Gel Filtration, Aggregation, and the Enzymatic Activity of Glycogen Phosphorylase*

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ABSTRACT: The association–dissociation properties of muscle glycogen phosphorylase were investigated by gel filtration and correlated with changes in the catalytic properties of the enzyme. Physical characterization of the enzyme was accomplished under conditions approximating those used in assaying enzymatic activity. Under these conditions, the dimeric forms of both phosphorylase *b* and phosphorylase *a* were more active and present in much greater quantities than their tetrameric counterparts. Changes in the aggregated state of the two forms of the enzyme could be correlated with changes in enzymatic activity. Conditions which favored the dissociation of phosphorylase *a* tetramer to dimer (dilution of the enzyme, addition of adenosine monophosphate (AMP) or substrates, high pH or temperatures) increased the activity of the enzyme. Phosphorylase *a* dimer could be dissociated to monomers at pH 5.5 and low protein concentrations. Phosphorylase *b* dimer could be dissociated to monomer at low enzyme concentrations by the addition of adenosine triphosphate (ATP); $NaBH_4$ reduction of the prosthetic group, pyridoxal 5′-phosphate, decreased the affinity of the monomers for one another. Light-scattering measurements were employed to measure the rate of dissociation of phosphorylase *a* tetramer caused by dilution or the addition of AMP.

The tetramer–dimer dissociation process was found to be rapid; AMP affected both the rate of attainment of equilibrium and the equilibrium constant between phosphorylase *a* tetramer and dimers. From the study of the aggregation phenomenon under a number of different conditions, it was concluded that the forces responsible for holding monomer units together to form dimers and dimer units together to form tetramers are different.

Glycogen phosphorylase (EC 2.4.1.1 α-1,4-glucan: orthophosphate glucosyltransferase) from rabbit skeletal muscle, a key enzyme in the regulation of glycogen metabolism, has been shown to exist in structures of varying molecular sizes. Reports from this laboratory and others have established the molecular weights of phosphorylase *a* tetramer and phosphorylase *b* dimer as being 360,000 and 180,000 g per mole, respectively (DeVincenzi and Hedrick, 1967; Seery *et al.*, 1967). Seery *et al.* (1967) also showed that dissociation of phosphorylase in urea or guanidine hydrochloride yielded a monomer subunit with a molecular weight of about 90,000. This finding was subsequently confirmed by Ullman *et al.* (1968) and Hedrick *et al.* (1969a).

Kinetic analyses and light-scattering studies have suggested that phosphorylase *a* tetramer dissociates into a dimeric species of higher catalytic activity in solutions of high ionic strength or of low protein concentrations (Wang and Graves, 1964; Huang and Graves, 1967; Graves *et al.*, 1967) and that activation by preincubation with glycogen is related to enzyme dissociation (Wang *et al.*, 1965). More recent experiments (Metzger *et al.*, 1967) have indicated that phosphorylase *a* tetramer cannot bind to glycogen and that the dimer is the only active form of the enzyme when glycogen is the substrate. In addition, experiments with disc gel electrophoresis performed in this laboratory have shown that phosphorylases *a* and *b* can exist in forms equal in size (dimers with molecular weights near 180,000 g/mole) under the conditions used for electrophoresis and both are enzymatically active (Hedrick *et al.*, 1969a). Hence, although phosphorylase can exist in various states of subunit aggregation (monomer, dimer, and tetramer), the effect of subunit interaction upon the catalytic properties of the enzyme has yet to be clearly defined since physical measurements have not been made under conditions approximating those of the enzymatic assay. Correlation of physical properties (state of aggregation) and enzymatic activity has usually been accomplished by extrapolation of the physical data obtained at high protein concentrations to the lower protein concentrations used in enzymatic activity measurements. This problem was circumvented in the present work by using gel filtration, a technique which permits physical characterization under assay conditions.

The results reported here indicate that the equilibria among the monomer, dimer, and tetramer forms of phosphorylase are affected by many factors. Protein concentration, temperature, pH, and the presence of activator and substrates have a marked effect upon the quaternary structure of this enzyme as well as upon its catalytic function. Under experimental conditions approximating those in the enzymatic assay, phosphorylase *a* exists as a catalytically active dimer.

* From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616. *Received December 19, 1969.* This work was supported in part by a grant from the National Science Foundation (GB-4946). A preliminary report of this work was given at the 154th Annual Meeting of the American Chemical Society at Chicago, Sept 1967. Due to financial support limitations, reprint requests from U. S. residents cannot be honored.

† Recipient of a U. S. Public Health Service predoctoral fellowship (No. 5-F1-GM-32940-02) from the National Institute of General Medical Sciences. The material presented is taken in part from a thesis submitted to the Graduate Faculty of the University of California, Davis, in partial fulfillment of Ph.D. degree requirements.

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Further, the data show a correlation between the extent of dissociation of subunits and the activity of the enzyme.

Experimental Procedure

Enzyme Preparation and Assay. Crystalline rabbit muscle phosphorylases *a* and *b* were prepared as described previously (DeVincenzi and Hedrick, 1967). Reduced phosphorylase *b* was prepared according to the method of Strausbauch *et al.* (1967) and apophosphorylase as described by Shaltiel *et al.* (1966). The modified forms contained less than 5% of the native enzyme.

Phosphorylase assays were carried out as previously described (DeVincenzi and Hedrick, 1967) except that incubation times were varied from 25 min (at 0.002 mg of enzyme/ml) to 15 sec (at 1.5 mg of enzyme/ml). The decreased activities observed in assays at high enzyme concentrations were not due to substrate diffusion limitations or to reduced substrate levels (less than 20% of the initial glucose-1-P was hydrolyzed at the highest enzyme levels).

Some gel filtration experiments were carried out in a buffer which contained an equilibrium mixture of substrates and products of the enzymatic reaction. When effluent concentrations of enzyme were too low to detect by ultraviolet absorption measurements, a modification of the coupled spectrophotometric assay described by Maddiah and Madsen (1966) was used to determine the elution volume. When the coupling enzymes were added to samples of column effluent, a very rapid nucleotide reduction occurred due to the presence of glucose-1-P in the column buffer. This was followed by a slower reaction, the rate of which was proportional to the amount of phosphorylase present. Samples of column eluate (0.2 ml) containing glucose-1-P and other buffer components together with phosphorylase were mixed with 2.8 ml of reaction mixture at pH 7.5 containing 9 mM Tris, 23 μg of phosphoglucomutase, 7 μg of glucose 6-phosphate dehydrogenase, 1 mM $NADP^+$, 6 mM imidazole, 0.07 mM magnesium acetate, 7 mM P_i, 0.14% glycogen, and 0.3 mM AMP. Assays were carried out with a recording spectrophotometer at 340 mμ. The two rate processes were always sufficiently different so that they could be distinguished.

The equilibrium constant for phosphorylase *b* ($K = [P_i]/[\text{glucose-1-P}]$) was determined at pH 6.5 and 30° with maltodextrin or bacteriological dextrin as the carbohydrate primer and found to be 8.0. The K_m of each primer for phosphorylase was found to be 0.1%.

Physical Techniques. Analytical gel filtration columns of Sephadex G-200 were prepared and operated as previously described (DeVincenzi and Hedrick, 1967). Unless otherwise noted, the buffer was 30 mM β-glycerophosphoric acid–1 mM EDTA adjusted to pH 6.5 with Tris ($\mu = 0.07$). Elution volumes were determined by continuous monitoring of the column eluate at 278 or 230 mμ, and at 290 mμ when AMP was added to the eluting buffer. When protein concentrations were too low to detect spectrophotometrically, eluate fractions were collected in tubes containing enzyme diluent (DeVincenzi and Hedrick, 1967) and assayed for phosphorylase activity.

Sedimentation velocity experiments were carried out at 50,740 or 59,780 rpm in a Spinco Model E ultracentrifuge equipped with an RTIC temperature control unit. At protein concentrations above 0.5 mg/ml, the schlieren optical system was used. Ultraviolet absorption optics at 278 mμ (or 290 mμ in the presence of AMP) were used at protein concentrations of 0.1–0.8 mg/ml. In these cases, it was necessary to correct for nonproportionality between blackening of the photographic plate and the protein concentration in the ultracentrifuge cell as described by Svedberg and Pedersen (1940). These corrections allowed accurate determination of the concentration boundary midpoint. Sedimentation coefficients calculated without applying the corrections were in error by as much as 0.7 S from the corrected values. A partial specific volume of 0.737 was used in molecular weight calculations (Seery *et al.*, 1967).

Light-scattering experiments were performed in a Brice-Phoenix light-scattering photometer equipped with a recorder. All solutions were clarified by filtration through 0.3 μ Millipore filters directly into the scattering cell. Measurements were made at room temperature in a 24-mm^2 cell at 436 mμ. Protein concentrations were determined spectrophotometrically on aliquots taken directly from the scattering cell. Instrument calibrations were checked with crystalline bovine serum albumin at 0.87 mg/ml in 0.15 M NaCl. Values for solvent refractive index and refractive index increment of the protein reported by Edsall *et al.* (1950) were used. Light-scattering data together with these constants yielded a value of 79,000 g/mole for the molecular weight of albumin. This value is in reasonable agreement with that reported by Jaenicke and Knof (1968) since the sample contained small amounts of albumin polymers. Experiments with phosphorylase *a* were carried out in 68 mM β-glycerophosphate–Tris–EDTA buffer at pH 6.5 and $\mu = 0.23$, a buffer used in gel filtration experiments. The refractive index of the buffer was 1.3443 and the refractive index increment of phosphorylase *a* was 0.186 ml/g. Turbidities and molecular weights were calculated as described by Bier (1957). As the molecular weight values obtained by light scattering were not corrected for all experimental variables, these values should be considered as only approximate.

Chemicals. β-Glycerophosphate (Sigma Chemical Co.) was purified as described earlier (DeVincenzi and Hedrick, 1967). Proteins used as standards for gel filtration were obtained from the sources noted in that report. Glucose-1-P was purified so that it contained <0.5% inorganic phosphate as a contaminant. Maltodextrin was a generous gift from Dr. W. J. Whelan. The mixture contained maltodextrins with degrees of polymerization between 6 and 12 glucose units (molecular weight of 1000–2000) with the hexasaccharide representing about 15% of the total. Bacteriological dextrin (Mann) was heterogeneous as determined by the ultracentrifuge. Chromatography on Sephadex G-200 resulted in a decidedly asymmetric peak and indicated the presence of large amounts of high molecular weight polymers (weight-average molecular weight of 13,000 g/mole). Shellfish glycogen (Sigma) was purified by alkali treatment and imidazole (Eastman) was recrystallized from ethyl acetate. All other chemicals were obtained from commercial sources and were used without further purification.

Results

Concentration-Dependent Dissociation of Phosphorylase a. Initial gel filtration experiments indicated that under certain conditions (low protein concentration, 30°, and pH 6.5)

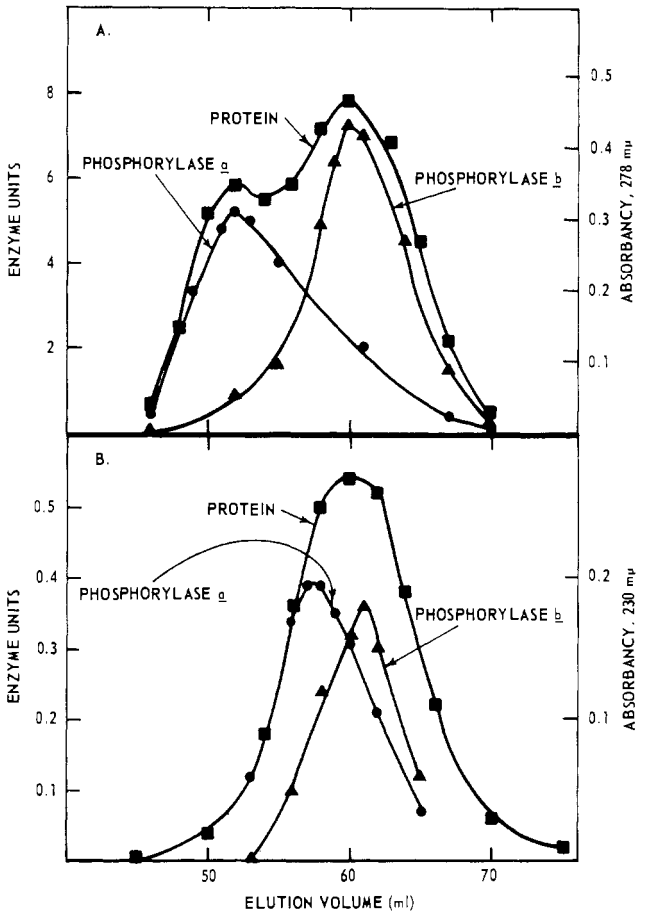

FIGURE 1: Gel filtration of phosphorylase *a* and *b* mixtures on Sephadex G-200. (A) A mixture containing 4.0 mg of each enzyme was applied to a 1.5 × 84 cm column at 30°. (■) Absorbancy curve. Aliquots (0.2 ml) of each 1-ml fraction were assayed for enzyme activity. One enzyme unit is defined as 1 μmole of glucose-1-P hydrolyzed per min. Phosphorylase activity was measured in the presence and absence of AMP. Phosphorylase *a* activity (●) was derived from the measurements in the absence of AMP. Phosphorylase *b* activity (▲) was calculated by subtracting the activity without AMP from the activity with AMP. The enzymes were separated by 8 ml. (B) Same as part A, except that 0.5 mg of each enzyme was applied. Phosphorylases *a* and *b* were separated by 3 ml.

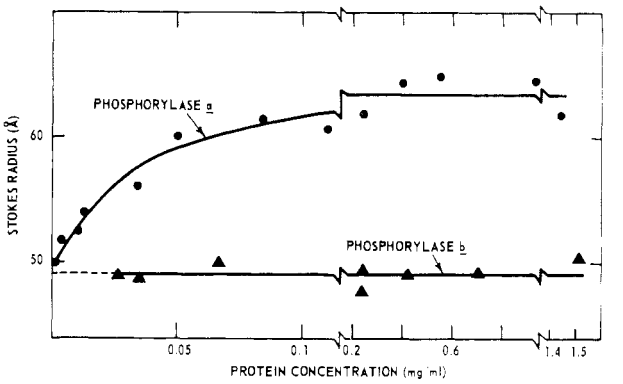

FIGURE 2: Variation in molecular size of phosphorylases *a* and *b* as a function of protein concentration. Each enzyme was chromatographed on 2.5 × 41 cm Sephadex G-200 columns under experimental conditions as in Figure 1. Protein concentrations refer to the maximum concentration of protein in the eluate. Stokes radii were calculated from elution volumes and other column parameters as previously described (DeVincenzi and Hedrick, 1967).

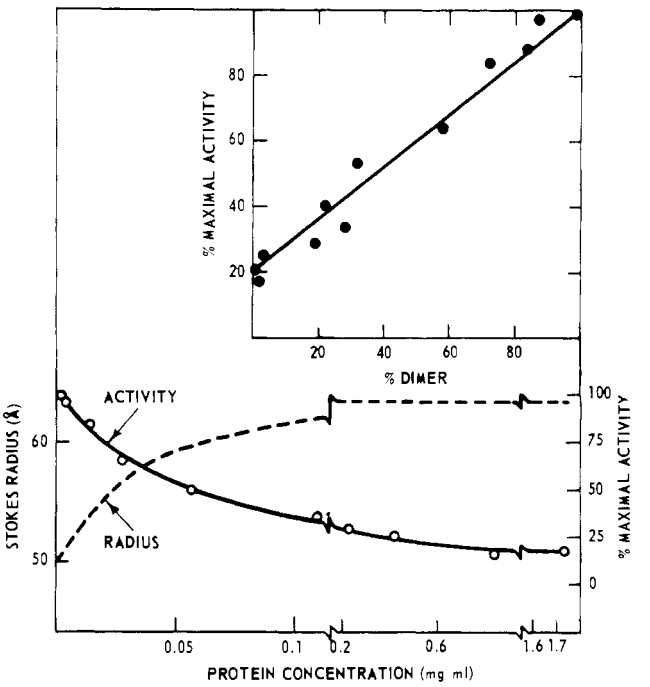

FIGURE 3: Variation of enzymatic activity of phosphorylase *a* as a function of protein concentration, and correlation between molecular size and activity. The specific activity of phosphorylase *a* (○) in the absence of AMP was measured as a function of protein concentration at 30°, pH 6.5, $\mu = 0.34$. The dashed line represents the dissociation curve for phosphorylase *a* from Figure 2 redrawn here for reference. The per cent dimer present at a particular protein concentration (insert) was calculated from the data of Figure 2.

phosphorylase *a* existed in a form similar in size to phosphorylase *b*, a dimer. The behavior of a phosphorylase *a* and *b* mixture on Sephadex G-200 is illustrated in Figure 1. At a high protein concentration (Figure 1A), the two protein peaks were clearly resolved on the basis of absorbance and enzymatic activity measurements. The elution volumes corresponded to those of phosphorylases *a* and *b* chromatographed individually. When the mixture was applied at lower concentration (Figure 1B), only one relatively broad protein peak was observed. However, the two enzyme forms were resolvable by their different enzymatic activities. Whereas the elution volume (and hence molecular size) of phosphorylase *b* in this mixture remained unchanged, the elution volume of phosphorylase *a* shifted markedly toward that of *b*. Although the phosphorylase *b* peaks were relatively symmetrical, the decidedly asymmetric phosphorylase *a* peaks were indicative of a dissociation to a smaller species (Gilbert, 1959).

The concentration-dependent dissociation of phosphorylase *a* to a species approaching the size of phosphorylase *b* was investigated by individually subjecting both enzymes to gel filtration over a wide range of protein concentrations; buffer, pH, and temperature were identical with conditions of enzymatic assay (Figure 2). Over the entire range of protein concentrations tested, the Stokes radius of phos-

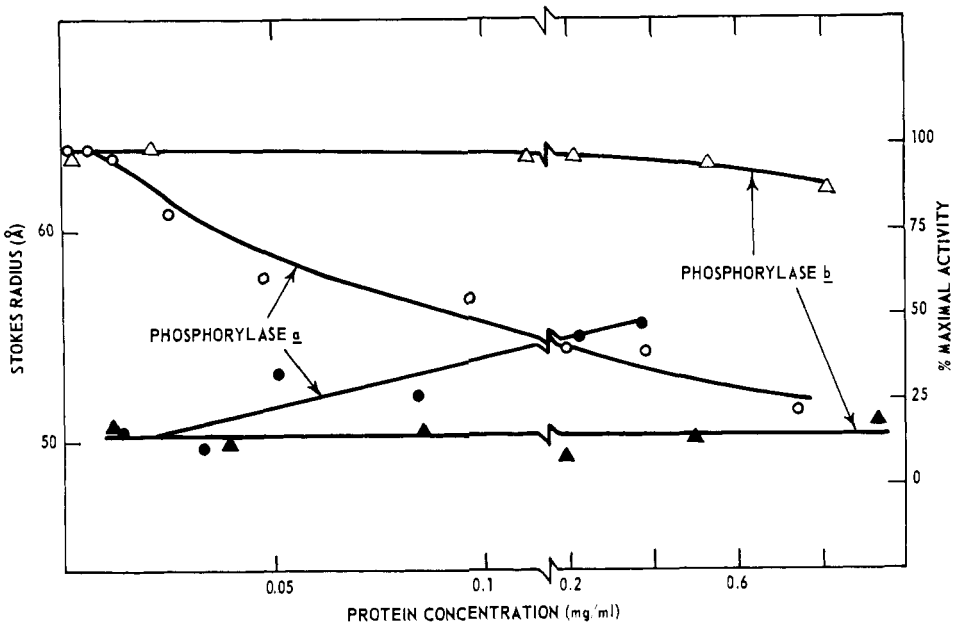

FIGURE 4: Effect of the allosteric activator AMP on molecular size and enzymatic activity of phosphorylases *a* and *b*. The Stokes radii of phosphorylases *a* (●) and *b* (▲) were determined under experimental conditions as in Figure 2, except that 1 mM AMP was added to the eluting buffer. The specific activities of phosphorylases *a* (○) and *b* (△) were measured in the presence of 1 mM AMP as in Figure 3.

phorylase *b* remained constant at 49 Å, corresponding to a molecular weight of 180,000 g/mole (DeVincenzi and Hedrick, 1967). At protein concentrations above 0.2 mg/ml, the gel filtration data for phosphorylase *a*, extrapolated to zero protein concentration, gave a Stokes radius of 63 Å. This represented a tetramer of molecular weight 360,000 g/mole. However, at protein concentrations below 0.1 mg/ml, phosphorylase *a* underwent a marked dissociation and approached the size of phosphorylase *b* at assay concentrations (0.01 mg/ml). These results indicated that under conditions of temperature, pH, and protein concentration identical with those in the enzymatic assay, phosphorylase *a* existed in a form essentially the same size as phosphorylase *b*, a dimer.

In the series of experiments summarized in Figure 2, the elution profiles for phosphorylase *b* were relatively symmetrical. The profiles for phosphorylase *a*, however, were decidedly asymmetric toward smaller molecular weight components. At very low concentrations, these elution patterns assumed a nearly symmetrical shape; only one elution peak was observed. Since the two forms (dimer and tetramer) were not resolved, the association–dissociation equilibrium between these two forms was rapid (*i.e.*, rapid in comparison to the time required for separation on the column (Gilbert, 1959)).

The protein concentration-dependent dissociation of phosphorylase *a* was found to correlate well with changes in enzymatic activity as a function of enzyme concentration. The specific activity of phosphorylase *a* was measured in the absence of AMP and expressed as per cent maximal activity (Figure 3). At protein concentrations above 0.2 mg/ml, the specific activity of phosphorylase *a* was relatively constant and low. Below 0.1 mg/ml, there was a marked increase in specific activity and maximal activity was obtained at assay concentrations of the enzyme. When the per cent dimer was plotted against activity (Figure 3), a linear relation was observed. The intercept indicated that the tetramer had 20% of the activity of the dimer.

Effect of Allosteric Effector AMP on Dissociation and Activity. Since phosphorylases *a* and *b* are both activated by AMP, its effect on the concentration-dependent dissociation and enzymatic activity was investigated. Phosphorylases *a* and *b* were chromatographed at various protein concentrations on G-200 columns equilibrated with 1 mM AMP (Figure 4). The elution profile of phosphorylase *b* in the presence of AMP was not significantly different from that in its absence. In contrast, AMP had a marked effect on

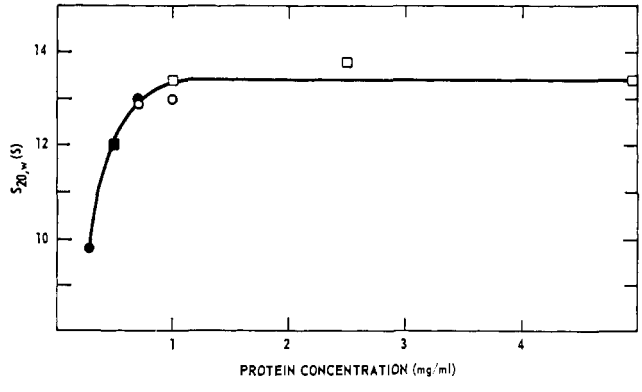

FIGURE 5: Effect of AMP on the sedimentation velocity of phosphorylase *a*. The sedimentation coefficient of phosphorylase *a* was measured as a function of protein concentration under different experimental conditions in the presence of 1 mM AMP. (□, ■) In 50 mM β-glycerophosphate–1 mM dithioerythritol, pH 7.0, 23°. (○, ●) In 68 mM β-glycerophosphate–Tris–EDTA, pH 6.5, 30°. Open symbols refer to sedimentation coefficients determined from schlieren photographs. Closed symbols refer to those by absorption optics at 290 mμ.

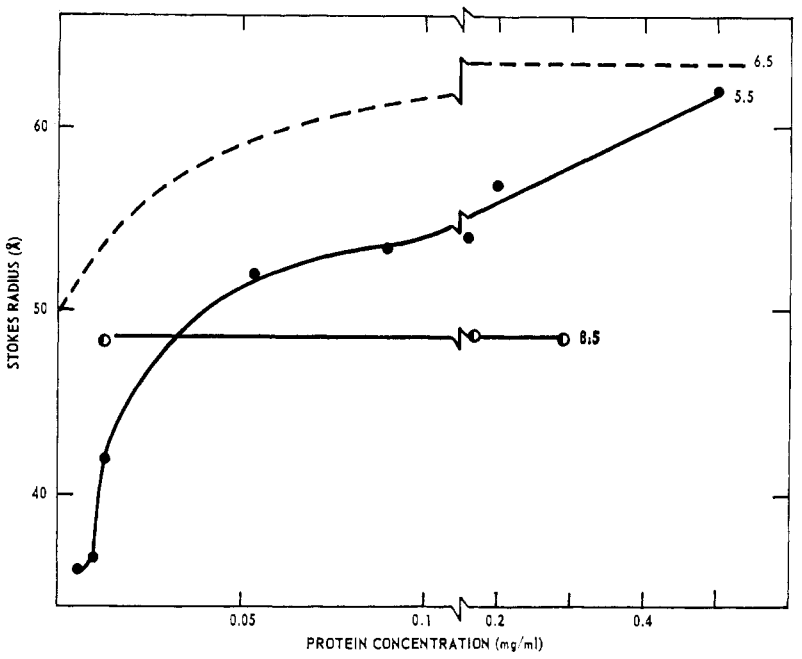

FIGURE 6: Effect of pH on the concentration-dependent dissociation of phosphorylase *a*. The enzyme was subjected to gel filtration on 2.5 × 41 cm Sephadex G-200 columns at the indicated pH values. Other experimental conditions including buffer components (β-glycerophosphate–Tris–EDTA), temperature (30°), and ionic strength (0.07) were held constant. The dashed line represents the dissociation curve for phosphorylase *a* from Figure 2 redrawn here for reference.

phosphorylase *a*. At any one protein concentration, phosphorylase *a* was always more dissociated in the presence of AMP than in its absence (compare with Figure 2). As before, the dissociation was characterized by the appearance of a single asymmetric peak.

The effect of AMP on the enzymatic activities of phosphorylases *a* and *b* as a function of enzyme concentration is also shown in Figure 4. Below 0.2 mg/ml, the specific activity of phosphorylase *b* remained constant and maximal as did its size on gel filtration. Above this concentration, the activity decreased slightly; but aggregation of phosphorylase *b* was not apparent in this experiment. Other gel filtration data (see data at pH 5.5) have shown that at high protein concentrations in the presence of AMP, phosphorylase *b* does associate. Furthermore, ultracentrifugation experiments indicated that phosphorylase *b* was aggregated under similar conditions.

The activity of phosphorylase *a* increased as the enzyme concentration decreased. At any one enzyme concentration the activity of phosphorylase *a* was significantly higher in the presence of AMP than in its absence (compare Figures 2 and 4).

The specificity of the AMP effect on the dissociation of phosphorylase *a* was tested by gel filtration in the presence of 1 mM ATP. Morgan and Parmeggiani (1964) have shown that at this concentration ATP has no effect on the enzymatic activity of phosphorylase *a*. Consistent with this observation, we found that ATP had no effect on the dissociation of the enzyme. At protein concentrations below 0.2 mg/ml, gel filtration of phosphorylase *a* resulted in a dissociation profile superimposable on that shown in Figure 2. This was interpreted as evidence that the AMP effect was specific.

On the other hand, ATP is a competitive inhibitor of phosphorylase *b* activity (Morgan and Parmeggiani, 1964) and ATP was found to effect the size of this form of the enzyme (DeVincenzi, 1968). At protein concentrations above 0.1 mg/ml, the Stokes radius of *b* was normal at 50 Å. Below this concentration phosphorylase *b* began to dissociate and reached 43 Å at a concentration of 0.015 mg/ml. The significance of this ATP-induced dissociation of phosphorylase *b* dimer toward monomer is not clear.

The dissociation behavior of phosphorylase observed using gel filtration was verified by ultracentrifugation. Sedimentation coefficients were obtained over the range of 0.1–7.0 mg/ml. Neither enzyme showed any concentration-dependent sedimentation over this range and $s_{20,w}$ values of 8.4 ± 0.25 S for phosphorylase *b* and 13.5 ± 0.35 S for phosphorylase *a* were obtained (DeVincenzi and Hedrick, 1967). However, when 1 mM AMP was present, phosphorylase *a* exhibited concentration-dependent sedimentation (Figure 5). At protein concentrations less than 0.6 mg/ml, phosphorylase *a* was significantly dissociated. Due to limitations of the optical system available to us, we were unable to work at protein concentrations less than 0.1 mg/ml. However, in the concentration range where the Sephadex and ultracentrifugation experiments overlapped, the results correlated very well. Furthermore, with both techniques only one asymmetric component was detectable, indicative of a rapid equilibrium (rapid relative to the separation time of the components involved) between dimer and tetramer species.

Effect of pH on Dissociation and Activity of Phosphorylase a. Variations in the Stokes radius of phosphorylase *a* as a function of protein concentration at three pH values are summarized in Figure 6. In contrast to the results at pH 6.5, phosphorylase *a* at pH 8.5 existed as a dimer independent of protein concentration. This correlates with the results of disc gel electrophoresis of phosphorylase *a* at pH 8.5 where it has been shown that the enzyme migrates exclusively as dimer (Hedrick *et al.*, 1969b).

At pH 5.5, on the other hand, three aggregated forms of phosphorylase *a* (tetramer, dimer, and monomer) were present depending upon the protein concentration. Assuming that the monomer had a molecular weight of 90,000 and

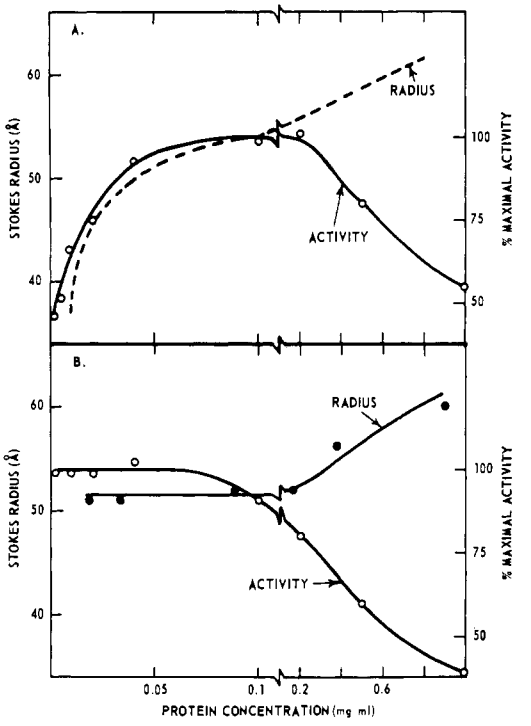

FIGURE 7: Effect of AMP on molecular size and enzymatic activity of phosphorylase *a* at pH 5.5. (A) The specific activity of phosphorylase *a* in the absence of AMP (○) was measured as a function of protein concentration at pH 5.5, 30° and μ = 0.18. The dashed line represents the dissociation curve for phosphorylase *a* at pH 5.5 from Figure 6 redrawn here for reference. (B) The dissociation behavior of phosphorylase *a* at pH 5.5 (●) was determined as in Figure 6, except that 1 mM AMP was included in the elution buffer. The specific activity of phosphorylase *a* (○) was determined as in A, except that 1 mM AMP was added to the substrate.

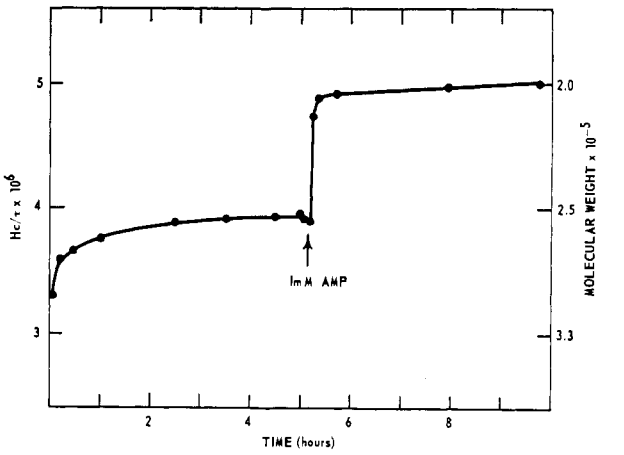

FIGURE 8: Effect of AMP on the dissociation of phosphorylase *a* as measured by light scattering. Dilution due to the addition of AMP was negligible as the volume added was <1% of the cell volume. Turbidities and molecular weights were calculated as described in Experimental Procedure. As a first approximation, *Hc/T* was identified with 1/molecular weight. This neglects the contribution of the term 2*Bc* which, however, should be small under these experimental conditions (Edsall *et al.*, 1950).

an $s_{20,w}$ of 5.6 S (Madsen and Cori, 1956), the Stokes radius of monomer was calculated to be 37 Å. The data in Figure 6 can be interpreted as indicating that phosphorylase *a* dissociated to monomer at low protein concentrations.

In view of the effect of low pH on the structure of phosphorylase *a*, we examined the effect of AMP on dissociation and correlated enzymatic activity with molecular size at this pH (Figure 7). In the absence of AMP (Figure 7A), phosphorylase *a* dissociated to dimer with decreasing protein concentration. Complete dissociation to monomer occurred at low concentrations. In the presence of AMP (Figure 7B), the pH-induced dissociation to monomer was prevented and the dimer form was stabilized. Again, there was a correlation between molecular size and enzymatic activity. In the absence of AMP (Figure 7A), activity increased as the tetramer form dissociated, plateaued at maximum activity as the dimer form predominated, and finally decreased again as phosphorylase *a* dimer dissociated to monomers. In the presence of AMP (Figure 7B), activity increased as tetramer dissociated to dimers, and then leveled off at maximum activity in the region of protein concentration where AMP stabilized the dimer form and prevented its dissociation to monomers.

The concentration-dependent dissociation of phosphorylase *a* was eliminated at pH 8.5 (in the absence of AMP), and the enzyme existed as dimer at all concentrations tested (Figure 6). In a similar manner, the specific activity of phosphorylase *a* in the absence of AMP at pH 8.5 showed no concentration dependence and remained maximal over the concentration range tested (DeVincenzi, 1968).

In the absence of AMP, the Stokes radius of phosphorylase *b* showed no concentration dependence and remained constant at about 49 Å at all three pH values. When phosphorylase *b* was chromatographed at pH 5.5 in the presence of 1 mM AMP the enzyme showed partial aggregation at high protein concentrations (Stokes radius reached 55 Å at 1 mg/ml, DeVincenzi, 1968).

Dissociation of Phosphorylase a as Characterized by Light Scattering. Dissociation of phosphorylase *a* tetramer to dimer can be represented as

$$T \underset{k_2}{\overset{k_1}{\rightleftharpoons}} 2D$$

$K_{equil} = [D]^2/[T]$, where T represents the tetramer and D the dimer form of the enzyme. The apparent AMP-induced increase as detected by gel filtration of phosphorylase *a* dimer could be due to a change of the equilibrium between tetramer and dimer (effect on K_{equil}) or to a change in the rate at which the equilibrium was attained (compensating changes in k_1 and k_2 which would allow a more rapid attainment of equilibrium but not change the value of the equilibrium constant). Thus either situation could cause a retardation of the enzyme on Sephadex. Light-scattering experiments were performed to distinguish between these two possibilities.

The conditions used were analogous to those in the gel filtration and ultracentrifugation studies. The scattering ratio (ratio of light scattered at 90° to that of the transmitted light at 0°) was initially measured as a function of time after dilution of phosphorylase *a* to 0.22 mg/ml. As shown in Figure 8, dilution resulted in an initial rapid dissociation of phosphorylase *a*. The half-life of this process is in the range of tens of seconds to minutes. Experimental limitations precluded an accurate estimate of the rate. Equilibrium was

TABLE I: Effect of Substrates and Products on Stokes Radii of Phosphorylases *a* and *b*.[a]

Expt	Phosphorylase	Bacteriological Dextrin	Maltodextrin	AMP	Stokes Radius (Å)
1	*b*	+		+	56
	a	+		+	57
2	*b*		+	+	56
	a		+	+	56
3	*b*			+	59
	a			+	60
4	*b*			+	56
	a			+	66
5	*b*				49
	a				68

[a] Phosphorylases *a* and *b* were chromatographed on 2.5 × 41 cm Sephadex G-200 columns in the presence of the substrate buffer. In all experiments, buffers contained 41 mM P_i, 5.4 mM glucose-1-P, 1 mM EDTA, 30 mM β-glycerophosphate, and were adjusted to pH 6.5 with Tris. Carbohydrate, when added, was 0.1%. AMP, when added, was 1 mM. Protein concentrations ranged from 0.02 to 0.2 mg per ml.

TABLE II: Stokes Radii of $NaBH_4$-Reduced and Apophosphorylase *b* as a Function of Protein Concentration and Temperature.[a]

Temp (°C)	Enzyme	Protein Concn (mg/ml)	Stokes Radius (Å)
30	Reduced phosphorylase *b*	0.18	50.0
		0.03	48.8
		0.01	42.7
	Apophosphorylase *b*	0.06	36.0
		0.01	35.8
15	Reduced phosphorylase *b*	0.15	48.5
		0.01	46.6
	Apophosphorylase *b*	0.02	44.6, 31.4
		0.006	48.3, 32.7

[a] $NaBH_4$-reduced and apophosphorylase *b* were chromatographed on 2.5 × 41 cm Sephadex G-200 columns in 68 mM β-glycerophosphate–Tris–EDTA, pH 6.5, $\mu = 0.23$. Stokes radii were calculated as in Figure 2.

attained at a weight-average molecular weight of 254,000 g/mole. Thus, under the conditions used here, equilibrium in the absence of AMP was attained rapidly and the equilibrium position was such that both dimers and tetramers were present. This result agrees with the gel filtration and ultracentrifugation experiments.

Addition of AMP to the equilibrated solution caused an almost instantaneous further dissociation of the enzyme and a new equilibrium was reached with a number-average molecular weight of 201,000 g/mole. The rate of attainment of the equilibrium appeared to be faster in the presence of AMP than in its absence; but again, experimental limitations precluded an accurate estimate of the rate. Thus, in this experiment, AMP affected the rate at which equilibrium was attained and also resulted in displacement of the equilibrium toward the dimer.

Effect of Substrates on the State of Aggregation of Phosphorylases a and b. In addition to the effects of protein concentration, AMP, etc., it was desirable to determine what effect the combined substrates and products of the enzymatic reaction might have on the quaternary structure of the enzymes as determined by gel filtration. A workable combination of assay components to use in the elution buffer for this experiment was an equilibrium mixture of substrates and products. Although there would be no net enzymatic reaction, the enzyme would be in an active conformation in the presence of all the components necessary for activity. Experimental conditions were chosen so as to minimize synthesis of high molecular weight polysaccharides which could affect the observed size of the enzyme. Bacteriological dextrin and low molecular weight maltodextrin were used as the polysaccharide primers in these gel filtration experiments instead of macromolecular glycogen.

The results of gel filtration of phosphorylases *a* and *b* in the presence of substrates are shown in Table I. In expt 1, the Stokes radii of both enzymes in the range of assay protein concentrations (0.015 mg/ml) corresponded to those expected for a trimeric species. Also, the eluted peaks were nearly symmetrical.

Experiments 2 and 3 indicated that the observed Stokes radii did not reflect changes in molecular size due to carbohydrate binding. The Stokes radii corresponded to trimer in the presence and absence of low molecular weight maltodextrin. Furthermore, the sedimentation coefficient for phosphorylase *b* in the presence of only bacteriological dextrin (no other substrates present) was nearly identical with that in its absence.

That these results were not simply an artifact of the Sephadex polysaccharide gel itself binding the enzyme was shown by expt 4. The high Stokes radii were also obtained in the presence of substrates on Bio-Gel P-300, a polyacrylamide gel.

This effect of combined substrates and products on the size of phosphorylase was also investigated by light scattering. In the presence of buffers identical with expt 3 and 4, the molecular weight of phosphorylase *b* was 20% higher than in the absence of substrates.

The results of expt 5 indicate that AMP, in combination with glucose-1-P and P_i, was important for the aggregation process resulting in Stokes radii intermediate between dimer and tetramer. In the presence of glucose-1-P and P_i, phosphorylase *b* exhibited a normal dimer Stokes radius of 49 Å. The value for phosphorylase *a* was likewise near the Stokes radius for tetramer.

Similar results were obtained from ultracentrifugation studies. In the presence of glucose-1-P and P_i, phosphorylase *b* had a sedimentation coefficient of 7.8. When AMP was added, the sedimentation coefficient increased to 10.5 which corresponds to that expected for a trimer. In this case, only

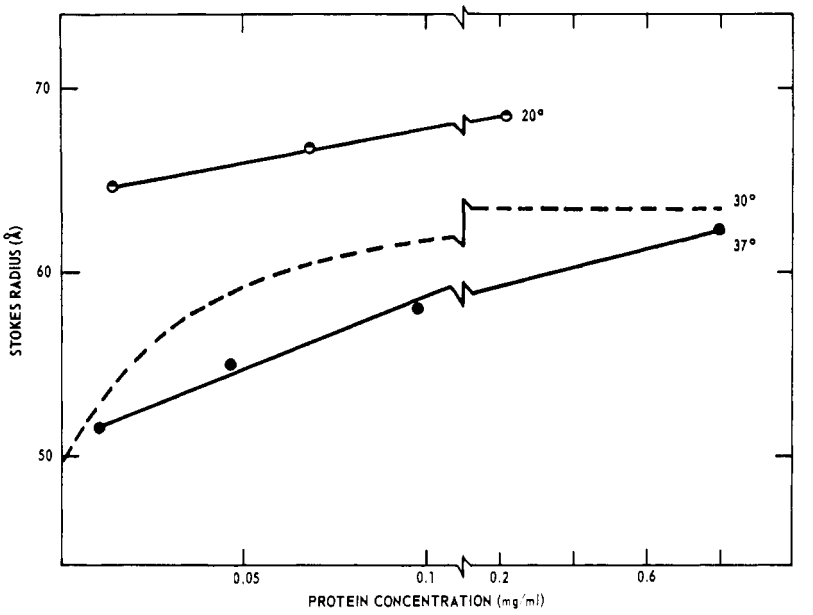

FIGURE 9: Effect of temperature on the concentration-dependent dissociation of phosphorylase *a*. The enzyme was chromatographed (pH 6.5 and μ = 0.23) on 2.5 × 41 Sephadex G-200 columns. Temperatures were maintained in the jacketed columns by means of a circulating water pump. The dashed line represents the dissociation curve for phosphorylase *a* at 30° from Figure 2 redrawn here for reference.

one symmetrical peak was observed which was suggestive of a rapid equilibrium between different polymeric forms.

Association–Dissociation Behavior of $NaBH_4$-Reduced and Apophosphorylase b. On the basis of ultracentrifugation studies, apophosphorylase *b* possesses a very fragile quaternary structure easily modified by changes in its environment (*e.g.*, pH, ionic strength, and temperature). For instance, by varying only the parameter of temperature with all other experimental conditions held constant, the apoenzyme exists as a monomer at 35°, predominately a dimer at 23°, and a mixture of dimers and tetramers plus higher aggregates at 1° (Hedrick *et al.*, 1966). Similar temperature-dependent association–dissociation behavior was observed when apophosphorylase *b* was chromatographed on Sephadex G-200 (Table II). At 30°, the enzyme was completely dissociated to monomers and its elution profile was symmetrical. At 15°, the apoenzyme monomers associated and two clearly resolved peaks corresponding to monomer and dimer were evident. Thus, the dissociation of apophosphorylase *b* was apparently a relatively slow process as two distinct symmetrical peaks were observed. The quaternary structure of native phosphorylase *b* exhibits no such temperature dependency. These data confirm the involvement of pyridoxal 5′-phosphate in ordering the quaternary structure of glycogen phosphorylase.

$NaBH_4$-reduced phosphorylase *b* has previously been shown to have essentially the same physicochemical characteristics as does phosphorylase *b* (Strausbach *et al.*, 1967). However, gel filtration experiments suggest that reduction of pyridoxal 5′-phosphate in phosphorylase affects the association–dissociation properties of the enzyme (Table II). At 30° and high protein concentrations the reduced enzyme was a dimer like native phosphorylase *b;* however, unlike native phosphorylase *b*, the elution profile of the reduced enzyme was asymmetric toward a smaller molecular weight species. As the protein concentration was decreased, the dimer partially dissociated to monomers. Qualitatively, this behavior was similar to the dissociation of phosphorylase *a* tetramer to dimer. In both instances, dissociation was relatively rapid since only one asymmetric peak was observed.

Effect of Temperature and Ionic Strength on Dissociation and Activity. The dissociation of phosphorylase *a* was temperature dependent (Figure 9); temperature increases promoted dissociation of the tetramer to dimer. Changes in enzymatic activity also paralleled the extent of dissociation (DeVincenzi, 1968). Thus, at any given protein concentration, phosphorylase *a* was more dissociated and had a higher specific activity at elevated temperatures. The Stokes radius observed for phosphorylase *b*, however, was independent of the temperature used.

Over the ionic strength range of 0.07–0.23 (assay ionic strength), the dissociation behavior of phosphorylase *a* did not vary from that shown in Figure 2. Phosphorylase *b* was also unaffected by changes in ionic strength and its gel filtration behavior remained protein concentration independent (Stokes radius of 49 Å, DeVincenzi, 1968).

Discussion

The results presented here demonstrate that there is a difference in the nature of the forces holding phosphorylase monomeric units together to form a dimer and dimeric units together to form a tetramer. For convenience, these forces will be referred to as "dimeric" and "tetrameric bonds," respectively. Table III summarizes the effects of different environmental factors on the quaternary structure of phosphorylase.

It is to be anticipated that any reversible aggregating system would show a dependence on concentration of the reacting species due to mass action effects. As indicated in Figure 2, dissociation occurs in the concentration range of 0–0.2 mg/ml. Chignell *et al.* (1968) have stated that dissociation occurs in the concentration range of 0–3.0 mg/ml; however, they have not made precise measurements of the dissociation process.

The allosteric effector AMP affected both the dimeric

TABLE III: Summary of Effects of Environmental Parameters on the Quaternary Structure of Phosphorylase.[a]

Bonds Affected	Dimeric	Tetrameric
Protein concentration	Dissociation[b]	Dissociation
AMP	Association	Dissociation[c]
ATP	Dissociation	No effect
pH 8.5	No effect	Dissociation
pH 5.5	Dissociation	Dissociation
Temperature	Dissociation[d]	Association[e]
PLP reduction	Dissociation	
PLP removal	Dissociation	
Substrates	No effect	Dissociation

[a] Only the conditions studied in this report are considered in this tabulation. Effects on dimeric bonds are from phosphorylase *b* measurements except as a function of protein and pH 5.5. Effects on tetrameric bonds are from phosphorylase *a* measurements except as a function of protein and AMP. [b] The combination of low protein concentration and pH 5.5 caused rupture of dimeric phosphorylase *a* bonds. [c] AMP caused association of phosphorylase *b* dimers. [d] Low temperatures affected dimeric bonds (dissociation) in reduced and apophosphorylase *b*. [e] Low temperatures affected tetrameric bonds (association) of phosphorylase *a*.

and tetrameric bonds. In the case of the dimeric bonds of phosphorylase *a*, it tended to increase the association of monomers to dimer. In the case of the tetrameric bonds, AMP decreased the association of dimers (Wang and Graves, 1964). In the case of phosphorylase *b*, AMP strengthened both the dimeric and tetrameric bonds by preventing dissociation of the dimer to monomers at pH 5.5 and by promoting the association of dimers to tetramer at neutral pH and high enzyme concentrations. There are at least two mechanisms that can be proposed to explain the AMP effect: (1) an AMP induced conformational change which affects the affinity of the subunits for one another, (2) displacement of the equilibrium caused by the binding of AMP to only a single aggregated state. These two options are essentially restatements of the two currently leading hypotheses to explain allosteric transformations (Monod *et al.*, 1965; Haber and Koshland, 1967). From the data presented here, it is not possible to differentiate between these two concepts. Attempts have been made by others to fit phosphorylase to the Monod *et al.* hypothesis (Metzger *et al.*, 1967; Helmreich *et al.*, 1967; Kastenschmidt *et al.*, 1968). However, as clearly stated by these authors, the results obtained "are not sufficiently discriminatory to exclude other possible explanations for the allosteric properties of phosphorylase." Indeed, results of Wang and Black (1968) on the antagonistic effects of AMP and glucose on phosphorylase *a* activity are best explained by assuming the "induced-fit" model of Haber and Koshland (1967). Since "model fitting" of the allosteric properties of phosphorylase clearly has serious interpretational limitations, we have not attempted it here.

Helmreich *et al.* (1967) have stated that AMP promotes the association of phosphorylase *a* dimers to tetramer in the absence of the substrate glycogen; this conclusion was based on kinetic evidence. Chignell *et al.* (1968) have also stated that AMP promotes association of phosphorylase dimers to tetramer; their conclusions were based on ultracentrifugation experiments at relatively high (milligram per milliliter) enzyme concentrations. Our results disagree with these two reports. As clearly shown here by Sephadex, ultracentrifugation, and light-scattering experiments AMP promotes the dissociation of phosphorylase *a* tetramer to dimers. This process is rapid ($t_{1/2} \cong$ tens of seconds) as shown by light scattering, and affects both the rate at which equilibrium is attained as well as the equilibrium constant.

The effect of ATP on the state of aggregation was to cause dissociation of phosphorylase *b* dimer to monomers. That the effect is specific for dimers of phosphorylase *b* but not *a* is consistent with the ATP inhibition of phosphorylase *b* but not *a*. Whether or not dissociation is actually responsible for inhibition remains to be shown. Again, in terms of the mechanism of dissociation, the same possibilities exist as described for AMP.

The effect of pH on the gel filtration behavior of phosphorylase *a* suggests that electrostatic interactions may be involved in the dimeric and tetrameric bonds. A number of reports have implicated the involvement of ionic interactions between the dimer and tetramer forms (Wang and Graves, 1964; Huang and Madsen, 1966; Sealock and Graves, 1967; Graves *et al.*, 1968; Wang *et al.*, 1968). The observation that the dimer form of phosphorylase *a* predominates at high pH shows that the prediction of Wang and Graves (1964) based on enzyme kinetics is incorrect. They stated that: "The lack of dependence of activity with protein concentration at pH 8.0 suggests that under these conditions only the tetramer form of the enzyme is present."

Gel filtration of phosphorylase *a* as a function of pH also indicates that the forces involved in keeping monomer units associated as dimers and dimer units as tetramers differ in nature and/or stability. The combination of very low protein concentration and low pH resulted in rupture of the dimeric bonds. These experiments also indicate that dimeric bonds of phosphorylase *a* differ from those of phosphorylase *b*. Phosphorylation of the protein alters this attraction such that phosphorylase *a* dimer can be dissociated to monomers at low pH and low protein concentration. Under these same conditions, phosphorylase *b* dimer showed no tendency to dissociate. It is of course knowledge of long standing that phosphorylases *a* and *b* differ predominately in their protein conformation as reflected by a difference in various physical properties (*e.g.*, solubility, $s_{20,w}$, and optical rototary dispersion) since the only chemical difference between the two is the presence of two additional phosphate groups in phosphorylase *a*.

Dissociation at low temperatures seems best interpreted as an effect on the hydrophobic forces holding the subunits together (Nemethy and Scheraga, 1962). This interpretation agrees with that suggested by Graves *et al.* (1965) to explain the cold sensitivity of phosphorylase *b* and with the effect of temperature on the aggregation of apophosphorylase *b* (Hedrick *et al.*, 1966).

Previous work on the role of pyridoxal 5′-phosphate in phosphorylase indicated that this cofactor has a function different from other classical pyridoxal 5′-phosphate-enzymes in that it had predominately a structural rather than

a catalytic role (Illingworth *et al.*, 1958; Hedrick and Fischer, 1965; Hedrick *et al.*, 1969b). Removal of this cofactor from phosphorylase *b* caused dramatic changes in the physical structure of the enzyme. These results have been confirmed by the observations reported here using gel filtration.

The effect of pyridoxal 5′-phosphate reduction onto phosphorylase *b* has heretofore not resulted in any detectable changes in the enzyme's hydrodynamic characteristics (Strausbach *et al.*, 1967). As shown here, reduction affects the dimeric bonds of the enzyme as indicated by its dissociation to monomers under conditions where the native enzyme did not dissociate. These results further emphasize the structural role of pyridoxal 5′-phosphate in phosphorylase and show that reduction as well as complete removal of the cofactor causes alterations in the quaternary structural forces of the enzyme.

The effects of substrates on the quaternary structure of phosphorylase are complex. The results obtained here indicate that the tetrameric bonds are affected. The gel filtration and ultracentrifugation experiments seemed best interpreted as a rapid equilibrium between dimers and tetramers rather than the presence of a trimer. Throughout this work a stable trimer has never been obtained; only one group has suggested that a phosphorylase trimer exists (Chignell *et al.*, 1968). However, the trimer was obtained after chemically modifying the enzyme. The presence of a single peak observed for both phosphorylases *a* and *b* in the presence of all substrates may represent the presence of nearly equal quantities of dimers and tetramers in rapid equilibrium. This would contrast to other conditions (temperature, pH, AMP, and cofactor) which shifted the equilibrium to extreme positions. This unifying interpretation is consistent with the experimental results but is not the sole interpretation possible.

Changes in the enzymatic activity of both phosphorylases *a* and *b* could be directly correlated with the enzymes' state of aggregation. Any change in environmental conditions which increased the presence of the dimer also increased the activity of the enzyme. The dimer is the most active form of the enzyme and the form which predominates under the conditions of assay. We have found no evidence indicating that the monomer form of either phosphorylase *a* or phosphorylase *b* has any enzymatic activity. However, the tetramer form of phosphorylase *a* does possess appreciable activity (20% of dimer, Figure 3). Our results agree with the suggestion of Wang and Graves (1963) that the tetramer is active and disagrees with that of Metzger *et al.* (1967) that the tetramer is inactive.

Attempts to explain the *in vivo* functioning and control of glycogen phosphorylase from *in vitro* studies are becoming more difficult as our knowledge about the enzyme increases. The aggregated state of the enzyme is intimately associated with its enzymatic characteristics and, as shown here, factors such as protein concentration, pH, temperature, activator, inhibitor, pyridoxal 5′-phosphate, and substrates all affect the aggregated state. Precise measurements of these conditions as they exist *in vivo* and the *in vivo* variation of these parameters have in many instances not been carried out. Thus, much data have yet to be collected in order to define the *in vivo* functioning of phosphorylase, but it becomes more apparent that as the available information increases, so does the complexity of the problem.

Acknowledgments

We are grateful to Drs. R. J. Feeney, R. S. Criddle, and J. R. Whitaker for their helpful discussions and to Mrs. J. Tweedie and Mr. A. J. Smith for their excellent technical assistance in some aspects of this work.

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Mg^{2+}–Ca^{2+}-Activated Adenosine Triphosphatase System Isolated from Mammalian Brain*

Soll Berl and Saul Puszkin

ABSTRACT: A Mg^{2+}- or Ca^{2+}-activated adenosine triphosphatase (ATPase) has been isolated from whole brain of rat or cat. Its properties are similar, in many respects, to those of muscle actomyosin. Additional studies are presented which further characterize this protein. The Mg^{2+}-activated ATPase activity of the brain protein was dependent upon the relative concentrations of adenosine triphosphate (ATP) and Mg^{2+}. When the ATP concentration exceeded that of the Mg^{2+} the enzyme activity was inhibited. Polyethylenesulfonate also inhibited the Mg^{2+}-activated ATPase activity of the brain protein at concentrations effective with muscle actomyosin. Antisera prepared against the brain proteins showed single immunodiffusion bands against the specific antigen. There was no cross-reaction between rat and cat antigen and their respective antiserum. However, antiserum prepared against cat muscle actomyosin did cross-react with the cat brain protein but not with the rat brain protein. Proteins with actin-like and myosin-like properties have been isolated from whole bovine brain. Mixtures with each other or their counterparts from muscle resulted in a marked stimulation of the Mg^{2+}–ATPase activity of the myosin and myosin-like proteins. Such mixtures also showed increased relative viscosities which fell sharply upon the addition of ATP and rose again over periods of 30–60 min. Disc electrophoresis on acrylamide gel with and without urea revealed distinct differences among brain actomyosin-like protein, striated muscle actomyosin, and vascular actomyosin, as well as among brain actin-like protein, striated muscle actin, and vascular actin.

The brain actin-like protein contained bound nucleotide which exchanged with free [^{14}C]ATP in a fashion similar to muscle actin. For the brain actomyosin-like protein we have suggested the name, neurostenin, for the actin-like protein, neurin, and for the myosin-like protein, stenin.

The isolation of a Mg^{2+}- or Ca^{2+}-activated adenosine triphosphatase from whole brains of the rat and cat was recently described (Puszkin *et al.*, 1968). The enzyme presented properties similar to that of muscle actomyosin. This was shown in relation to its method of preparation, solubility, activation, and inhibition of ATPase activity and ability to demonstrate the phenomenon of superprecipitation in the presence of Mg^{2+} and ATP. The present communication compares further the brain protein with the muscle protein.

Perry and Grey (1956) had shown that the ATPase activity of myofibrils was dependent upon the concentration of the ATP and the Mg^{2+}. When the concentration of the former exceeds that of the latter, the enzyme activity of the myofibrils was inhibited. Similar experiments were attempted with the proteins isolated from cat and rat brain. Bárány and Jaisle (1960) reported that upon incubation of actomyosin with polyethylenesulfonate and low concentrations of ATP and Mg^{2+} the ATPase activity of the actomyosin decreased. They were able to demonstrate that this effect was due to splitting of the actomyosin into actin and myosin and they called the polyethylenesulfonate an "interaction inhibitor." The effect of polyethylenesulfonate on rat and cat brain protein was therefore studied. In immunological studies, antisera were prepared against these proteins and their purity and cross-reactivity assayed by immunodiffusion technique.

Furthermore, proteins were isolated from whole bovine brain which combined with each other and with their counterparts isolated from cat striated muscle. These interactions suggested actin-like and myosin-like properties. The actin-like protein contained bound nucleotide and its exchange with free [^{14}C]ATP was compared with that of cat muscle actin. Purity and electrophoretic mobility of the actin-like

* From the Department of Neurology and Parkinson's Disease Information and Research Center, College of Physicians and Surgeons, Columbia University, New York, N. Y. 10032. *Received August 1, 1969.* This work was supported in part by Public Health Service Grants NB-04064 and Research Career Program Award 5-K3-NB-5117 (S. B.) from the National Institute for Neurological Diseases and Blindness and by the Clinical Research Center for Parkinson's and Allied Diseases, NB 05184 and The Parkinson's Information Center, a part of the National Information Network of NINDB under Contract No. NIH 69-76.