levels of P_i. The velocity in OD units per min was obtained as described under "Methods" for the coupled assay system. For experimental conditions, see "Methods."

Kinetics

In Direction of Glycogen Degradation—Because of the many differences found between lobster and rabbit phosphorylase *b*, it was felt that a kinetic study of lobster phosphorylase would further aid in the understanding of the differences between these two enzymes and in evaluating whether allosteric transitions (if any) for this enzyme obey the fully "concerted" transition theory of Monod *et al.* (12).

With the use of the coupled assay conditions (see "Methods"), the kinetics of lobster phosphorylase *b* were studied at a fixed level of glycogen (0.30% final concentration) with varying AMP and P_i concentrations. A plot of velocity *versus* P_i was sigmoidal except at high concentrations of AMP; likewise sigmoidicity of the saturation curve was observed for AMP except at high levels of P_i. Because the double reciprocal plots were nonlinear, the apparent K_m and V_{max} values were evaluated from plots of $1/v$ *versus* $(1/P_i)^2$ or $(1/AMP)^2$ which were linear as shown in Figs. 7 and 8. Such plots were used by Okazaki and Kornberg (39) in the analysis of their kinetic data with deoxythymidine kinase and also by Madsen (40) on his studies of rabbit phosphorylase *b*. K_m values were also evaluated from Hill plots. The slope of the Hill plots, as well as the apparent K_m values are shown in Table III. It can be seen that the K_m values from the Hill plots agree with those obtained from the negative intercepts on the

TABLE III

Kinetic constants and Hill plot slopes in direction of glycogen degradation

P_i used	K_m for AMP[a]	Hill plot slopes	AMP used	K_m for P_i[b]	Hill plot slopes
M	*mM*		*mM*	*M*	
0.10	0.155 (0.162)	1.6	1.0	0.015 (0.012)	1.3
0.075	0.207 (0.191)	1.7	0.5	0.024 (0.024)	1.5
0.06	0.238 (0.234)	1.7	0.35	0.030 (0.029)	1.5
0.04	0.292 (0.295)	1.8	0.25	0.039 (0.039)	1.6
0.026	0.394 (0.407)	2.0	0.10	0.060 (0.055)	1.7
0.0065	0.645 (0.631)	2.0	0.05	0.070 (0.060)	1.8

[a] K_m values of AMP were taken from a plot of $1/v$ *versus* $(1/AMP)^2$ at fixed P_i concentration levels at the constant level of 0.30% glycogen. The K_m values in parentheses were taken from Hill plots.

[b] K_m values of phosphate were taken from a plot of $1/v$ *versus* $(1/P_i)^2$ at fixed AMP concentration levels at the constant level of 0.30% glycogen. The K_m values in parentheses were taken from Hill plots.

abscissa of $1/v$ *versus* $(1/\text{AMP})^2$ or *versus* $(1/P_i)^2$. These data agree in general with the kinetic studies on rabbit phosphorylase *b* (27, 40–42), *i.e.* the apparent K_m values for P_i decrease with increasing P_i concentration; and the values of the interaction coefficient n of the Hill plots increase with decreasing AMP or P_i. The apparent maximal velocities represented by the intersection points in Figs. 7 and 8 are low and are not tabulated because the glycogen concentration used is far from the K_m value (as observed under "In Direction of Glycogen Synthesis"). These activities, therefore, do not reflect the specific activity of lobster phosphorylase *b* under saturating conditions. The apparent V_{max} values obtained from these plots were used in the determination of the allosteric constant (L_0) and the dissociation constants for P_i and AMP. The data was analyzed according to the equation derived by Buc (13) from the exclusive binding model and is shown below

$$\sqrt{v/V_a - v} = \sqrt{1/L_0}\,(1 + P/K_P)\,(1 + A/K_A)$$

where v = initial velocity, V_a = velocity observed at a given concentration of phosphate, and at saturation of AMP for which $\bar{R} = 1$, and is $= V_{max}$, $L_0 = T/R$ in absence of both phosphate and AMP, the concentration of any other ligand remaining constant, P = phosphate concentration, A = AMP concentration, K_P = dissociation constant of enzyme-bound phosphate, and K_A = dissociation constant of enzyme-bound AMP. In this derivation the enzyme is assumed to belong to a K system of allosteric enzymes where an increase in substrate concentration (in this case P_i) should decrease the enzyme requirement for the allosteric effector (in this case AMP). As suggested by Buc (13), the data were plotted in the form of $\sqrt{v/V_{max}\text{-}v}$ *versus* AMP at various constant levels of P_i concentrations as shown in Fig. 9. Except for points at zero AMP which fall below the lines (not shown because they would obstruct the intercepts), all phosphate lines were linear and intersected at a common point on the abscissa which is equal to the dissociation constant for AMP (2.5×10^{-5} M). Each of the ordinate intercepts of the phosphate lines is equal to $\sqrt{1/L_0}\,(1 + P/K_P)$. These values are plotted *versus* phosphate concentration as shown in the *inset* to Fig. 9. The ordinate intercept is equal to 0.0285 giving an L_0 value of 1200. The abscissa intercept is equal to 0.025 which is the dissociation constant (K_P) for P_i.

These values differ from those reported by Buc (13) for rabbit phosphorylase *b* for which L_0 was found to be 600 and K_P and K_A were 4×10^{-3} M and 3×10^{-6} M, respectively. The fitness of the data to the equation derived by Buc (13) suggests that lobster phosphorylase *b* belongs to those allosteric enzymes which fit the general two-state exclusive binding model, with the exception of data in two cases: (*a*) at high P_i concentrations and (*b*) in the absence of AMP. Substrate inhibition was observed when P_i was > 0.075 M in the presence of AMP $> 5 \times 10^{-4}$ M. Similar substrate inhibition has been reported recently for rabbit phosphorylase *b* (43). In the absence of AMP, substrate inhibition was not detected at P_i concentrations up to 0.15 M.[1]

The velocities obtained in the absence of AMP and with 0.15, 0.12, 0.10, and 0.075 M P_i fell below the lines on the Buc plot of Fig. 9 and gave the following values of 1.1×10^{-3}, 6.5×10^{-4}, 5.0×10^{-4} and 3.3×10^{-4} OD units per min, respectively. Velocities in the absence of AMP obtained at lower P_i concentrations could not be accurately determined because of the uncertainty in evaluating the slopes of the Cary 15 lines. A model is presented in the appendix to account for deviation of velocities at zero AMP.

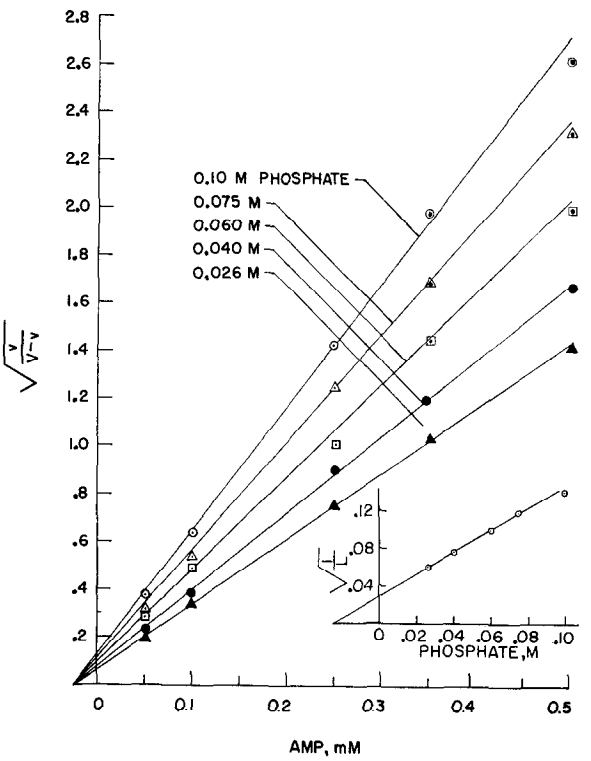

FIG. 9. Determination of the dissociation constant of AMP from five levels of fixed P_i. The zero AMP points for the various P_i lines are not shown because they fall below the lines and would obstruct the intercepts on the ordinate which were used in the replot shown in the *inset*. Details are in the text.

To calculate V'_m (maximal velocity) at zero AMP and infinite P_i concentration, Equation 3 in the appendix was used. With $\gamma = 0$, α = the concentration P_i divided by 0.025 (the dissociation constant for P_i obtained from Fig. 9), $L_0 = 1200$, and the observed velocities, V'_m values were determined. These calculations were performed for 0.15, 0.12, 0.10, and 0.075 M P_i and gave V'_m values of 0.033, 0.029, 0.031, and 0.034 OD unit per min, respectively. Based on the experimentally determined V_m at infinite P_i and AMP at 0.30% glycogen (0.062 OD unit per min), V'_m is 53, 46, 49, and 55% of V_m, respectively. With the model of Monod *et al.* (12), V'_m and V_m are the same, whereas this study shows V'_m to be only half of V_m. Therefore, this work suggests that the kinetics of lobster muscle phosphorylase *b* cannot be completely explained by a simple K system.

In Direction of Glycogen Synthesis—Since the preceding results showed that kinetic constants of lobster phosphorylase *b* are significantly different from that of rabbit phosphorylase *b* in the direction of glycogen degradation, initial velocity studies were carried out in the direction of glycogen synthesis. Experiments with lobster phosphorylase *b* were performed at pH 6.8 at two different temperatures (0° and 30°) and were compared to results with rabbit phosphorylase *b* to determine if the properties of the enzyme from this poikilothermic animal were related to its physiological activity in its environment.

[1] The kinetic data with P_i concentration > 0.2 M (concentrations which caused decrease in the sedimentation constant of lobster phosphorylase *b*) could not be analyzed because these P_i concentrations caused severe inhibition of the auxiliary enzymes.

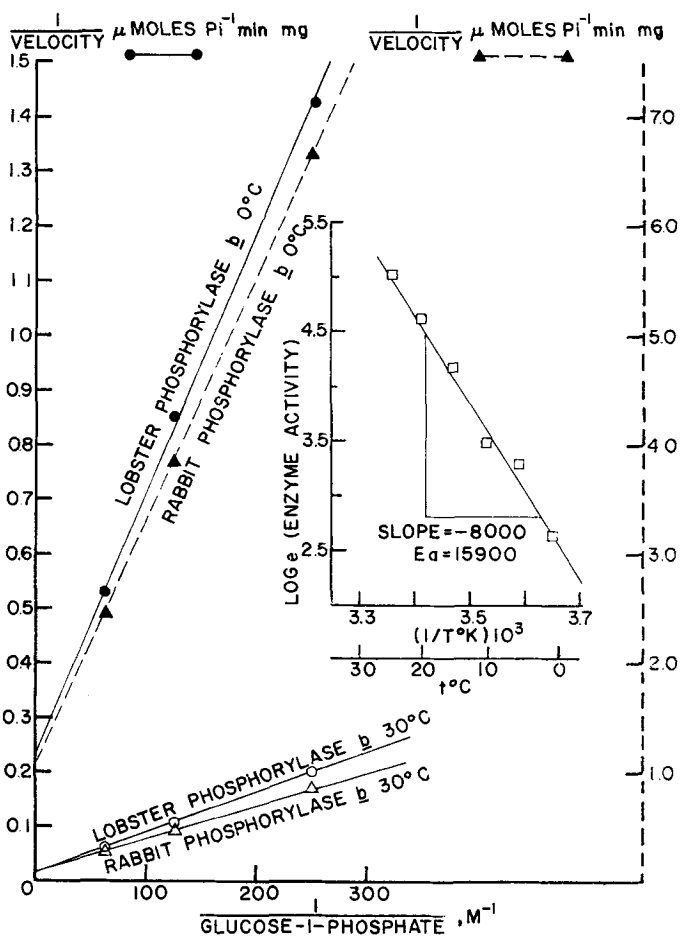

FIG. 10. Reciprocal plot for glucose-1-P at 30° and 0° for lobster and rabbit phosphorylase and Arrhenius plot at pH 6.8. For experimental conditions, see "Methods." The *ordinate* on the *right* refers only to data of rabbit phosphorylase *b* at 0°.

A plot of $1/v$ *versus* 1/glucose-1-P for both enzymes is shown in Fig. 10. From these data, the apparent K_m for glucose-1-P of lobster phosphorylase *b* was calculated to be 0.054 M at 30°. This value is similar to that reported earlier by Cowgill (11). The K_m at 0° was lowered to 0.021 M. The K_m values of glucose-1-P for rabbit phosphorylase *b* under these experimental conditions were 0.01 M and 0.015 M at 30° and 0°, respectively. The K_m values of lobster phosphorylase *b* for glycogen were also determined at pH 6.8 with substrate containing 4×10^{-3} M AMP and 0.048 M glucose-1-P (see "Methods"). These values were 0.9% and 0.25% at 30° and 0°, respectively.

A significant difference in the effect of temperature on the V_{max} of these two enzymes may be noted in Fig. 10. Whereas the V_{max}, *viz.* the turnover number, of lobster phosphorylase *b* is 71 μmoles of P_i released per min per mg at 30°, it was 4.5 at 0°. At 30° the V_{max} value of rabbit phosphorylase *b* was 60 μmoles of P_i released per min per mg which is closely similar to that of lobster phosphorylase *b*. However, the V_{max} value of lobster phosphorylase *b* at 0° was markedly different, *viz.* 5.6 times greater, than that for rabbit phosphorylase *b* under the same conditions of substrate, assay, and temperature. The *inset* shown in Fig. 10 is an Arrhenius plot of lobster phosphorylase *b* obtained from other studies on the effect of temperature on enzymic activity. The energy of activation at pH 6.8 was calculated and found to be 15.9 kcal which is lower than the 21.2 kcal reported for rabbit phosphorylase *b* at pH 6.8 (44). Thus, from the value of energy of activation and the turnover number at 0°, it seems reasonable to classify lobster phosphorylase *b* as one which belongs to the group of enzymes of poikilothermic animals (45) which has a great catalytic efficiency at cold temperature.

Lobster Phosphorylase b_{II}

Earlier in the paper under "Purification Procedure" it was stated that another form of phosphorylase *b* existed in some crude extracts (in 6 out of 15 preparations). This form, which is activated by high AMP (1 to 2×10^{-2} M) in the substrate-enzyme reaction mixture, was called phosphorylase b_{II} and may be related to the form of phosphorylase called *c* by Cowgill and Cori (10) except that the percentage of activation of the b_{II} form with high concentrations of AMP was much less than that described by Cowgill (11) for phosphorylase *c*. The b_{II} form was purified by ammonium sulfate fractionation and DEAE-cellulose chromatography as described earlier for phosphorylase *b*. During storage for a period of 10 days to 2 weeks, the percentage activation by high AMP concentrations decreased gradually, and inhibition instead of activation was observed. Phosphorylase b_{II} was also found to convert to *b* (*i.e.* inhibition by high concentrations of AMP was observed) upon freezing and thawing. In this study, the percentage inhibition of crystalline phosphorylase *b* by high AMP concentrations was sometimes as much as 60% whereas the highest activation of phosphorylase b_{II} by high AMP was found to be about 35%; activation was found to be pH-dependent and highest at pH 7.4. Cowgill (11), in his study of lobster crude extracts, reported little or no inhibition of phosphorylase *b* by high concentrations of AMP and in some cases reported as much as 10% activation. He also reported an activation of 500% for the form he called phosphorylase *c*. Possibly, our different results may be related to differences in the physiological condition of the lobsters used in the two studies.

Cold Inactivation Studies

Studies on the effect of cold temperature on the structure and activity of lobster muscle phosphorylase *b* were undertaken since it had previously been shown that phosphorylase from a warm blooded animal (rabbit) showed unusual sensitivity to cold temperature (46). Fig. 11 shows the effect of temperature on the stability of lobster and rabbit phosphorylase *b*. The enzymes were incubated at 0° and 20° at pH 6.0 and at various intervals aliquots were removed and tested for enzymic activity at 30° at pH 6.8. It should be noted that both enzymes are more stable at 20° than at 0°; under these experimental conditions, however, lobster phosphorylase *b* is more rapidly inactivated than rabbit phosphorylase *b* at 0°. The extent of inactivation of lobster phosphorylase *b* has been found to depend upon buffers, salts, and protein concentration as described previously (46).

Cold inactivation of the enzyme in the presence of the above mentioned pH 6.0 buffer was also studied in the analytical ultracentrifuge. The photograph is not shown here because it is similar to that previously published for rabbit phosphorylase under the same experimental conditions. As reported earlier for rabbit phosphorylase *b*, two peaks were observed, a slow symmetrical peak ($s_{20,w}$ = 8.4 S) and a fast moving, very broad peak corresponding to an aggregate of phosphorylase *b*. In the

earlier studies, the loss of activity was attributed to a conformational change followed by a loss of pyridoxal phosphate. Incubation of the cold-inactivated rabbit enzyme for 1 hour at 30° resulted in an enzyme with 80% of the original activity. This reversal was not observed in lobster phosphorylase *b* as only 4% of the activity was restored under the same conditions.

A different form of phosphorylase has also been reported in this paper (see "Lobster Phosphorylase b_{II}"). Although these studies suggested that lobster phosphorylase b_{II} is labile and converted to phosphorylase *b* upon freezing, a test for the interconversion of phosphorylase *b* to b_{II} was made under the cold inactivation conditions. Aliquots of enzyme were removed at different intervals and assayed at low (2×10^{-3} M) and high (2×10^{-2} M) AMP concentrations at pH 7.5. Inactivation was observed but no conversion of phosphorylase *b* to b_{II} was detected.

The reason for the higher sensitivity of lobster phosphorylase *b* to cold temperature in comparison to rabbit phosphorylase *b* is not clear. Earlier results with rabbit phosphorylase suggested that subunit structure of the enzyme was a determining factor, as dimeric rabbit phosphorylase *b* and *a* were cold-sensitive but the tetrameric form of rabbit phosphorylase *a* was not. The fact that lobster phosphorylase *b* is dissociable into a monomeric form by phosphate, whereas rabbit phosphorylase *b* is not, suggests differences in the interaction energy of the subunits of these two enzymes which might account for the differences in cold lability. It needs to be emphasized that although lobster phosphorylase *b* is inactivated with the above experimental conditions, it is quite stable for several months when frozen at higher pH values in a different buffer (0.04 M Tris-0.01 M EDTA, pH 6.8 to 7.5), and that inactivation *in vitro* does not imply anything in regard to the stability of lobster phosphorylase *b* in the lobster at different environmental temperatures.

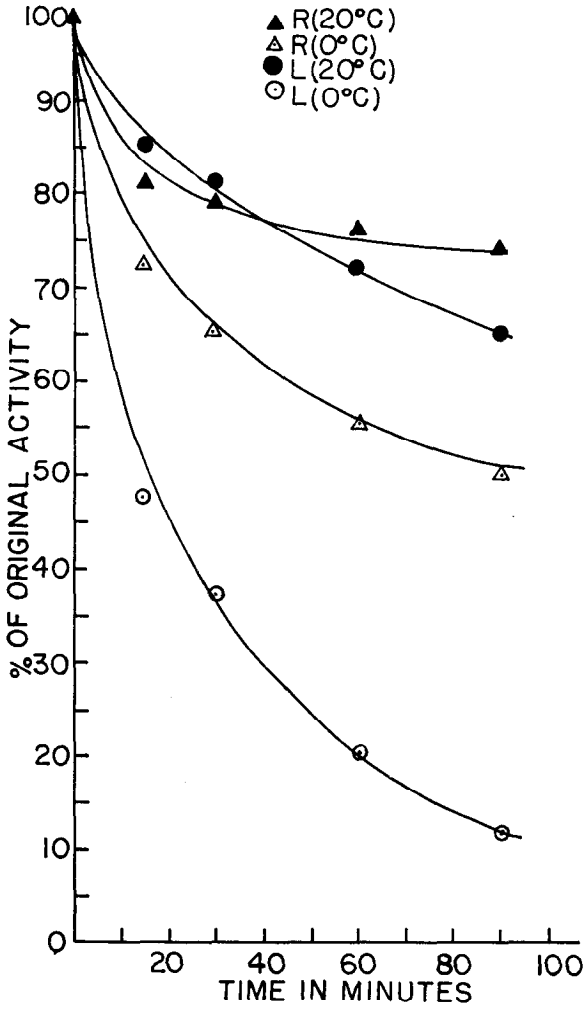

FIG. 11. Inactivation of lobster (*L*) and rabbit (*R*) phosphorylase *b* in cysteine-NaCl at pH 6.0. Lobster and rabbit phosphorylase *b* (1 mg per ml) were incubated in 0.2 M NaCl containing 0.04 M glycerophosphate-0.03 M cysteine, pH 6.0, for various intervals prior to 1:100 dilution in 0.15 M glycerophosphate-0.05 M mercaptoethanol, pH 6.8.

DISCUSSION

Significant differences in the ultracentrifugal properties of lobster and rabbit phosphorylase *b* and *a* were found in this work. Earlier results of Cowgill (11) indicated that, in contrast to rabbit muscle phosphorylase, there is no change in sedimentation constant when lobster phosphorylase *b* is converted to phosphorylase *a*. This has been confirmed in this study and it was also shown that lobster phosphorylase *a* ($s_{20,w}$ = 8.5 S) does not possess a dimer $\rightleftharpoons$ tetramer reaction even at high enzyme concentration and cold temperature, conditions which favor the association of rabbit phosphorylase *a* (32, 33). Similarly lobster phosphorylase *b* remains a dimer with a sedimentation constant equal to 8.5 S under conditions which promote association of rabbit phosphorylase *b* at cold temperature, *e.g.* with NaF and AMP (34), AMP and mercaptoethanol (35), and AMP and Mg^{++} (28). Presumably crystallization of other muscle phosphorylases (1, 3, 4) also involves tetramer formation. The failure of lobster phosphorylase *b* or *a* to crystallize with Mg^{++} and AMP is probably related to its inability to assume a tetrameric form. As it has been suggested that the region in the primary structure which is phosphorylated in the conversion of phosphorylase *b* to *a* is a key site in the subunit assembly of tetrameric phosphorylase (47–49), the differences in subunit structure of lobster and rabbit muscle phosphorylase may be related to differences in primary structure at this locus in the two proteins. Such a difference seems possible in view of the many differences found in the amino acid composition of these two proteins. Work is in progress in this laboratory to determine and compare the amino acid sequence of phosphorylated peptides obtained from a chymotryptic digest of lobster phosphorylase *a* with results already obtained on rabbit phosphorylase *a* (50).

Another important difference in sedimentation behavior between rabbit and lobster phosphorylase was found in the effect of different salt solutions. In contrast to rabbit phosphorylase *b* which in the presence of 0.5 M P_i, pH 7.5, was found to sediment mainly as a dimer with a slight tendency to associate, lobster phosphorylase *b* was converted in high phosphate to a form which sedimented with an $s_{20,w}$ value of 5.4 S which is characteristic of a monomeric form of phosphorylase (36). Also, whereas AMP favors formation of a tetrameric form of rabbit phosphorylase *b* at cold temperature, AMP was found to promote association of lobster phosphorylase *b* only to a dimer and only at warmer temperatures (20°) or low pH (6.5).

The dissociation of a tetramer of phosphorylase *a* to a more active dimer has been suggested as one of the mechanisms involved in the control of glycogen degradation by phosphorylase in skeletal muscle (9, 51). The interesting results of Metzger

et al. (9) with frog phosphorylase *a* show that the temperature dependence of the dimer ⇌ tetramer equilibrium predicts more active dimeric phosphorylase *a* than that for rabbit phosphorylase *a* at the same temperature. This difference was suggested to be related to the energy requirements of animals at low environmental temperatures. Since lobster phosphorylase *a* remains a dimer under conditions known to associate frog and rabbit phosphorylase, the dimer-tetramer association reaction must be ruled out as a mechanism for the control of glycogen degradation by phosphorylase in lobster muscle. Glycogen degradation in lobster muscle is no doubt regulated by the concentration of the substrates, *viz.* P_i, glucose-1-P, and glycogen, and the activator AMP. Since the Michaelis constants of lobster phosphorylase *b* for the substrates and the activator are very high, phosphorylase *b* may have only a very minor role in glycogen degradation. To better understand the control of glycogen degradation in lobster muscle, comparable kinetic data are needed on phosphorylase *a*. These experiments are presently being done in our laboratory.

Kinetic studies in the direction of glycogen degradation indicate heterotropic cooperativity between the binding sites of substrate (P_i) and those of the activator AMP. Also, these studies suggest homotropic cooperativity for each of the binding sites of the substrate (P_i) and the activator. Analysis of the kinetic data suggests that lobster phosphorylase *b* conforms to the fully concerted transition model at all concentrations of P_i and AMP except at high inorganic phosphate, *i.e.* over 0.075 M in the presence of $>5 \times 10^{-4}$ M AMP where substrate inhibition was observed, and at zero AMP and a given phosphate where only 50% of the activity predicted from this model was obtained. The equation presented in the appendix accounts for the deviation at zero AMP and is based on the assumption of a lower catalytic rate constant when AMP is absent.

Other possibilities which may account for the low velocities with zero AMP have been suggested for rabbit phosphorylase *b* by Kastenschmidt *et al.* (52). They indicated that a state of lower activity called *R′* may form when AMP concentrations approach zero. Black and Wang (53) found that their kinetic data in the presence of IMP deviated from the model of Monod *et al.* (12), and also suggested a third conformational state. These studies taken along with the recent results of Madsen and Engers (54) and Graves *et al.* (49) suggest that AMP does effect the maximal velocity. Therefore, it appears that lobster and rabbit phosphorylase *b* are similar in that their kinetics cannot be accurately described as a K system.

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APPENDIX

These derivations are based on the model of Monod *et al.* (12).

Assume $V_m > V'_m > 0$, where V_m is the maximal velocity in the presence of saturating P_i and saturating AMP at constant glycogen and V'_m is the maximal velocity in the presence of saturating P_i in the absence of AMP at constant glycogen; with the use of the terminology of Monod *et al.* (12), let R_0 = the R state unbound by AMP, T_0 = the T state unbound by AMP, (A) = concentration of AMP, (P) = concentration of P_i, K_A = the intrinsic dissociation constant for AMP, K_P = the intrinsic dissociation constant for P_i, E_T = total enzyme forms, v = velocity in OD units per min per known enzyme concentration, k = the catalytic rate constant in the presence of AMP, k' = the catalytic rate constant in the absence of AMP, then a general rate equation may be written in the following form

$$\frac{V}{E_T} = \frac{2k(RP_2A_2) + (k + k')RP_2A + \left(\frac{k}{2} + \frac{k'}{2}\right)RPA + k'RP + 2k'RP_2}{R_0 + RA + RA_2 + RP + RP_2 + RAP + RAP_2 + RA_2P + RA_2P_2 + T_0} \tag{1}$$

The statistical relations are given below

$$\frac{(R_0)(A)}{RA} = \frac{K_A}{2}, \qquad \frac{(R_0)(P)}{RP} = \frac{K_P}{2}, \qquad \frac{(RA)(A)}{RA_2} = 2K_A, \qquad \text{and} \qquad \frac{(RP)(P)}{RP_2} = 2K_P.$$

From these relations the concentration of the various terms of the enzyme can all be expressed in terms of R_0.

$$RA = 2R_0\frac{(A)}{K_A}, \qquad RA_2 = (RA)\left(\frac{A}{2K_A}\right) = \frac{R_0A^2}{K_A{}^2}, \qquad RP = 2R_0\frac{(P)}{K_P}, \qquad RP_2 = R_0\frac{P^2}{K_P{}^2}, \qquad \text{and} \qquad RPA = RP\frac{(A)}{K_A} = \frac{4R_0AP}{K_PK_A}.$$

Substituting these into the general rate equation and collecting terms, the following equation is obtained

$$\frac{V}{E_T} = \frac{2k\left(\frac{P}{K_P}\right)\left(1 + \frac{P}{K_P}\right)\frac{A}{K_A}\left(1 + \frac{A}{K_A}\right) + 2k'\left(\frac{P}{K_P}\right)\left(1 + \frac{P}{K_P}\right)\left(1 + \frac{A}{K_A}\right)}{\left(1 + \frac{P}{K_P}\right)^2\left(1 + \frac{A}{K_A}\right)^2 + L_0} \tag{2}$$

where L_0 is the allosteric constant equal to T/R in the absence of both P_i and AMP at constant glycogen.

$$\frac{P}{K_P} = \alpha, \qquad \frac{A}{K_A} = \gamma, \qquad 2kE_T = V_m, \qquad 2k'E_T = V'_m$$

Making these substitutions and collecting terms, one obtains the final equation in its final form as shown below

$$v = \frac{\alpha(1 + \alpha)(1 + \gamma)(\gamma V_m + V'_m)}{(1 + \alpha)^2(1 + \gamma)^2 + L_0} \tag{3}$$

When $V_m = V'_m$ as assumed by Buc (13) one obtains Buc's equation in a different form as shown below

$$v = \frac{2V_m\alpha(1 + \alpha)(1 + \gamma)^2}{(1 + \alpha)^2(1 + \gamma)^2 + L_0} \tag{4}$$

and when $V'_m = 0$, *viz.* the activity in the absence of AMP is equal to zero, then the equation shown below is obtained

$$v = \frac{V_m\alpha(1 + \alpha)\gamma(1 + \gamma)}{(1 + \alpha)^2(1 + \gamma)^2 + L_0} \tag{5}$$