Correlation between Subunit Interactions and Enzymatic Activity of Phosphorylase *a*. Method for Determining Equilibrium Constants from Initial Rate Measurements*

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ABSTRACT: Light-scattering measurements of molecular weight at different protein concentrations at 25 and 30° provided direct evidence for the concentration-dependent dissociation of phosphorylase *a*. The molecular weight of tetrameric phosphorylase *a* was estimated to be 380,000. Methods for evaluating the equilibrium constant and specific activities of the dimeric and tetrameric species from initial rate measurements were developed. It was found that the increase in specific activity upon dilution can be quantitatively accounted for by a decrease in molecular weight. Standard enthalpy and entropy changes of the dissociation reaction at pH 6.8 were calculated to be 60 kcal/mole and 170 eu, respectively. The concept of a catalytically more active dimer and a less active tetramer was reinforced by the findings that the dimeric form has a lower activation energy of 11.3 kcal and a greater affinity for glycogen, whereas the tetrameric form has a higher activation energy of 22.6 kcal and a lower affinity for glycogen. The hypothesis of inactive tetramer was also examined. When tetramer was assumed inactive, the change in specific activity no longer correlated with the change in molecular weight. The protein concentration-dependence of the apparent Michaelis constant for glycogen also indicated that tetrameric phosphorylase *a* is capable of catalysis. A more general treatment for determining equilibrium constants and kinetic parameters from activity measurements for an enzyme undergoing a one-step dissociation–association reaction is presented in the Appendix. This method permits the study of dissociation at low protein concentrations which are inaccessible to ordinary means of molecular weight measurements, provided the two states of enzyme aggregation differ in their catalytic activity and/or ligand binding affinity.

Rabbit skeletal muscle glycogen phosphorylase *a* (EC 2.4.1.1), molecular weight 367,000–380,000 (Oncley, 1943; Seery *et al.*, 1967; DeVincenzi and Hedrick, 1967; also see Results), is generally designated as a tetramer because treatment of this enzyme with *p*-hydroxymercuribenzoate resulted in four subunits (Madsen and Cori, 1956). In addition, adenosine 5′-monophosphate (AMP) binding studies revealed four equivalent sites per enzyme molecule (Madsen and Cori, 1957; DeVincenzi and Hedrick, 1967; Helmreich *et al.*, 1967). For some time it was thought that the active form of phosphorylase *a* was the tetramer. Wang and Graves (1964) found that phosphorylase *a* can exist in two states of aggregation: a catalytically more active dimer and a less active tetramer. In 2.8 M NaCl (pH 7.0), phosphorylase *a* was transformed into a dimeric species (Wang and Graves, 1963). Rapid assay following dilution of this sample yielded high initial enzymatic activity that decayed to the activity of samples not incubated in NaCl (Wang and Graves, 1964). The decay conformed to second-order kinetics, indicating that the enzymatically more active species is the dissociated dimer. Further support for the existence of a more active dimeric form was obtained from studies of activation of phosphorylase *a* by α-1,4-glucosyl compounds. Incubation of this enzyme with glucose resulted in a species of high enzymatic activity, which sediments as a dimer (Wang *et al.*, 1965a). Preincubation with glycogen and hydrolyzed amylose also generated the more active species (Wang *et al.*, 1965b). It was also shown (Wang and Graves, 1964) that the two subunit forms are in equilibrium with each other since decreasing protein concentration is accompanied by increasing specific activity, by consideration of the law of mass action, dilution would favor the formation of the dissociated component. Metzger *et al.* (1967) also found that the dimeric species is more active when assayed in the direction of glycogen degradation by way of arsenolysis and that in the presence of amyloheptaose the enzyme sediments as a dimer. From centrifugal experiments with phytoglycogen at 15°, they also concluded that the tetrameric form of phosphorylase *a* cannot bind to glycogen and is, therefore, catalytically inactive with glycogen as substrate.

Our report is concerned with several aspects of the dissociation–association of phosphorylase *a:* (1) to obtain physical evidence for the concentration-dependent dissociation–association of this enzyme and to correlate enzymatic activity with the state of subunit aggregation; and (2) to examine further the model of dynamic equilibrium between a more active dimer and a less active tetramer. Methods for evaluating

* From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50010. *Received June 20, 1969.* Journal Paper J-6154 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Project No. 1510. This research was supported by a research grant (GM-09587) from the National Institutes of Health, U. S. Public Health Service. Part of this investigation has been presented at the 154th National Meeting of the American Chemical Society, Chicago (Huang and Graves, 1967), and at the 4th Federation of European Biochemical Societies, Symposium on the Control of Glycogen Metabolism (Graves *et al.*, 1968).

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‡ Recipient of a Research Career Development Award of the U. S. Public Health Service (Grant GM6753).

equilibrium constants for enzymes undergoing one-step dissociation–association from activity measurements are also presented.

Experimental Section

Materials. Phosphorylase *a* from rabbit skeletal muscle was prepared from crystalline phosphorylase *b* by using phosphorylase *b* kinase as described by Fisher and Krebs (1962). Phosphorylase *b* was prepared according to the procedure of Fischer and Krebs (1958) except that dithiothreitol was used in place of cysteine throughout the preparation. The enzyme used was recrystallized at least four times and freed of AMP by treatment with acid-washed Norit A. Enzyme stock solutions were dialyzed against the buffer to be used for at least 24 hr at 5°. Enzymatic activity was assayed before each experiment to ensure that the enzyme was active.

Shellfish glycogen and glucose 1-phosphate were purchased from Sigma Chemical Co. Glycogen was made AMP free according to the procedure of Sutherland and Wosilait (1956). Frozen rabbit muscle was purchased from Pel-Freez Biologicals. Glycerophosphate[1] was obtained from either Calbiochem or Sigma Chemical Co., dithiothreitol from Calbiochem, and AMP from Pabst Laboratories. Ludox was the product of E. I. du Pont. All other chemicals were reagent grade.

Methods. Molecular weight measurements were performed with a Sofica light-scattering photometer (Wippler and Scheibling, 1954) equipped with a thermostated benzene vat. The instrument was calibrated with Ludox twice, once according to the modified method of Goring *et al.* (1957). It was found that, when pure benzene was used as reference, a correction factor of (refractive index of solvent/refractive index of benzene)^2 for excess scattering volume as derived by Hermans and Levinson (1951) was essentially correct. Rayleigh ratios for benzene at 546 nm used were 15.7×10^{-6} cm^{-1} at 20° (*cf.* Kratohvil *et al.*, 1962), 16.3×10^{-6} cm^{-1} at 25°, and 16.9×10^{-6} cm^{-1} at 30° (Carr and Zimm, 1950). Specific refractive increments (dn/dc) were measured by a Brice–Phoenix differential refractometer, Model BP-2000-V, equipped with temperature control. The instrument was calibrated with KCl solutions prepared from samples dried in a vacuum oven at 110° over Drierite. The data of Kruis (1936) were used to calculate the correction factor. The refractive index of water was calculated from the International Critical Tables. The specific refractive index of phosphorylase *a* in 40 mM glycero-*P*, 1 mM dithiothreitol, and 0.5% NaCl (pH 6.8), at 546 nm, 25°, is 0.173 ml/g. Phosphorylase *a* concentrations were measured by absorbance at 280 nm using an absorbance index of 1.27, for 1 mg/ml at pH 6.8, determined by dry weight (Huang, 1968). Weight-average molecular weights were calculated according to the equation given by Townend and Timasheff (1960) for systems undergoing one-step dissociation–association

$$\frac{Kc}{R_{90°}} = \frac{1}{\bar{M}_W} + \frac{2Bc}{M_D} = \frac{1}{M_{app}}$$

where $K = 2\pi^2 n_0^2 (dn/dc)^2 / N\lambda^4$; n_0 is refractive index of solvent; N is Avogadro's number, λ is the wavelength of incident beam *in vacuo;* c is the protein concentration; $R_{90°}$ is the Rayleigh ratio measured at an angle of 90° to the incident beam and is equal to $(I_{solution} - I_{solvent})(R_B/I_B) \times (n_0/n_B)^2$, where I is the intensity of scattered light, I_B is the intensity of scattered light by pure benzene, R_B is the Rayleigh ratio for benzene, n_B is the refractive index for benzene, and $(n_0/n_B)^2$ is the correction factor for excess scattering volume; $\bar{M}_W$ is the weight-average molecular weight; M_D is the molecular weight of the nonaggregated dimer; $2B/M_D$ is the second virial coefficient of dimer; M_{app} is the apparent weight-average molecular weight. Since the second virial coefficient appeared small (see Figure 3), M_{app} should be close to $\bar{M}_W$.

Protein samples were dedusted by filtration through either 0.45 μ pore size HA type or 0.22 μ GS type Millipore filters directly into 2-cm diameter cylindrical cells and recycled through the same filter three times. The average dissymmetry ratio, $I_{45°}/I_{135°}$, was 1.16.

Enzymatic activity was measured in the direction of glycogen synthesis according to the procedure of Illingworth and Cori (1953). Assay time varied with protein concentration and temperature as stated in the figure legends. The activity of enzyme preincubated with glycogen was measured with samples which were incubated with 2% glycogen for at least 2 hr at room temperature and then at least 30 min at the desired temperature before assay. Arsenolysis was carried out according to the method described by Kent (1959). In kinetic studies, progress curves of phosphate release were run in duplicates for each sample and a tangentimeter was employed to estimate initial velocities.

Theory

Determination of Equilibrium Constant and Specific Activities from Initial Rate Measurements. The dissociation–association of phosphorylase *a* can be expressed as

$$E_T \rightleftharpoons 2E_D$$

and

$$K_d = \frac{E_D^2}{E_T} = \text{dissociation constant} \qquad (1)$$

where E_T and E_D represent the tetrameric and dimeric species. The subscripts D and T will be used to denote dimer and tetramer throughout the text.

It can be shown that the observed specific activity (defined as activity per unit of protein), $\bar{\phi}$, is the weight-average value of the specific activities of the dimer, ϕ_D, and the tetramer, ϕ_T:

$$\bar{\phi} = \frac{v}{E_0} = \frac{v_D + v_T}{E_0} = \frac{v_D}{E_D} \times \frac{E_D}{E_0} + \frac{v_T}{E_T} \times \frac{E_T}{E_0} = \frac{E_D\phi_D + E_T\phi_T}{E_0} \qquad (2)$$

where v = observed initial velocity and E_0 = total weight con-

[1] The abbreviation used is: glycero-*P*, glycerophosphate.

[2] All specific activities are expressed as μmoles of P_i released per minute per milligram of protein.

centration of the enzyme = $E_D + E_T$. Substitution of $E_D = E_0 - E_T$ and $E_T = E_0 - E_D$ into eq 2 yields

$$E_D = \frac{E_0(\bar{\phi} - \phi_T)}{\phi_D - \phi_T} \tag{3}$$

and

$$E_T = \frac{E_0(\phi_D - \bar{\phi})}{\phi_D - \phi_T} \tag{4}$$

Although it has been shown that glycogen activates phosphorylase *a* by causing the dissociation of tetramer into dimer, this induced dissociation process is slow compared with the enzymatic reaction (Wang *et al.*, 1965b; Metzger *et al.*, 1967). Thus the relative amounts of the two forms remain essentially unchanged during initial rate measurement. In other words, the equilibrium between the two forms is not significantly affected by the preferential binding of the substrate glycogen. On substituting eq 3 and 4 into eq 1 the dissociation constant can be expressed in terms of specific activities:

$$K_d = \frac{E_0(\bar{\phi} - \phi_T)^2}{(\phi_D - \phi_T)(\phi_D - \bar{\phi})} \text{ g/l.} \tag{5}$$

or

$$K_d = \frac{2E_0(\bar{\phi} - \phi_T)^2}{M_D(\phi_D - \phi_T)(\phi_D - \bar{\phi})} \text{ moles/l.} \tag{5a}$$

Upon rearrangement, eq 5 becomes

$$\frac{1}{\bar{\phi} - \phi_T} = \frac{1}{K_d(\phi_D - \phi_T)^2} \times E_0(\bar{\phi} - \phi_T) + \frac{1}{\phi_D - \phi_T} \tag{5b}$$

A plot of $1/(\bar{\phi} - \phi_T)$ *vs.* $E_0(\bar{\phi} - \phi_T)$, then, will yield $1/(\phi_D - \phi_T)$ as intercept on the ordinate and $-K_d(\phi_D - \phi_T)$ as intercept on the abscissa. If ϕ_T is known, K_d and ϕ_D can be evaluated. From the law of mass action, at infinite protein concentration only the tetrameric form is present, and we have

$$\lim_{(1/E_0)\to 0} \bar{\phi} = \phi_T \tag{6}$$

By plotting $\bar{\phi}$ *vs.* $1/E_0$ and extrapolating to $1/E_0 = 0$, the value of ϕ_T can be estimated.

If one or both forms of the enzyme exist in more than one conformational states of different catalytic activities and/or substrate affinities as the study by Helmreich *et al.* (1967) indicates, the equations derived above can be shown to be still valid. Suppose there are three conformational states of the same molecular weight, M_1, M_2, and M_3, in equilibrium with one another:

$$M_1 \overset{K_{12}}{\rightleftharpoons} M_2 \overset{K_{23}}{\rightleftharpoons} M_3$$

Let the total weight concentration of the enzyme $E_0 = E_1 + E_2 + E_3$, where E_1, E_2, and E_3 are the weight concentrations for M_1, M_2, and M_3 at equilibrium, and let K_{12} and K_{23} be the equilibrium constants for the transitions $M_1 \rightleftharpoons M_2$ and $M_2 \rightleftharpoons M_3$, then

$$K_{12} = \frac{E_2}{E_1}$$

$$K_{23} = \frac{E_3}{E_2}$$

hence $E_2 = K_{12}E_1$ and $E_3 = K_{12}K_{23}E_1$. The specific activity for such a system is therefore

$$\bar{\phi} = \frac{E_1\phi_1 + E_2\phi_2 + E_3\phi_3}{E_0} = \frac{\phi_1 + K_{12}\phi_2 + K_{12}K_{23}\phi_3}{1 + K_{12} + K_{12}K_{23}} = \text{constant}$$

where ϕ_1, ϕ_2, and ϕ_3 are the specific activities for M_1, M_2, and M_3. It should be noted that whether the transition between different conformations is slow or rapid relative to the overall enzymatic reaction has no effect on the outcome of the derivation shown here since E_1, E_2, and E_3 represent equilibrium concentrations at a given E_0 under a given assay condition.

This simple derivation shows two things: (1) As long as there is no molecular weight change, the specific activity of the system at a given substrate level can be represented by a single constant. The existence of several conformational states, for instance, the R and T states popularized by Monod *et al.* (1965), does not alter the appearance of eq 2–5. (2) Specific activity is independent of protein concentration if the system does not involve subunit dissociation–association. Therefore, concentration dependence of specific activity (or kinetic properties) is indicative of molecular weight change, barring nonspecific denaturation, or the presence of *small* amounts (relative to the amount of enzyme) of activator or inhibitor in the assay mixture.

If we simultaneously solve eq 2 and the expression for weight-average molecular weight of phosphorylase *a*

$$\bar{M}_w = \frac{E_D M_D + E_T M_T}{E_0}$$

where M_T = molecular weight of tetramer = $2M_D$, we can express $\bar{M}_w$ in terms of specific activities:

$$\bar{M}_w = \frac{M_D(2\phi_D - \phi_T - \bar{\phi})}{\phi_D - \phi_T} \tag{7}$$

The weight-average molecular weight of phosphorylase *a* at any given protein concentration E_0, then, can be computed from the observed specific activity at that concentration.

If one of the subunit forms is inactive, *e.g.*, the inactive tetramer proposed by Metzger *et al.* (1967), then $\phi_T = 0$, and eq 5b and 7 reduce to

$$\frac{1}{\bar{\phi}} = \frac{1}{K_d\phi_D^2} \times E_0\bar{\phi} + \frac{1}{\phi_D} \tag{5c}$$

$$\bar{M}_w = \frac{M_D(2\phi_D - \bar{\phi})}{\phi_D} \tag{7a}$$

Consequently, theoretical lines of $\bar{M}_w$ (or $1/\bar{M}_w$) as a function of protein concentration can be calculated by assuming active or inactive tetrameric phosphorylase *a* and compared with the experimentally determined molecular weights to test the validity of either model.

Results

Concentration-Dependent Dissociation of Phosphorylase a. Figures 1A and 2A show plots of reciprocal apparent weight-average molecular weight of phosphorylase *a* obtained from light-scattering measurements at 546 nm at 25 and 30°, respectively, as a function of protein concentration. These plots are typical of dissociation–association systems and demonstrate that molecular weight decreases directly with the decrease in concentration. At 25°, the extent of dissociation is moderate. The change is, however, quite significant, considering that, in weight-average molecular weight, the contribution from large molecule is more influential. The lowest molecular weights obtained at this temperature shown in Figure 1A were 306,000–317,000, representing the presence of 37–43% dimer. As the temperature was elevated to 30°, the change in molecular weight was much more pronounced (Figure 2A). At low protein concentrations, the molecular weight seems to approach that of the dimer rapidly. From either plot it can be seen that at higher protein concentrations, molecular weight reaches a finite value of 380,000 ± 10,000, indicating that the aggregation is not a random phenomenon, but a rather specific reaction.

Molecular Weight of Phosphorylase a. The molecular weight of tetrameric phosphorylase *a* measured by light scattering was determined by extrapolating molecular weights obtained at higher protein levels to zero concentration. From the intercept on the ordinate in Figure 3, a molecular weight of 380,000 is obtained. This value agrees quite well with the 370,000 and 367,000 redetermined by Seery *et al.* (1967) and by DeVincenzi and Hedrick (1967). Since these latter values were determined by ultracentrifugation or gel filtration in which the knowledge of partial specific volume is vital, the molecular weight reported here, using dn/dc and extinction coefficient repeatedly determined by us, is a good support for the validity of the new values.

Correlation between Molecular Weight and Enzymatic Activity. Wang and Graves (1964) have shown that specific activity of phosphorylase *a* increases directly with the decrease in protein concentration. If the dimeric and tetrameric forms of phosphorylase *a* have distinctly different intrinsic activities, the change in enzymatic activity should parallel the change in molecular weight. Thus activities of phosphorylase *a* were measured under conditions identical with those of the light scattering measurements, except for the presence of substrates, to make the correlation more relevant. Molecular weight of phosphorylase *a* cannot be measured in the presence of substrates because of the huge molecular weight of glycogen and the multiple binding of the enzyme to glycogen. The specific activity profiles so obtained at 25 and 30° are shown in Figures 1B and 2B. Although the enzymatic assays were carried out in the direction of glycogen synthesis, assays done in the direction of glycogen degradation by arsenolysis yielded similar results. It was assumed that activation by glycogen was negligible during assay because progress curves

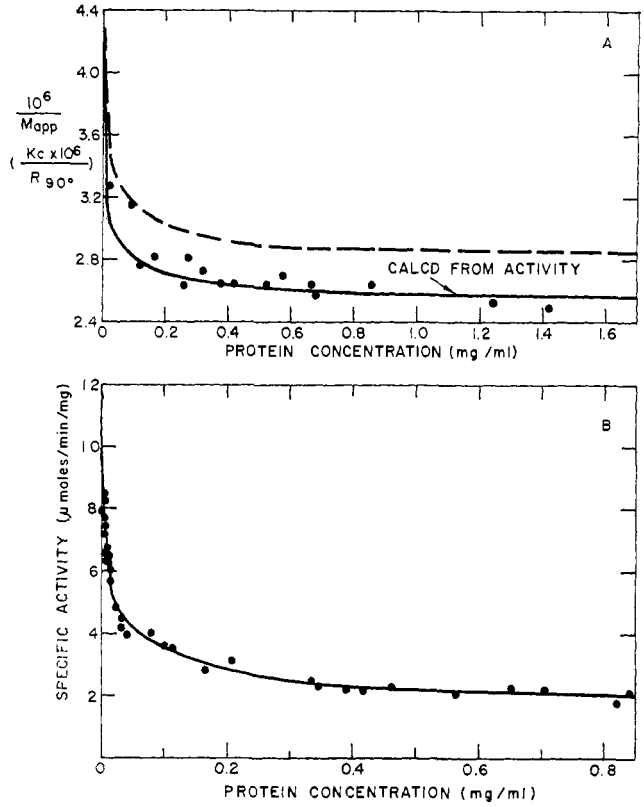

FIGURE 1: Molecular weight and specific activity of phosphorylase *a* as a function of protein concentration at 25°. All experiments were carried out in 40 mM glycero-*P*, 1 mM EDTA and 1% KCl buffer (pH 6.8). (A) Reciprocal apparent molecular weight of phosphorylase *a* as a function of protein concentration. Molecular weights were measured by light scattering at 546 nm as described in Methods. Different enzyme preparations were used: (——) theoretical line of molecular weight as a function of protein concentration calculated from specific activity curve shown in B. Values used for the calculation: specific activity of tetramer = 1.86, specific activity of dimer = 11.7. All values obtained by least-squares method as described in the test: (– – –) theoretical line of molecular weight as a function of protein concentration calculated from specific activity curve shown in B by assuming inactive tetramer. Specific activity of dimer used for the calculation = 9.48, obtained from least-squares method as described in the text. (B) Specific activity of phosphorylase *a* as a function of protein concentration. Assay was carried out in AMP-free substrate. Assay time varied from 20 sec to 10 min as protein concentration decreased from 1.71 mg/ml to 0.0024 mg/ml. Specific activity is expressed as micromoles of P_i released per minute per milligram of protein. Data were obtained from the same enzyme preparation.

of phosphate release and of glucose formation by arsenolysis always appeared linear.

If one compares the light-scattering data with the specific activity curves, one can see the first sign of correlation between them since they both level off at approximately the same enzyme concentration, around 0.4 mg/ml at 25° and around 0.8 mg/ml at 30°, and both seem to asymptotically approach finite values. By plotting specific activity, $\bar{\phi}$, observed at high protein concentrations against the reciprocal enzyme concentration, the specific activity for tetramer, $\bar{\phi}_T$, was obtained by extrapolating to $1/E_0 = 0$. An example of such a plot is shown in the inset of Figure 4. The ϕ_T values resulting from such extrapolations were 1.86 and 3.30[2] at 25 and 30°,

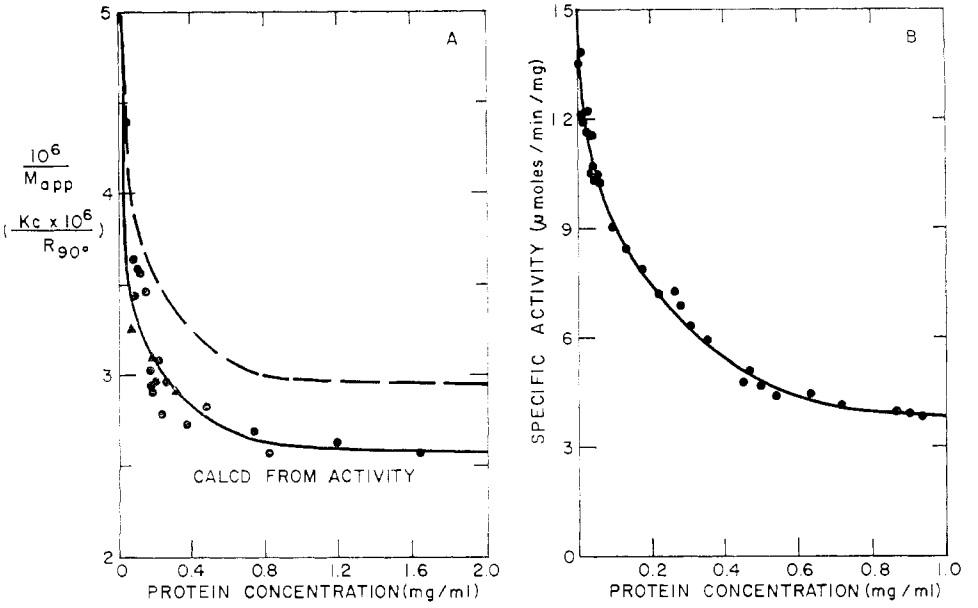

FIGURE 2: Molecular weight and specific activity of phosphorylase *a* as a function of protein concentration at 30°. (A) Reciprocal apparent molecular weight of phosphorylase *a* as a function of protein concentration. Molecular weights were measured by light scattering at 546 nm as described in Methods. Different enzyme preparations were used. (●) Data obtained in 40 mM glycerol-*P*, 1 mM dithiothreitol, and 0.5% NaCl buffer (pH 6.8); (▲) data obtained in 40 mM glycerol-*P*, 1 mM EDTA, and 1% NaCl buffer (pH 6.8): (——) theoretical line of molecular weight a as function of protein concentration calculated from specific activity curve shown in B. Values used for the calculation: specific activity of tetramer = 3.30, specific activity of dimer = 15.8; all values obtained by least-squares method as shown in Figure 4: (– – –) theoretical line of molecular weight as a function of protein concentration calculated from specific activity curve shown in B by assuming inactive tetramer. Specific activity of dimer used for the calculation = 13.5, obtained from least-squares method as shown in Figure 4B. (B) Specific activity of phosphorylase *a* as a function of protein concentration. Assay was carried out in AMP-free substrate. Assay time varied from 10 sec to 5 min as protein concentration varied from 1.42 mg/ml (data at high protein concentration shown in inset of Figure 4) to 0.0056 mg/ml. Specific activity is expressed as micromoles of P_i released per minute per milligram of protein. Data were obtained from same enzyme preparation.

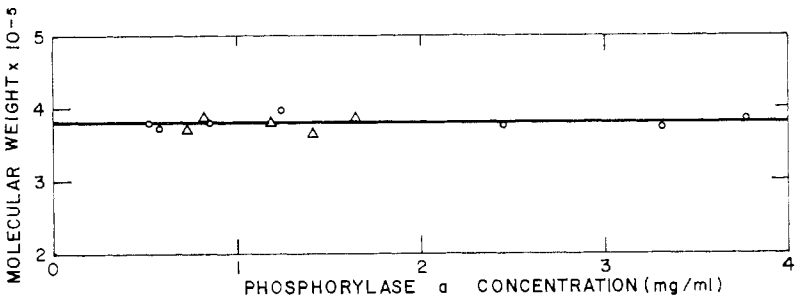

FIGURE 3: Molecular weight of phosphorylase *a* measured by light scattering at 546 nm: (○) in 40 mM glycerol-*P*, 1 mM EDTA and 1% KCl (pH 6.8) at 25°; (Δ) in 40 mM glycerol-*P*, 1 mM EDTA and 0.5% NaCl (pH 6.8) at 30°.

respectively. The values of specific activity for dimer, ϕ_D, and dissociation constant, K_d, were then obtained by a $1/(\bar{\phi} - \phi_T)$ *vs.* $E_0(\phi - \phi_T)$ plot by using eq 5b. The values of ϕ_D so determined were 11.7 at 25° and 15.8 at 30°. From values of these specific activities, theoretical lines of molecular weight as a function of protein concentration (solid lines, Figures 1A and 2A) were calculated according to eq 7 and compared with actual molecular weights obtained by light scattering. In both cases, the theoretical lines fit the experimental points, demonstrating that activity change can be quantitatively accounted for by a change in molecular weight. The correlation between molecular weight and enzymatic activity strongly suggests that the increase in activity is indeed the consequence of the formation of a catalytically more active species. It also supports the concept that the dissociated form is a dimer since eq 7, which is derived on the assumption of dimer–tetramer equilibrium, seems to describe the system quite adequately.

The hypothesis of inactive tetramer was tested by using eq 5c and 7a. The specific activity data obtained at 25 and 30° were plotted according to eq 5b or eq 5c (for $\phi_T \neq 0$ and $\phi_T = 0$, respectively) and least-squares methods were employed to obtain specific activities and dissociation constants. As an example, graphical analyses of data at 30° are shown in Figure 4A,B. For $\phi_T = 0$, the ϕ_D values calculated were 9.48 at 25° and 13.5 at 30°. Theoretical lines of molecular weight as a function of protein concentration were then calculated from eq 7a by using these values. From Figures 1A and 2A (dashed lines), it can be seen that the lines calculated by assuming $\phi_T = 0$ do not fit the experimental points. It is evident that the proposed inactive tetramer is unlikely since the enzyme would have to undergo more dissociation than seen from

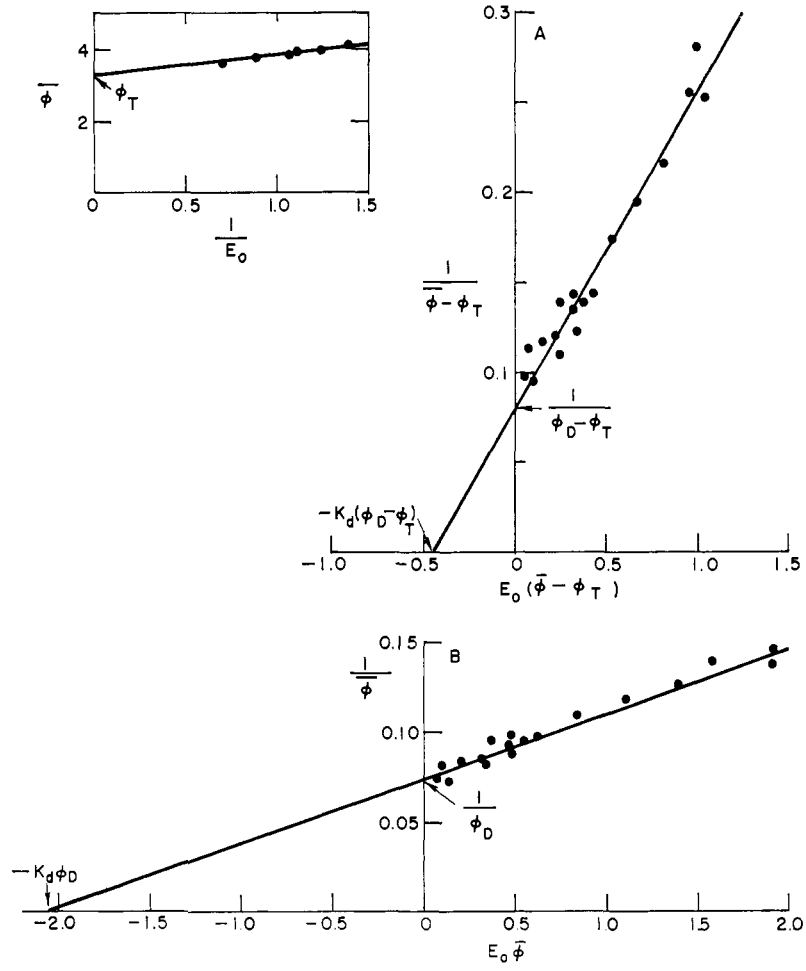

FIGURE 4: Graphical analyses of specific activity data at 30° (shown in Figure 2B). (A) Determination of specific activities and dissociation constant by least-squares method, assuming active phosphorylase *a* tetramer. Inset: determination of specific activity for tetramer, ϕ_T by extrapolation to $1/E_0 = 0$. The ϕ_T value so obtained, 3.30, was used for calculation of $1/(\bar{\phi} - \phi)$ and $E_0(\bar{\phi} - \phi_T)$ as shown in A (*cf.* eq 5b). Specific activity of dimer, $\phi_D = 15.8$, was obtained from *y* intercept. Dissociation constant, $K_d = 3.53 \times 10^{-2}$ g/l., was calculated from *x* intercept. (B) Determination of specific activity of dimer and dissociation constant by least-squares method, assuming inactive phosphorylase *a* tetramer (*cf.* eq 5c). Specific activity of dimer, $\phi_D = 13.5$, and dissociation constant, $K_d = 1.53 \times 10^{-1}$, were obtained from *y* and *x* intercepts.

molecular weight measurements to account for the observed specific activities; this is manifested by the fact that K_d's obtained by assuming $\phi_T = 0$, 1.43×10^{-2} g/l. at 25° and 1.53×10^{-1} g/l. at 30°, are considerably larger than their corresponding values 3.59×10^{-3} g/l. and 3.53×10^{-2} g/l. obtained by assuming active tetramer.

Activation Energies and Thermodynamic Parameters of Dissociation. We have shown that the equilibrium between tetrameric and dimeric phosphorylase *a* can be studied by activity measurements. It is feasible, then, to determine activation energies of the two forms and thermodynamic parameters of dissociation from specific activity curves. Since specific activity varies somewhat with different enzyme preparations and with age, it is necessary to carry out assays at different temperatures in a short period using the same batch of enzyme. Figure 5A–D show specific activity profiles for the same enzyme preparation at 35, 30, 25, and 20°. Specific activities of enzyme preincubated in glycogen in the same temperature range were also measured. The specific activities of dimer calculated from the graphic methods were in good agreement with those obtained from preincubation with glycogen experiments (Figure 6, solid circles and open circles). Theoretical specific activity curves calculated from the dissociation constants, obtained also from graphic methods, in general fit the experimental points (Figure 5), indicating that the dissociation reactions obey the law of mass action and that the K_d's are of the right magnitude. The results also suggest that the species generated by incubation with glycogen is the dimeric form, confirming the work of Wang *et al.* (1965b).

Figure 6 shows Arrhenius plots for the two species of phosphorylase *a*. The activation energies for dimer and tetramer in the temperature range of 20–30° are 11.3 kcal and 22.6 kcal, respectively. This again is in line with the model of dimer being more active and tetramer being less active.

As shown in Figure 7A,B, the standard enthalpy change, ΔH_d°, and entropy change, ΔS_d°, of dissociation for phosphorylase *a* at pH 6.8, in 40 mM glycero-*P*–1 mM dithiothreitol, and 0.5% NaCl, in the temperature range 20–35°, calculated from activity measurements are 60.0 kcal/mole and 170 entropy units, respectively. The thermodynamic parameters of dissociation are summarized in Table I.

The magnitude of ΔH_d° and ΔS_d° was checked by measuring molecular weight change by light scattering in the temperature range 15–35°. Two enzyme samples at 0.095 mg/ml and 0.262 mg/ml were subject to cooling, warming, and recooling. The dissociation was found to be completely reversible, indicating a truly equilibrating system. Although the dissociation constants calculated from the two samples show deviations, the ΔH_d° values, 58.5 and 60.0 kcal, and the ΔS_d° values, 165 and 172 eu, agree very well with those determined from rate measurements (Figure 7A,B). These results and

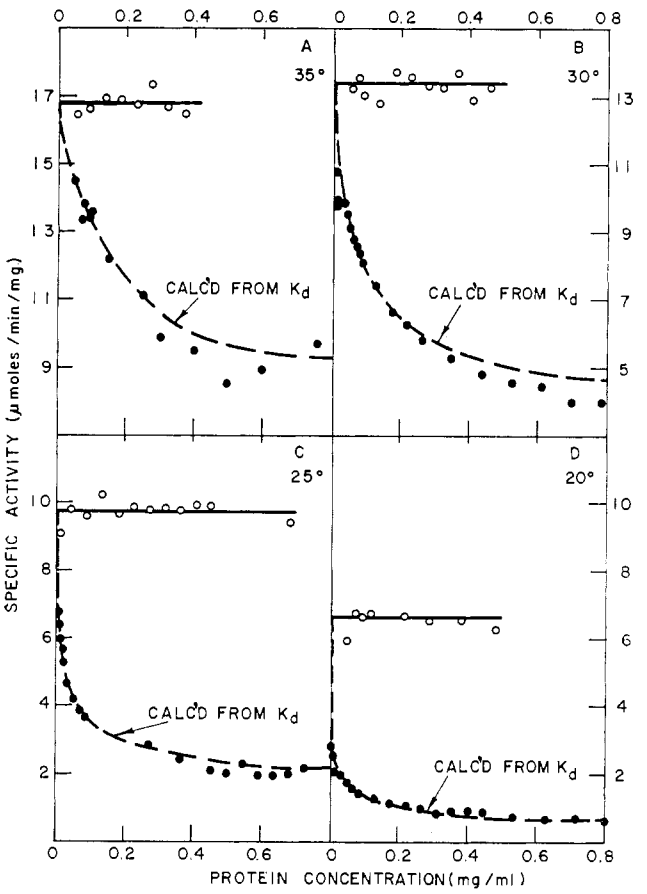

FIGURE 5: Specific activities of phosphorylase *a* with and without preincubation with glycogen at different temperatures. All data were obtained in 40 mM glycero-*P*, 1 mM dithiothreitol and 0.5% NaCl, pH 6.8 buffer, using same enzyme preparation: (○) preincubated in 2% glycogen before assay; (●) without preincubation; (- - -) theoretical lines calculated from dissociation constants obtained by graphical method as described in the text. Data obtained at protein concentration >0.8 mg/ml are not shown. (A) (35°) Assay time varied from 10 to 30 sec as enzyme concentration decreased from 0.366 mg/ml to 0.055 mg/ml in preincubation with glycogen experiments; without preincubation, assay time varied from 5 to 40 sec as enzyme concentration decreased from 2.19 mg/ml to 0.050 mg/ml. $K_d = 1.56 \times 10^{-1}$ g/l. (B) (30°). Assay time varied from 12 to 40 sec as enzyme concentration decreased from 0.453 mg/ml to 0.054 mg/ml in preincubation with glycogen experiments; without preincubation, assay time varied from 12 sec to 2 min as enzyme concentration decreased from 1.05 mg/ml to 0.0087 mg/ml. $K_d = 4.50 \times 10^{-2}$ g/l. (C) (25°). Assay time varied from 12 sec to 2 min as enzyme concentration decreased from 0.685 mg/ml to 0.018 mg/ml, in preincubation with glycogen experiments; without preincubation, assay time varied from 15 sec to 5 min as enzyme concentration decreased from 1.37 mg/ml to 0.0091 mg/ml. $K_d = 6.69 \times 10^{-3}$ g/l. (D) (20°). Assay time varied from 15 to 80 sec as enzyme concentration decreased from 0.446 mg/ml to 0.0045 mg/ml in preincubation with glycogen experiments; without preincubation, assay time varied from 1 to 10 min as enzyme concentration decreased from 0.892 mg/ml to 0.0036 mg/ml. $K_d = 1.17 \times 10^{-3}$ g/l.

the fact that all the plots shown in Figures 6 and 7 seem to fall on straight lines further support the validity of the equations derived for the phosphorylase *a* system, which is based on the model of a more active dimer and a less active tetramer.

Concentration Dependence of Apparent Michaelis Constant for Glycogen for Phosphorylase a. To further demonstrate that both subunit species of phosphorylase *a* have affinity for glycogen, apparent Michaelis constants were determined at four different enzyme concentrations, ranging from 0.0060 mg/ml to 0.302 mg/ml, at 25°. On the basis of slow equilibration between dimer and tetramer in the presence of glycogen, the kinetic equation for the phosphorylase *a* system is

$$v = v_D + v_T = \frac{k_D E_D S}{K_D + S} + \frac{k_T E_T S}{K_T + S}$$

where K_D and K_T are rates of breakdown, K_D and K_T are Michaelis constants, for dimer and tetramer; S is substrate concentration. This equation is analogous to the case of two enzymes acting on the same substrate (Dixon and Webb, 1964) except that the relative amounts of dimer and tetramer (E_D and E_T) will be dependent on the total protein concentration E_0. As has been shown by Dixon and Webb, theoretical plots of $1/v$ *vs.* $1/S$ for the two-enzyme system yield hyperbolic curves which are often indistinguishable from ordinary Michaelis–Menten kinetics because the bending of the curves occurs mainly in the imaginary negative substrate concentration range. If only dimeric *a* is capable of binding glycogen, one would expect the apparent Michaelis constant, K_{app}, to show no protein concentration dependence since the kinetic equation will contain only the K_D term. If the two forms can both bind glycogen but differ in their affinities, then at high enzyme concentrations, K_{app} would be expected to approach

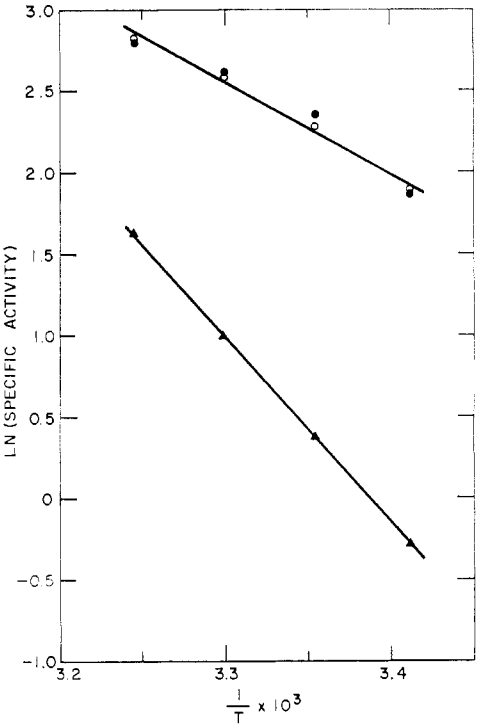

FIGURE 6: Activation energies of dimeric and tetrameric phosphorylase *a*. All specific activities were obtained from data shown in Figure 5: (●) specific activities of dimer calculated according to eq 5b; (○) specific activities of dimer obtained from preincubation with glycogen experiments. Activation energy for dimer = 11.3 kcal; (▲) specific activities of tetramer obtained from extrapolation using eq 6. Activation energy for tetramer = 22.6 kcal.

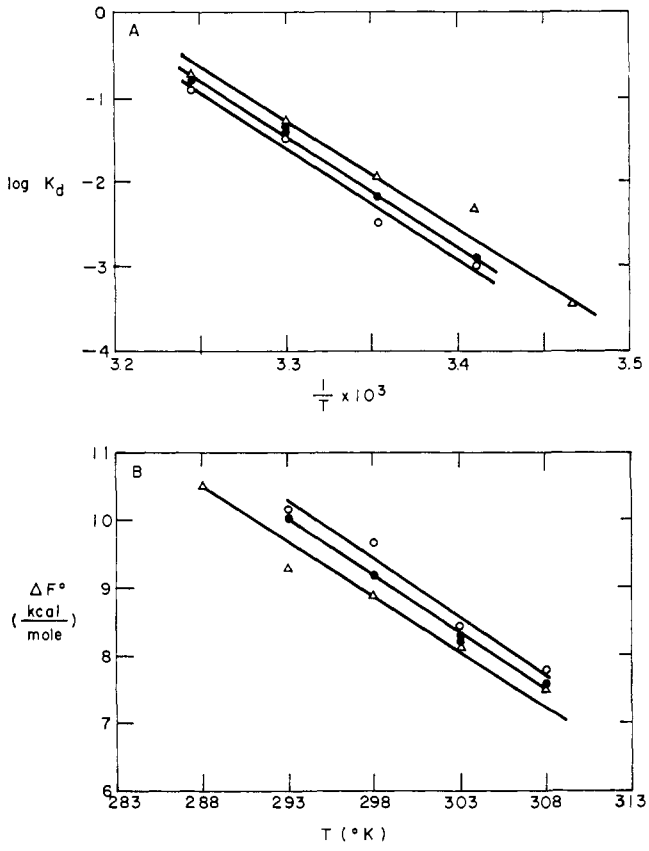

FIGURE 7: Standard enthalpy (A) and entropy (B) changes of dissociation of phosphorylase *a* at pH 6.8. All experiments were carried out in 40 mM glycero-*P*, 1 mM dithiothreitol, and 0.5% NaCl buffer: (●) data obtained from specific activity curves shown in Figure 5 and Figure 2B, $\Delta H_d° = 60.0$ kcal/mole, $\Delta S_d° = 170$ eu; (△) data obtained from molecular weight measurements by light scattering, enzyme concentration = 0.095 mg/ml, $\Delta H_d° = 58.5$ kcal/mole, $\Delta S_d° = 165$ eu; (○) data obtained from molecular weight measurements by light scattering, enzyme concentration = 0.262 mg/ml, $\Delta H_d° = 60.0$ kcal/mole, $\Delta S_d° = 172$ eu.

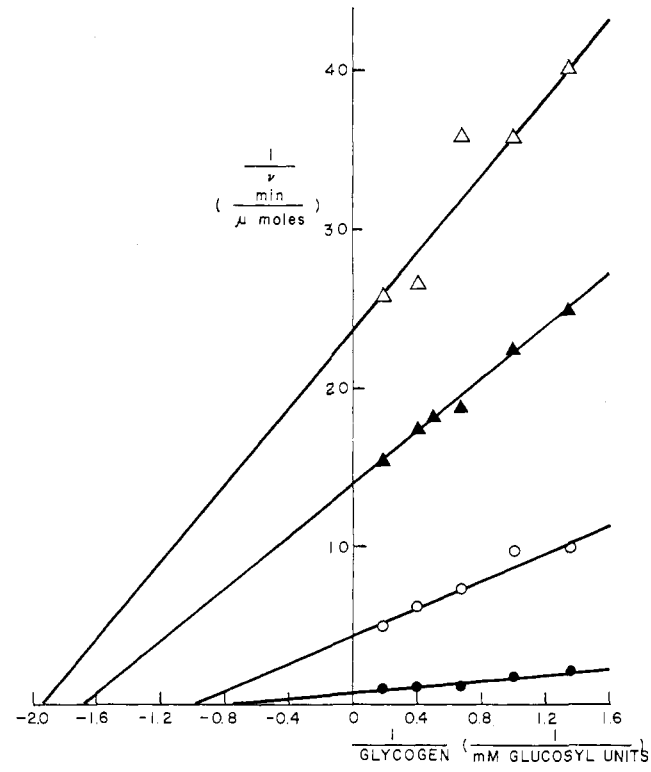

FIGURE 8: Protein concentration dependence of apparent Michaelis constant for glycogen for phosphorylase *a*. Assay was carried out in AMP-free substrate, in 40 mM glycero-*P*–1 mM dithiothreitol pH 6.8 buffer, at 25°. Enzyme concentrations are as follows: (●) 0.302 mg/ml; (○) 0.0302 mg/ml; (▲) 0.0071 mg/ml; (△) 0.0060 mg/ml.

K_T; at very low enzyme concentrations, K_{app} would approach K_D. At protein concentrations intermediate to the two extremes, extrapolation of the curve would yield a value somewhere between K_D and K_T. In Figure 8, Lineweaver–Burk plots show that the K_{app}'s vary from 1.39 to 0.52 mM as enzyme concentration decreases, which is compatible with the concept that both subunit species are catalytically active, with dimer having higher affinity and tetramer having lower affinity for glycogen.

To show that the change in K_{app} is due to the presence of different molecular weight forms, the Michaelis constant for phosphorylase *b*, a dimer, was determined at five different protein concentrations. As shown in Figure 9, a value in the vicinity of 0.50 mM was obtained at all five concentrations, showing virtually no protein concentration dependence of Michaelis constant for this enzyme.

Discussion and Conclusion

That correlations between molecular weight and enzymatic activity were demonstrated at several temperatures seems to rule out coincidence and strongly suggests the following: (1) Each state of aggregation of this enzyme is associated with a well-defined catalytic activity under a given set of conditions (pH, temperature, ionic strength, buffer salts, substrate level, etc.). (2) The model of a catalytically more active dimer and a less active tetramer in dynamic equilibrium is valid. In fact, the tetramer is fairly active, its specific activity ranges from 12 to 33% that of the dimer as temperature increases from 20 to 35°.

Metzger *et al.* (1967) have proposed that tetrameric phos-

TABLE I: Thermodynamic Parameters of Dissociation for Phosphorylase *a* at pH 6.8.[a]

Temp (°C)	$\Delta F_d°$ (kcal/mole)	$\Delta H_d°$ (kcal/mole)	$\Delta S_d°$ (eu)
20	10.0	60.0	170
25	9.18		
30	8.21		
	8.34[b]		
35	7.56		

[a] Buffer used: 40 mM glycero-*P*, 1 mM dithiothreitol, and 0.5% NaCl. [b] Calculated from the K_d obtained from Figure 4 using a different enzyme preparation.

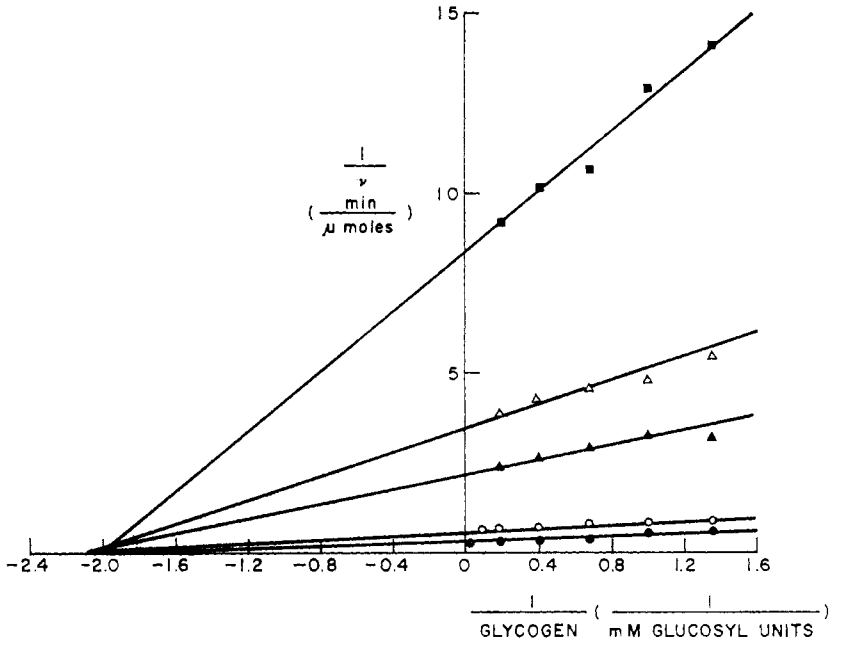

FIGURE 9: Michaelis constant for glycogen for phosphorylase *b* at different protein concentrations at 25°. Assay was carried out in substrate containing 10^{-3} M AMP, in 40 mM glycero-*P*-1 mM dithiothreitol pH 6.8 buffer. Enzyme concentrations are as follows: (●) 0.250 mg/ml; (○) 0.100 mg/ml; (▲) 0.0250 mg/ml; (△) 0.0156 mg/ml; (■) 0.0082 mg/ml.

phorylase *a* is inactive with glycogen as substrate. Judged from existing evidence, the proposed inactive tetramer seems unlikely: (1) The specific activities observed at high protein concentrations at four temperatures all seem to approach finite values. The specific activity *vs.* $1/E_0$ plots could not be extrapolated to zero activity. (2) When tetramer was assumed to be inactive, the change in specific activity did not correlate with the change in molecular weight. The dissociation constants obtained by assuming inactive tetramer were too high. (3) The K_{app}'s for glycogen for phosphorylase *a* showed protein concentration dependence. If dimer is the sole active form, only one Michaelis constant should be obtained at all concentrations. Since Helmreich *et al.* (1967) have shown that the dimer may exist in R and T states, the question may be raised whether the variation in K_{app} may be ascribed to the presence of two or more active conformations of the dimeric species.[3] However, Helmreich *et al.* (1967) also reported that even in the absence of reactive ligands phosphorylase *a* exists to a large extent in the active R form. This is supported by the fact that phosphorylase *a* ordinarily does not yield cooperative-type kinetics. The study by Ullmann *et al.* (1964) on the binding of bromothymol blue to phosphorylase *a* also implies that the enzyme exists predominantly in one form. Thus the possibility that the concentration dependence of K_{app} is the result of R and T states of the dimeric form appears remote. (4) Wang and Graves (1964) have shown that the decay of activity upon dilution of phosphorylase *a* preincubated in 3.0 M NaCl followed second-order kinetics, *i.e.*, $1/(\bar{\phi} - \phi_T)$ *vs.* time plot yielded a straight line, which is consistent with a dimer → tetramer process. We have replotted the same data by assuming ϕ_T to be zero; the decay did not fit either second-order or first-order kinetics. (5) When active tetramer was assumed and ϕ_T values were determined according to eq 6, the specific activities of dimer calculated from eq 5b agreed very well with those obtained from preincubation with glycogen experiments at four temperatures. All these observations indicate that tetrameric phosphorylase *a* is capable of catalysis. The observation by Metzger *et al.* (1967) of the inability of tetramer to bind phytoglycogen is probably due to the fact that phytoglycogen is a much larger molecule than glycogen and may encounter greater steric hindrance in approaching the tetramer.

The thermodynamic parameters reported here show clearly that the driving force for polymerization of phosphorylase *a* is enthalpic, a phenomenon shared by several other self-associating proteins, *e.g.*, fibrin (Sturtevant *et al.*, 1955), flagellin (Erlander *et al.*, 1960), β-lactoglobulin (Townend and Timasheff, 1960), and lactic dehydrogenase (Millar, 1962). Although hydrogen bond formation has been proposed to account for the large enthalpy change (Sturtevant *et al.*, 1955) observed in the case of fibrin, it seems unlikely in view of later findings that heat of formation or of disruption of amide hydrogen bond is zero in aqueous solution (Klotz, 1965) and that the free energy of transferring such a bond from aqueous to apolar surrounding is also zero (Klotz and Farnham, 1968). The large enthalpy change cannot be explained by ionic or hydrophobic interactions either, since these interactions presumably are characterized by large entropy effects (Kauzmann, 1959). Sinanoglu and Abdulnur (1965) have proposed that the energy required to form cavities to accommodate macromolecules in aqueous solution may result in a large enthalpy effect. Subsequent studies by Crothers and coworkers (Crothers *et al.*, 1968; Crothers and Ratner, 1968), however, showed that while surface tension has some influence on the dimerization of actinomycin, it does not seem to be the dominant factor in the binding of deoxyguanosine to actinomycin. Thus the large enthalpy change observed in the dissociation-association of phosphorylase *a* is difficult to interpret with available information

[3] Theoretically, kinetics of conformation transitions should be independent of protein concentration (*cf.* Theory). In practice, $1/v$ *vs.* $1/s$ plots may result in apparent enzyme concentration dependence since V_m varies with the fraction of dimer present.

from model compound studies, particularly when the thermodynamic parameters in different solvents are lacking.

The preferential binding of glycogen to phosphorylase *a* dimer implies that subunit interaction may serve as a regulatory mechanism (*cf.* Metzger *et al.*, 1967, 1968) in addition to the already complicated and subtle system controlling the metabolism of glycogen. It can be envisioned that as the glycogen molecule is debranched to a certain degree, K_m's for dimer and tetramer may become closer, resulting in association of the enzyme and functioning as an intermediate brake in reducing the rate of glycogen breakdown prior to the slower dephosphorylation of the enzyme by phosphorylase phosphatase (half-time of dephosphorylation was shown to be about 13 min; *cf.* Fischer and Krebs, 1966) and thereby preventing the depletion of glycogen.

Acknowledgments

The authors are indebted to Dr. Malcolm A. Rougvie for advice on light-scattering experiments, to Dr. Carl Frieden for looking into the kinetic derivations, and to Sister Burcharda for performing part of the specific activity measurements.

Appendix

Equilibrium constant for dissociating–associating enzyme systems in general can be determined by molecular weight measurements. However, if the dissociation takes place only at low protein concentration, molecular weight measurement becomes difficult. The determination of equilibrium constant by activity measurement, in the case of phosphorylase *a*, has been shown to agree with the result of molecular weight measurement closely. Hence initial rate measurement should be a useful addition to the available methods for studying subunit dissociation of enzymes; at very low protein concentration, it may be the only tool.

For a polymeric enzyme E_p which undergoes one-step dissociation to a subunit form E_m

$$E_p \overset{K_d}{\rightleftharpoons} pE_m$$

if E_m and E_p differ in their intrinsic maximum velocity and/or ligand binding affinity, the equilibrium constant of such a system can be determined by initial rate measurements.

If the readjustment of equilibrium between E_p and E_m caused by the preferential binding of a substrate or a modifier during assay is slow compared with the overall enzymatic reaction, or if the two molecular weight forms differ only in their intrinsic maximum velocity (*cf.* Kurganov, 1967), the equations and methods described in Theory can be utilized with appropriate alteration. For example, eq 5b can be generalized to the following form:

$$\frac{1}{\bar{\phi} - \phi_p} = \frac{1}{K_d(\phi_m - \phi_p)^p} - [E_0(\bar{\phi} - \phi_p)]^{p-1} + \frac{1}{\phi_m - \phi_p} \quad (8)$$

where ϕ_m and ϕ_p are the specific activities for the dissociated and the aggregated forms.

If the reequilibration between the two forms is rapid compared to the overall enzymatic reaction, by assuming n equivalent and independent active sites on the dissociated subunit,[4] the following equation can be obtained by extending the equation derived by Frieden (1967) for the dimerization case:

$$\bar{\phi} = \frac{v}{E_0} = \frac{k_m(E_m)(S/K_m)(1 + S/K_m)^{n-1} + k_p(E_p)(S/K_p)(1 + S/K_p)^{pn-1}}{(E_m)(1 + S/K_m)^n + (E_p)(1 + S/K_p)^{pn}} \quad (9)$$

where k_m and k_p = rates of breakdown for E_m and E_p, K_m and K_p = microscopic substrate dissociation constants for E_m and E_p, and (E_m) and (E_p) = weight concentrations of free E_m and E_p.

The expression for the dissociation constant for this rapid equilibrium case, $K_{d(S)}$, is

$$K_{d(S)} = \frac{(E_{m_0})^p}{(E_{p_0})} = \frac{[(E_m)(1 + S/K_m)^n]^p}{(E_p)(1 + S/K_m)^{pn}} = K_d\left(\frac{1 + S/K_m}{1 + S/K_p}\right)^{pn} (g/l.)^{p-1} \quad (10)$$

where (E_{m_0}) and (E_{p_0}) = total weight concentrations of E_m and E_p. It is evident that the dissociation constant obtained by use of the plots utilizing eq 8, for rapid equilibrium between the two molecular species, is the dissociation constant of the system under the influence of substrate, $K_{d(S)}$. To obtain the intrinsic dissociation constant in the absence of substrate, K_d, it is necessary to measure activity at suitable substrate levels where the enzyme concentration dependence of specific activity can be detected.

Two methods can be used to evaluate K_d. First, from

$$\phi_p = \frac{v_p}{(E_{p_0})} = \frac{k_p S}{K_p + S}$$

we have

$$\frac{1}{\phi_p} = \frac{1}{k_p} + \frac{K_p}{k_p}\left(\frac{1}{S}\right)$$

By plotting reciprocal ϕ_p values obtained at different substrate concentrations against $1/S$, k_p and K_p can be determined. k_m and K_m can be similarly determined (ϕ_m values can be determined from eq 8). From the values of K_m and K_p, K_d can be calculated from eq 10. Although K_m and K_p are dissociation constants for one-substrate systems, steady-state treatment would yield similar equations, and K_m and K_p could also be Michaelis constants.

Alternatively, a method similar to the treatment of Klapper and Klotz (1968) may be used. From eq 9 we can write

$$\frac{\bar{\phi}}{S} = \frac{k_m[(E_m)/K_m](1 + S/K_m)^{n-1} + k_p[(E_p)/K_p](1 + S/K_p)^{pn-1}}{(E_m)(1 + S/K_m)^n + (E_p)(1 + S/K_p)^{pn}}$$

[4] The term subunit means the dissociated species which may be a monomer or may contain several monomeric units.

If we define

$$\lim_{s\to 0} \frac{\bar{\phi}}{S} = \frac{k_m[(E_0)/K_m] + k_p[(E_{p_0})/K_p]}{(E_{m_0}) + (E_{p_0})} = \bar{\phi}' \qquad (11)$$

(ϕ' can be estimated by a log (ϕ/S) *vs.* S plot. It should be noted that at $S = 0$, (E_m) and (E_p) equal (E_{m_0}) and (E_{p_0}) since all enzyme species are void of substrate.)

$$\lim_{E_0\to 0} \bar{\phi}' = \frac{k_m}{K_m} = \phi_m' \qquad (12)$$

and

$$\lim_{(1/E_0)\to 0} \bar{\phi}' = \frac{k_p}{K_p} = \bar{\phi}_p' \qquad (13)$$

by making an appropriate plot to obtain ϕ_m' or ϕ_p' (depending on which value can be easily obtained), K_d can be calculated from eq 8 by replacing $\bar{\phi}$ with $\bar{\phi}'$, $\bar{\phi}_m$ with $\bar{\phi}_m'$, and $\bar{\phi}_p$ with ϕ_p'. It should be noted that eq 11–13 are also applicable to the case where E_m and/or E_p can exist in more than one conformational state.

For two-substrate systems, if one of the substrates binds equally well to both molecular species so that the equilibrium between the two species is not affected by this substrate, they can be treated as one-substrate systems and the equations presented here are still valid. If both substrates show preferential binding to one of the molecular species, the kinetic expression and method for evaluating K_d would be more complicated and will not be discussed here.

If there is interaction among sites on the same enzyme molecule, *i.e.*, the binding of a ligand on one site alters the binding and/or intrinsic maximum velocities on the other sites, for the Adair model where all the unoccupied sites are initially identical, it can be shown that

$$\bar{\phi} = \frac{v}{E_0} = \frac{(E_m)(S/K_1)(k_1 + \ldots + k_n S^{n-1}/K_2 \ldots K_n) + (E_p)(S/K_1')(k_1 + \ldots + k_{pn}' S^{pn-1}/K_2' \ldots K_{pn}')}{(E_m)(1 + \ldots + S^n/K_1 \ldots K_n) + (E_p)(1 + \ldots + S^{pn}/K_1' \ldots K_{pn}')} \qquad (14)$$

where $K_1, K_2, \ldots K_n$ are microscopic substrate dissociation constants for $E_mS, E_mS_2, \ldots E_mS_{n'}$; $K_1', K_2', \ldots K_{pn}'$ are microscopic substrate dissociation constants for $E_pS, E_mS_2, \ldots E_pS_{pn}$; $k_{1'} \ldots k_n$ are rates of breakdown for $E_mS, \ldots E_mS_{n'}$; and $k_{1'} \ldots k_{pn}'$ are rates of breakdown for $E_pS, \ldots E_pS_{pn}$.

$$\lim_{S\to 0} \frac{\bar{\phi}}{S} = \frac{(E_{m_0})(k_1/K_1) + (E_{p_0})(k_1'/K_1')}{(E_{m_0}) + (E_{p_0})} = \bar{\phi}'' \qquad (15)$$

$$\lim_{E_0\to 0} \bar{\phi}'' = \frac{k_1}{K_1} \qquad (16)$$

and

$$\lim_{(1/E_0)\to 0} \bar{\phi}'' = \frac{k_1'}{K_1'} \qquad (17)$$

It is evident that K_d for the interacting-site case can be evaluated by procedures similar to the second method described in the noninteracting-site case.

Limitations. Like all kinetic approaches, the methods described here are governed by the usual assumptions of enzyme kinetics. Besides, as has been pointed out by Nicol *et al.* (1964), some physicochemical study on the enzyme is often necessary before this method can be applied. First, one must establish that the dissociation–association essentially involves two molecular species. Second, the number of active sites on the enzyme may have to be determined. In the absence of such information, one can best fit the kinetic data to a certain model. Another limitation is that a suitable way of assay which is effective over a rather wide range of protein concentration may not be available. For instance, in coupled assays, at high enzyme concentration the required high levels of auxiliary enzyme(s) may interfere with the result or render it impracticable. Also, when the degree of polymerization is great, *i.e.*, the number p is large, the accuracy and sensitivity of this method greatly declines.

References

Carr, C. I., Jr., and Zimm, B. H. (1950), *J. Chem. Phys. 18*, 1616.

Crothers, D. M., and Ratner, D. I. (1968), *Biochemistry 7*, 1823.

Crothers, D. M., Sabol, S. L., Ratner, D. I., and Müller, W. (1968), *Biochemistry 7*, 1817.

DeVincenzi, D. L., and Hedrick, J. L. (1967), *Biochemistry 6*, 3489.

Dixon, M., and Webb, E. C. (1964), Enzymes, New York, N. Y., Academic pp 87–90.

Erlander, S. R., Koffler, H., and Foster, J. F. (1960), *Arch. Biochem. Biophys. 90*, 139.

Fischer, E. H., and Krebs, E. G. (1958), *J. Biol. Chem. 231*, 65.

Fischer, E. H., and Krebs, E. G. (1962), *Methods Enzymol. 5*, 369.

Fischer, E. H., and Krebs, E. G. (1966), *Fed. Proc. 25*, 1511.

Frieden, C. (1967), *J. Biol. Chem. 238*, 3286.

Goring, D. A. I., Senez, M., Melanson, B., and Hugue, M. M. (1957), *J. Colloid Sci. 12*, 412.

Graves, D. J., Huang, C. Y., and Mann, S. A. S. (1968), Control of Glycogen Metabolism, New York, N. Y., Academic, pp 35–47.

Helmreich, E., Michaelides, M. C., and Cori, C. F. (1967), *Biochemistry 6*, 3695.

Hermans, J. J., and Levinson, S. (1951), *J. Optical Soc. Am. 41*, 460.

Huang, C. Y. (1968), Ph.D. Thesis, Iowa State University, Ames, Iowa.

Huang, C. Y., and Graves, D. J. (1967), 154th National Meeting of the American Chemical Society, Chicago, Ill.

Illingworth, B., and Cori, G. T. (1953), *Biochem. Prepn. 3*, 1.

Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.

Kent, A. B. (1959), Unpublished Ph.D. Thesis, University of Washington, Seattle, Washington.

Klapper, M. H., and Klotz, I. M. (1968), *Biochemistry 7*, 223.

Klotz, I. M. (1965), *Fed. Proc. 24*, S-24.

Klotz, I. M., and Farnham, S. B. (1968), *Biochemistry 7*, 3879.
Kratohvil, J. P., Dezelic, Gj., Kerker, M., and Matijevic, E. (1962), *J. Polymer Sci. 57*, 59.
Kruis, A. (1936), *Z. Physik. Chem. (Leipzig) 34B*, 13.
Kurganov, B. I. (1967), *Mol. Biol. 1*, 17.
Madsen, N. B., and Cori, C. F. (1956), *J. Biol. Chem. 223*, 1055.
Madsen, N. B., and Cori, C. F. (1957), *J. Biol. Chem. 224*, 899.
Maron, S. H., and Lou, R. L. H. (1954), *J. Polymer Sci. 14*, 29.
Metzger, B., Glaser, L., and Helmreich, E. (1968), *Biochemistry 7*, 2021.
Metzger, B., Helmreich, E., and Glaser, L. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 994.
Millar, D. B. S. (1962), *J. Biol. Chem. 237*, 2135.
Monod, J., Wyman, J., and Changuex, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Nicol, L. W., Bethune, J. L., Kegeles, G., and Hess, E. L. (1964), *Proteins 2*, 396.
Oncley, J. L. (1943), *J. Biol. Chem. 151*, 21.
Seery, V. L., Fischer, E. H., and Teller, D. C. (1967), *Biochemistry 6*, 3315.
Sinanoglu, O., and Abdulnur, S. (1965), *Fed. Proc. 24*, 2 S-12.
Sturtevant, J. M., Laskowski, M., Jr., Donnelly, T. H., and Sheraga, H. A. (1955), *J. Am. Chem. Soc. 77*, 6168.
Sutherland, E. W., and Wosilait, W. D. (1956), *J. Biol. Chem. 218*, 459.
Townend, R., and Timasheff, S. N. (1960), *J. Am. Chem. Soc. 82*, 3168.
Ullmann, A., Vagelos, P. R., and Monod, J. (1964), *Biochem. Biophys. Res. Commun. 17*, 86.
Wang, J. H., and Graves, D. J. (1963), *J. Biol. Chem. 238*, 2386.
Wang, J. H., and Graves, D. J. (1964), *Biochemistry 3*, 1437.
Wang, J. H., Shonka, M. L., and Graves, D. J. (1965a), *Biochem. Biophys. Res. Commun. 18*, 131.
Wang, J. H., Shonka, M. L., and Graves, D. J. (1965b), *Biochemistry 4*, 2296.
Wippler, C., and Scheibling, G. (1954), *J. Chem. Phys. 51*, 201.

Studies on an Aged Preparation of Nucleoside Diphosphatase. Kinetics and Reaction Mechanism*

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ABSTRACT: A kinetic study has been made of the reaction catalyzed by a purified preparation of nucleoside diphosphatase which has been subjected to prolonged storage at −10°.

Such treatment has been shown to affect some, but not all, of the properties of the enzyme. Thus both preparations have approximately the same molecular weight and are activated by the allosteric modifier, magnesium adenosine triphosphate ($MgATP^{2-}$). However, whereas the freshly prepared enzyme exhibits nonlinear double-reciprocal plots of velocity as a function of the magnesium inosine diphosphate ($MgIDP^-$) concentration and its maximum velocity is reduced in the presence of the modifier, the aged enzyme gives linear plots of $1/v$ *vs.* $1/MgIDP^-$ and its maximum velocity is unaffected by the addition of modifier. The kinetic data are consistent with a reaction mechanism in which it is proposed that the aged enzyme possesses distinct substrate and modifier sites, that there is random addition of one molecule (or independent combinations of multiple molecules) of each of these reactants to the enzyme under rapid equilibrium conditions and that the modifier facilitates the combination of substrate without influencing the rate of product formation. Values for the various kinetic constants have been determined.

It has been shown previously (Schramm and Morrison, 1968) that, after prolonged storage, the kinetic properties of the allosteric enzyme, nucleoside diphosphatase, are altered in that plots of $1/v$ *vs.* $1/MgIDP^-$ are linear, rather than curvilinear, as obtained with freshly prepared enzyme. Further, it was reported that the aged enzyme is still capable of being activated by the allosteric modifier, $MgATP^{2-}$. These results have prompted an investigation of the mechanism of the reaction as catalyzed by the aged enzyme. It was considered that since the reaction of substrate with this enzyme, both in the absence and presence of modifier, obeys Michaelis–Menten kinetics, the analysis and interpretation of the kinetic data would be less difficult than for those derived from investigations with the freshly prepared enzyme. Apart from their intrinsic interest, it was also of interest to compare the results with those obtained from studies on the freshly prepared enzyme.

The results of the present work lend support to the sug-

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