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Influence of Allosteric Ligands on the Activity and Aggregation of Rabbit Muscle Phosphofructokinase†

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ABSTRACT: The Stokes' radius of rabbit skeletal muscle phosphofructokinase at a concentration of 0.15 mg/ml in 0.1 M phosphate buffer, pH 7.0, 5°, was determined by frontal gel chromatography in the presence of substrates and allosteric effectors. Strong activators of the enzyme such as fructose 6-phosphate and fructose 1,6-bisphosphate stabilize a tetramer with a Stokes' radius of 67 Å, while citrate, a strong inhibitor of the enzyme, stabilizes smaller aggregates, probably monomers and dimers, with a weight-average Stokes' radius of 37 Å. Weaker activators such as Mg^{2+} and mixtures of citrate and fructose 6-phosphate stabilize aggregates with an intermediate average Stokes' radius. The specific activity of the enzyme could be quantitatively correlated with the aggregation state of the enzyme. The tetramer and larger aggregates have the maximum possible specific activity, while smaller aggregates possess very little, if any, enzymatic activity. Although MgATP is an inhibitor of the enzyme, it does not depolymerize the enzyme, suggesting its mode of inhibition differs from that of citrate. The rates of tetramer depolymerization and polymerization of smaller aggregates are rapid in the presence of citrate and activators, respectively, with typical equilibration times of a few minutes at 5°. The depolymerization is reversible for limited time periods, providing dithiothreitol is present. The depolymerization rate of higher aggregates to the tetramer after dilution to a protein concentration of 0.15 mg/ml is pH dependent with half-times of 42 and 10 min at pH 7.0 and 8.0, respectively, at 5°. On the other hand, the rate of tetramer depolymerization following dilution to a protein concentration of 0.15 mg/ml or below at pH 7.0, 5°, is slower, with a reaction half-time of about 1.5 hr. These results establish that the specific activity of phosphofructokinase is directly related to its aggregation state and that under physiological conditions the polymerization processes are quite rapid, with equilibration times of a few minutes or less.

Rabbit skeletal muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is of primary importance in the regulation of glycolysis. It is susceptible to activation and inhibition by a number of effectors (Passonneau and Lowry, 1962) and exhibits sigmoidal initial velocity–substrate concentration isotherms (Hofer and Pette, 1968). The enzyme exhibits concentration- and pH-dependent aggregation. At concentrations of about 0.2 mg/ml, pH 8.0, 0.1 M phosphate buffer, the enzyme exists as a fully active tetramer (mol wt 320,000, Stokes' radius 67 Å), while at lower pH values and concentrations dimers (and probably monomers) of lower specific activity are formed (Pavelich and Hammes, 1973). Aggregates larger than the tetramer, having the same specific activity as the tetramer, are formed at higher pH values and concentrations (Leonard and Walker, 1972; Aaronson and Frieden, 1972).

In this work, the influence of substrates and allosteric effectors on the aggregation state of the enzyme is reported. The results indicate that activators of the enzyme stabilize the tetramer, whereas citrate, an inhibitor of the enzyme (Passonneau and Lowry, 1963), stabilizes smaller aggregates, probably monomers and dimers. Moreover, a quantitative correlation between the Stokes' radius and specific activity of the enzyme is found. Although MgATP is an inhibitor of the enzyme, it does not extensively depolymerize the enzyme, suggesting the inhibition by MgATP operates through a mechanism other than disaggregation. The rates of polymerization and depolymerization are relatively rapid at a protein concentration of 0.15 mg/ml (pH 7.0, 5°) in the presence of activators and citrate, respectively, with typical equilibration times of a few minutes. However, the rate of depolymerization following dilution and the rate of polymerization under steady-state assay conditions are quite slow, with typical equilibration times of hours.

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Experimental Section

Materials. The ATP, ADP, AMP, fructose 6-phosphate, fructose 1,6-bisphosphate, aldolase, α-glycerophosphate de-

TABLE I: Stokes' Radii of Phosphofructokinase.[a]

Ligand	R_s (Å)
Citrate (5 mM)	37 (±1.5)
Potassium phosphate (pH 7.0, 0.1 M)	57 (±1.5)
Mg^{2+} (10 mM)	57 (±1.5)
MgAMP (5 mM)	61 (±3.0)
Mg^{2+} (0.5 mM)–ATP (5 mM)	61.5 (±3.0)
MgATP (5 mM)	65 (±3.0)
Potassium phosphate (pH 8.0, 0.1 M)	67 (±1.5)
Fructose 6-phosphate (0.1–10 mM)	66 (±1.5)
Fructose 1,6-bisphosphate (5 mM)	68 (±1.5)

[a] 0.1 M Tris-Cl (pH 7.0); Tris-Cl is not present when potassium phosphate is the ligand.

hydrogenase, triosephosphate isomerase, albumin (bovine), β-lactoglobulin, and thyroglobulin were purchased from Sigma Chemicals. All other reagents were the best available commercial products. Distilled deionized water was used in all experiments.

Phosphofructokinase. Rabbit skeletal muscle phosphofructokinase was purified by the procedure of Ling *et al.* (1966). The ammonium sulfate precipitate was taken up in pH 8.0, 0.1 M potassium phosphate–1.0 mM EDTA, and dialyzed against this buffer to give a stock solution of 10–14 mg/ml. The ratio of the absorbance at 280–260 nm varied from 1.5 to 1.6. This ratio was unchanged after passing the enzyme through a charcoal-cellulose (1:1) column (0.8 cm i.d. × 3 cm). The enzyme was generally used without charcoal treatment. However, some kinetic and Stokes' radius measurements were also made with the charcoal-treated enzyme. The results were essentially identical for the treated and untreated enzyme. The enzyme concentration was determined from the absorbance at 280 nm, using an extinction coefficient of 1.02 ml/(mg cm) (Parmeggiani *et al.*, 1966). The specific activity of the enzyme at 23° was 120–135 units/mg. The specific activity of the enzyme begins to decline after about 4 weeks. Only enzyme of high specific activity was employed for the measurements reported here.

Assays. The coupled assay procedure (Ling *et al.*, 1966) was used with minor modifications. The presence of ammonium sulfate in the auxiliary enzymes was found to cause a loss of sigmoidal kinetic behavior of the enzyme. Accordingly the auxiliary enzymes were dissolved in 0.01 M Tris Cl (pH 8.0), with 2 mg/ml of bovine serum albumin, and dialyzed overnight against the buffer. With dialyzed enzymes, the coupled assay showed the expected sigmoidal behavior for the enzyme at pH 6.9–7.0. The observed kinetic behavior of phosphofructokinase was in excellent agreement with that previously reported (Hofer and Pette, 1968).

Standard assays were run under the following conditions. At pH 8.0 the reaction mixture contained 33 mM Tris-Cl, 2 mM ATP, 5 mM $MgCl_2$ or $MgSO_4$, 2 mM fructose 6-phosphate, 0.1 mM NADH, 1 mM dithiothreitol, 0.25 unit/ml of aldolase, 3.2 units/ml of α-glycerophosphate dehydrogenase, 35 units/ml of triosephosphate isomerase, and 0.1 μg/ml of phosphofructokinase in a total volume of 3 ml (assay A). At pH 6.9–7.0 the reaction mixture contained 3 mM ATP, 3 mM $MgCl_2$ or $MgSO_4$, and fructose 6-phosphate at a concentration of 2.5 mM (assay B) or 1.2 mM (assay C). All other reagent concentrations were identical with those used in the pH 8.0 assay. The assays were initiated by addition of phosphofructokinase to the assay mixtures and the velocity of the enzymatic reaction was recorded spectrophotometrically using a thermostated Cary 14 spectrophotometer. When the cuvettes were maintained at 5°, the temperature was monitored with a thermocouple, and dry nitrogen was flushed through the cell compartment to prevent condensation. Assay velocities were unaltered by increasing the concentrations of the auxiliary enzymes. The concentration of NADH was sufficiently low to avoid inhibition of the auxiliary enzymes (Newsholme *et al.*, 1970).

Gel Chromatography. Column chromatography on agarose (1.5m resin, Bio-Rad Laboratories, lot 100543, 1.5 cm i.d. × 50 cm) was used to determine the Stokes' radius of phosphofructokinase. The top and bottom of each column contained 0.8-cm plugs of Sephadex G-25 to ensure stable agarose interfaces. A fixed concentration of the enzyme, 0.15 mg/ml, was used for all elutions which were carried out at 5°. Elution profiles were analyzed by measuring the absorbance at 280 nm. In cases where the buffer contained adenine nucleotides, the profiles were analyzed by determining the protein concentrations (Lowry *et al.*, 1951). The details of the experimental procedure and the analysis of the results were as previously presented (Pavelich and Hammes, 1973). A frontal elution procedure was employed (Winzor, 1969). The distribution coefficient was calculated by the method of Laurent and Killander (1964) and the Stokes' radius was determined, by comparison with proteins of known Stokes' radii (Ogston, 1958).

Light-scattering measurements were made with a Bausch and Lomb Photo Gonio diffusometer thermostated at 6° using the 546-nm filter. Cells were cleaned with chromic acid, rinsed extensively with dust-free water, and oven dried before use. Solutions were rendered dust free with a 0.45 μ Millipore filter. Phosphofructokinase was diluted from a concentrated stock solution (>10 mg/ml) directly into the light-scattering cell containing 0.1 M potassium phosphate (pH 7.0), 1.0 mM EDTA, and 1 mM dithiothreitol. An alternative dilution procedure was to mix a phosphofructokinase solution (0.3–0.15 mg/ml) equilibrated for 3 hr in 0.1 M potassium phosphate (pH 8.0), 1.0 mM EDTA, 5.0 mM dithiothreitol with an equal volume of 0.1 M potassium phosphate (pH 6.44), 1.0 mM EDTA, and 5.0 mM dithiothreitol to give a final solution of pH 7.0. Changes of light-scattering intensity with time were followed at 90°. In cases where small changes in scattering intensity were to be measured, a tenfold increase in sensitivity was achieved with a Preston Scientific 8300XWB differential amplifier, together with a Keithly Instruments Model 260 nanovolt source as a stable offset voltage.

Results

The Stokes' radii of phosphofructokinase at 0.15 mg/ml, 5°, obtained by agarose chromatography in the presence of activators and inhibitors are given in Table I. Attempts to determine a Stokes' radius in 0.1 M Tris-Cl in the absence of any effectors were made, but under these conditions the enzyme rapidly loses activity and precipitates from solution. Some experiments were done with 1 mM dithiothreitol present in the elution buffer. This had no significant effect on the Stokes' radius. The variation of Stokes' radius with the concentration of the activator, fructose 6-phosphate, was also studied. The Stokes' radius was found to be constant as the concentration of the activator was varied from 10 mM to about 0.1 mM. At concentrations less than 0.1 mM, which is approximately the Michaelis constant for fructose 6-phosphate, the enzyme became unstable, lost activity, and precipitated before the

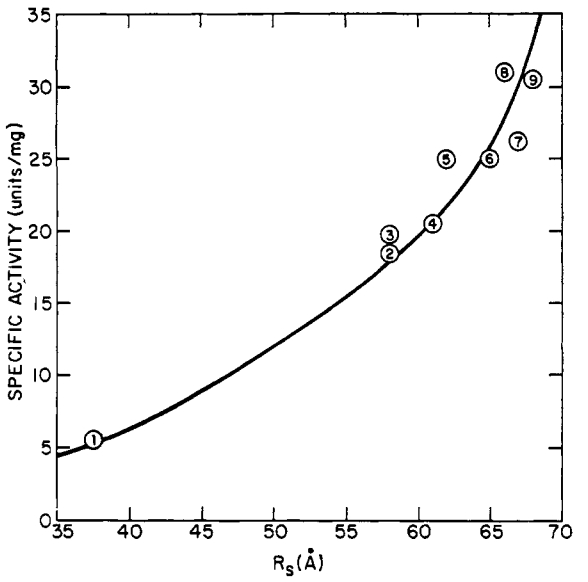

FIGURE 1: Correlation of the Stokes' radius, R_s (determined by gel chromatography at 5°), with specific activity. The enzyme was incubated at a concentration of 0.15 mg/ml in 0.1 M Tris-Cl (pH 7.0), in the presence of the ligands at the concentrations given below. The specific activities were determined by dilution into assay mixture A (see Experimental Section) at pH 8.0, 5°. The concentration of phosphofructokinase in the assay mixture was 0.1 μg/ml. The numbers indicate the following ligands: 1, 5 mM citrate; 2, 0.1 M phosphate, pH 7.0; 3, 10 mM Mg^{2+}; 4, 5 mM MgAMP; 5, 5 mM ATP–0.5 mM Mg^{2+}; 6, 5 mM MgATP; 7, 0.1 M phosphate, pH 8.0; 8, 10 mM fructose 6-phosphate; and 9, 5 mM fructose 1,6-bisphosphate.

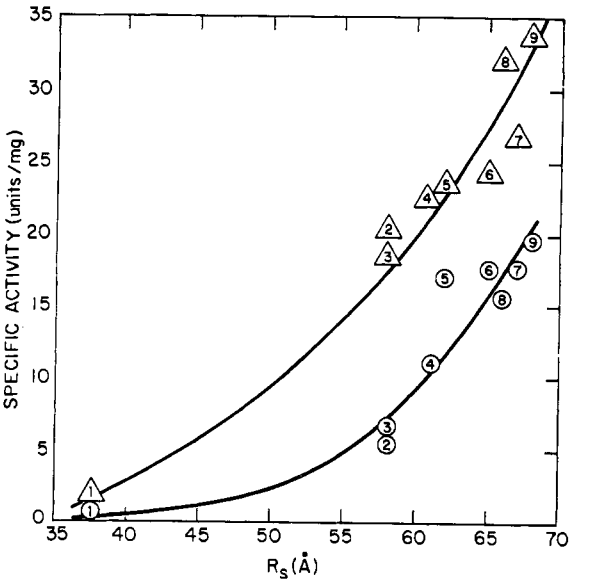

FIGURE 2: Correlation of Stokes' radius, R_s (determined by gel chromatography at 5°), with specific activity. The enzyme was incubated at pH 7.0, 5°, at a concentration of 0.15 mg/ml in the presence of the ligands at the concentrations given in the legend of Figure 1. Specific activities were determined by dilution of the enzyme into assay mixtures B (△) and C (○). The numbers indicate the same ligands given in the legend of Figure 1.

chromatographic run (requiring about 6 hr) could be completed.

The correlation between the Stokes' radius and activity is shown in Figures 1 and 2. For these activity measurements the enzyme was incubated for about 5 hr at 5°, 0.15 mg/ml in 0.1 M Tris-Cl (pH 7.0), with the concentration of the effectors given in Table I. The specific activity was then measured by dilution of the enzyme to 0.1 μg/ml in the standard assay mixtures at pH 7.0 and 8.0. At pH 7.0 two different concentrations of fructose 6-phosphate were used in the assay mixture. At a concentration of 2.5 mM fructose 6-phosphate a maximal velocity has been reached (assay B); at a concentration of 1.2 mM fructose 6-phosphate, the velocity is about half-maximal value (assay C). The velocity–fructose 6-phosphate curve is quite sigmoidal under the assay conditions employed. In assay B the correlation is between the Stokes' radius and the maximal velocity, whereas in assay C the correlation involves both the maximal velocity and the apparent Michaelis constant for fructose 6-phosphate. At pH 8.0 assays were done at 23° with results similar to those shown in Figure 1 except that larger velocities were measured.

The implicit assumption is made that activities measured in this manner reflect the state of the enzyme in the incubation mixture, rather than in the assay mixture. Two pieces of evidence in support of this assumption are that increasing the enzyme concentration threefold in the assay mixture gives the same specific activity, and the assay is linear with time for 5–6 min. This matter will be considered further in the Discussion section.

The results presented indicate that 5 mM citrate causes the formation of a species with a relatively small Stokes' radius possessing very little activity. This effect of citrate can be readily reversed by activators. In Figure 3, the change in specific activity and Stokes' radius with increasing fructose 6-phosphate concentrations is shown. The solutions contained 5 mM citrate and 0.1 M Tris-Cl (pH 7.0). The Stokes' radii were determined after incubation of the enzyme for about 2 hr at a concentration of 0.15 mg/ml in 5 mM citrate, the desired amount of fructose 6-phosphate and 0.1 M Tris-Cl (pH 7.0). The specific activities, also presented in Figure 3, were measured as described before (assays B and C). The enzyme is transformed from a relatively small species to a fully active aggregate with a Stokes' radius identical with that found at fructose 6-phosphate concentrations greater than 0.1 mM in the absence of citrate. This transition takes place over a narrow concentration range and is sigmoidal in nature.

Rates of Polymerization and Depolymerization. As shown in Figure 4 the specific activity of phosphofructokinase decreases when it is diluted from a concentrated stock solution (>10 mg/ml in 0.1 M potassium phosphate (pH 8.0)–1 mM EDTA) into 0.1 M potassium phosphate (pH 7.0), 1 mM EDTA, and 1 or 5 mM dithiothreitol to a final enzyme concentration of 0.15 mg/ml or less. Both the final equilibrium specific activity and the rate of approach to equilibrium are dependent on the final protein concentration. Plots of ln ($SA_t - SA_\infty$) *vs.* time are linear (SA_t is the specific activity at time t) and the apparent first-order rate constants are 0.551, 0.505, and 0.410 hr^{-1} at final protein concentrations of 0.029, 0.072, and 0.15 mg per ml, respectively.

Since the Stokes' radii are concentration dependent under these conditions and aggregates smaller than the tetramer (R_s = 67 Å) have low specific activities (Pavelich and Hammes, 1973; Hofer and Pette, 1968; Aaronson and Frieden, 1972), the changes in specific activity on dilution can be reasonably attributed to changes in the aggregation state of the enzyme. This hypothesis was tested directly by measuring changes in the light-scattering intensity at 90°, i_{90}, following dilution of the enzyme. The i_{90} of dust-free spectroscopic grade benzene was adjusted to 0.8 before each measurement. Dilution by 100-fold from concentrated stock solutions of enzyme

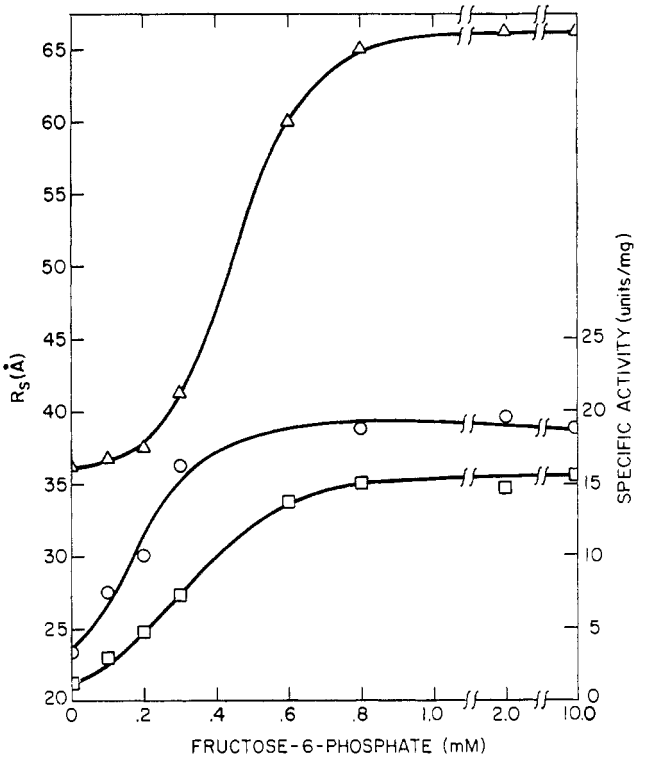

FIGURE 3: Variation of the Stokes' radius, R_s (△), and specific activity (○,□) of phosphofructokinase incubated at a phosphofructokinase concentration of 0.15 mg/ml in 0.1 M Tris-Cl (pH 7.0)–5 mM citrate, with varying fructose 6-phosphate concentrations. The specific activities were measured by dilution to 0.1 μg/ml in assay mixtures B (○) and C (□).

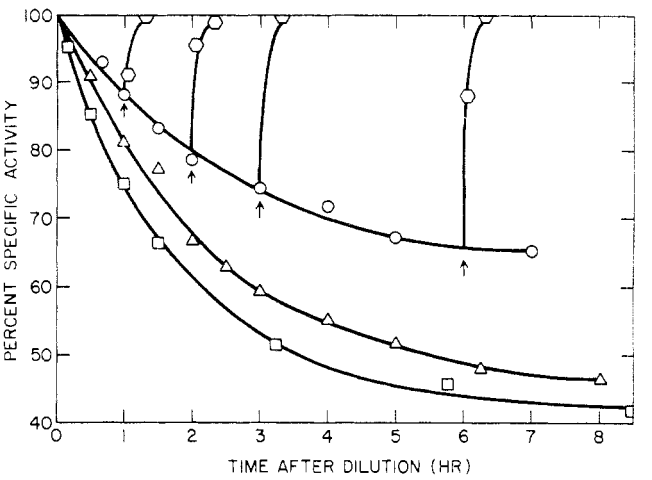

FIGURE 4: The change in specific activity with time following dilution of phosphofructokinase from a concentrated stock solution (>10 mg/ml) in 0.1 M potassium phosphate–1 mM EDTA (pH 8.0), into 0.1 M potassium phosphate, 1 mM EDTA, and 1 or 5 mM dithiothreitol, pH 7.0 at 5°. The final enzyme concentrations and the Stokes' radii at the equilibrium specific activity (Pavelich and Hammes, 1973) were 0.15 mg/ml (R_s = 57 Å), ○; 0.072 mg/ml (R_s = 53 Å), △; and 0.029 mg/ml (R_s = 50 Å), □. The assay used was identical with that described in the legend of Figure 1. The initial specific activity was 37.5 units/mg. The arrows indicate the times at which concentrated fructose 1,6-bisphosphate (final concentration 5 mM) was added to 1-ml aliquots of the 0.15-mg/ml enzyme sample containing 5 mM dithiothreitol. The subsequent change in specific activity of the aliquots is indicated by ○. The rate of activity loss was identical in the presence of both 1 and 5 mM dithiothreitol, but full reactivation with fructose 1,6-bisphosphate required 5 mM dithiothreitol.

(>10 mg/ml) caused much larger changes in i_{90} than possible for depolymerization of a tetramer. Moreover, first-order plots of the data were quite nonlinear. This is due to the fact that phosphofructokinase exists as aggregates larger than the tetramer at protein concentrations greater than 1 mg/ml (Aaronson and Frieden, 1972; Leonard and Walker, 1972). The apparent reaction half-life for depolymerization of higher aggregates at a final protein concentration of 0.15 mg/ml was 42 and 10 min for pH 7.0 and 8.0, respectively.

In order to measure the rate of depolymerization of the tetramer, the enzyme was initially diluted to 0.15 or 0.30 mg per ml at pH 8.0 (0.1 M potassium phosphate–1 mM EDTA–5 mM dithiothreitol) and allowed to equilibrate for 3 hr. Under these conditions the enzyme is a tetramer (Pavelich and Hammes, 1973). The equilibrated enzyme was then mixed with an equal volume of pH 6.44 buffer (0.1 M potassium phosphate–1 mM EDTA–5 mM dithiothreitol) to give either 0.075 or 0.15 mg per ml of enzyme at pH 7.0. The change in light scattering following mixing for a typical experiment is shown in Figure 5. The change in light scattering follows first-order kinetics with rate constants of 1.79 and 1.53 hr^{-1} at 0.15 and 0.075 mg per ml, respectively. The changes in light scattering, which reflect changes in the weight-average molecular weight, correlate well with the specific activity changes as shown in Figure 6.

The rate of change of specific activity was the same for 100-fold dilution or 2-fold dilution to the same final protein concentration at pH 7.0. On the other hand, 100-fold dilution from a concentrated stock solution to give 0.15 mg/ml of enzyme at pH 8.0 (0.1 M potassium phosphate buffer–1 mM EDTA–1 mM dithiothreitol) resulted in no loss of activity with time. This indicates that the tetramer and higher aggregates have the same specific activity (*cf.* Aaronson and Freiden, 1972) and that the dissociation of higher aggregates to give the tetramer is much faster than dissociation of the tetramer. These conclusions are also supported by the quantitative rate data presented above.

Some information also can be obtained about the rate of polymerization of the enzyme by adding the allosteric activator, fructose 1,6-bisphosphate, to dilute solutions of phosphofructokinase. In Figure 4, the effect on the specific activity of adding fructose 1,6-bisphosphate (to a final concentration of 5 mM) to aliquots of 0.15 mg/ml of enzyme in 0.1 M potassium phosphate (pH 7.0), 1 mM EDTA, and 5 mM dithiothreitol is shown at various times after 100-fold dilution of the enzyme from a concentrated stock solution. The aggregation equilibria are completely reversible up to 6 hr, but become irreversible at longer times. The rate of polymerization is quite rapid. The half-life of the reaction is certainly less than 5 min. The presence of dithiothreitol is an absolute requirement for reversibility; a concentration of 1 mM is sometimes sufficient, and 5 mM dithiothreitol is always adequate. Curiously, the final equilibrium specific activity reached upon dilution is identical in the presence or absence of dithiothreitol. As a final control, addition of 5 mM fructose 1,6-bisphosphate to the diluted protein gave the constant specific activity of the tetramer for over 10 hr.

The rate of depolymerization of phosphofructokinase also was measured by dilution to 0.15 mg/ml at pH 8.0 and 7.0 in 0.1 M Tris-Cl, 5 mM citrate, and 5 mM dithiothreitol. The decrease in specific activity as a function of time at pH 7.0 is shown in Figure 7. The final equilibrium specific activity is reached in about 3 min. Reactivation by the addition of 5 mM fructose 1,6-diphosphate led to 95% recovery of activity 10 min after dilution and to only 60% recovery of activity after 1

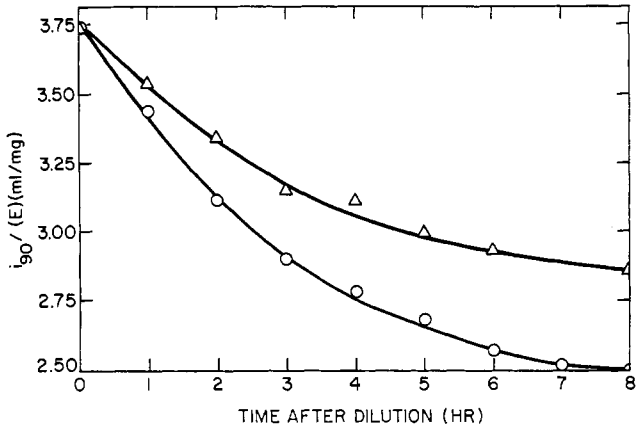

FIGURE 5: The change in light-scattering intensity at 90° divided by the enzyme concentration in mg/ml, i_{90}/(E), with time after mixing 0.15 or 0.30 mg per ml of phosphofructokinase in 0.1 M potassium (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol with an equal volume of 0.1 M potassium phosphate (pH 6.44), 1 mM EDTA, and 5 mM dithiothreitol to give a final enzyme concentration of 0.075 mg/ml, ○, or 0.15 mg/ml, △, at pH 7.0 and 6°. The i_{90} of spectroscopic grade benzene was adjusted to 0.800 for all measurements.

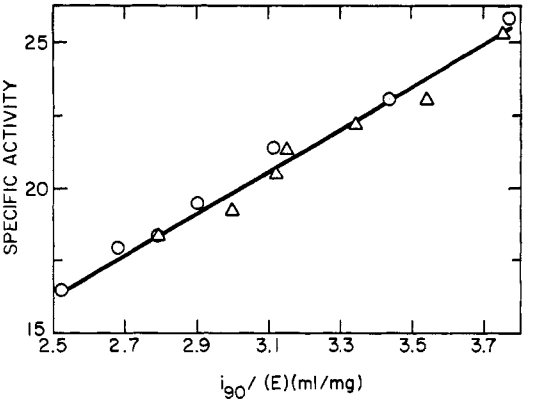

FIGURE 6: The correlation of specific activity with the specific light-scattering intensity at 90°, i_{90}/(E), at phosphofructokinase concentrations of 0.075 mg/ml, ○, and 0.15 mg/ml, △. The light-scattering data were taken from Figure 5 and assays were done as described in Figure 1.

hr, as shown in Figure 7. The half-time for reactivation is again less than 5 min. The omission of dithiothreitol led to much lower levels of the recovery of activity. Again, however, the final specific activity reached is independent of the amount of dithiothreitol present. Only the extent of reactivation is altered. The final specific activity is constant for more than 6 hr.

Discussion

Rabbit muscle phosphofructokinase is known to undergo aggregation reactions that are concentration and pH dependent (*cf.* Aaronson and Frieden, 1972; Pavelich and Hammes, 1973). In the pH range 6–8 in 0.1 M phosphate buffer, the dependence of the Stokes' radius on concentration can be semiquantitatively described by a dimer–tetramer equilibrium below enzyme concentrations of 0.2 mg/ml (Pavelich and Hammes, 1973). The tetramer has a Stokes' radius of 67 Å, while that of the dimer is postulated to be 44 Å. Kinetic studies have indicated that a number of ligands alter the activity of the enzyme at pH 7.0 (*cf.* Passonneau and Lowry, 1962; Hofer and Pette, 1968). The results shown in Table I and Figures 1–3 indicate that a good correlation exists between the Stokes' radius of the enzyme and its specific activity in the presence of various activators and inhibitors. At a protein concentration of 0.15 mg/ml, pH 7.0, strong activators such as fructose 6-phosphate and fructose 1,6-bisphosphate stabilize an essentially tetrameric species with a Stokes' radius of approximately 67 Å. Activators have been reported to stabilize a trimer in the case of yeast phosphofructokinase (Hofmann *et al.*, 1972). Citrate, a potent inhibitor of the enzyme (Passonneau and Lowry, 1963), stabilizes a species with an average Stokes' radius of only 37 Å. These results clearly indicate that the maximum specific activity of the enzyme is attained with the tetrameric species, while smaller aggregates have very little activity. The assay at pH 8 indicates only 15% of the maximal activity in the presence of citrate, while at pH 7, only 2–5% of the maximal activity is found.

The nature of the aggregation states smaller than the tetramer is uncertain. The species stabilized by citrate has too small a Stokes' radius for a dimer (mol wt 160,000). The data in Figure 3 indicate the correlation between Stokes' radius and specific activity is continuous, and it seems most likely that the tetramer depolymerizes to both monomer and dimer in the presence of citrate, both possessing very little enzymatic activity. The detailed nature of the aggregates in the presence of citrate is currently under investigation.

The idea that specific ligand binding is directly responsible for the stabilization of particular aggregation states is supported by the fact that reducing the concentration of fructose 6-phosphate below its apparent Michaelis constant results in dissociation and inactivation of the enzyme. The inactivation and depolymerization by citrate can be reversed by fructose 6-phosphate as shown in Figure 3. This reversal is sigmoidal in nature, suggesting cooperative binding and/or polymerization. Binding studies have indicated citrate is a noncompetitive inhibitor of fructose 6-phosphate (Kemp and Krebs, 1967).

A surprising result is that MgATP stabilizes the tetrameric species, although MgATP is known to inhibit the enzyme at high concentrations. Essentially a tetramer is observed even when the Mg^{2+} concentration is ten times less than that of ATP, indicating the tetramer is not being stabilized by free

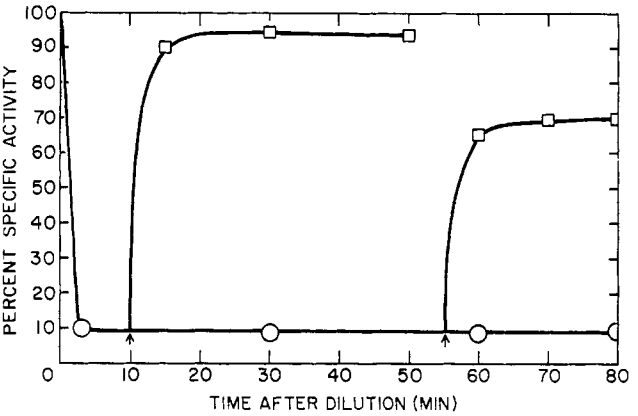

FIGURE 7: The rate of specific activity loss upon dilution of phosphofructokinase from a concentrated stock solution (>10 mg/ml) to an enzyme concentration of 0.15 mg/ml in 0.1 M Tris-Cl, 5 mM citrate, and 5 mM dithiothreitol (pH 7.0) at 5°. The assays were carried out by dilution to 0.1-μg/ml enzyme at pH 7.0, 5° in assay mixture C (see Experimental Section). The initial specific activity was 25 units/mg and the equilibrium Stokes' radius was 37 Å. The arrows indicate times at which concentrated fructose 1,6-bisphosphate (final concentration 5 mM) was added to 1-ml aliquots of the enzyme. The subsequent changes in specific activity of the aliquots are indicated by □.

Mg^{2+} when equimolar concentrations are used. This result suggests that the mechanism of inhibition of enzyme activity by MgATP probably does not involve depolymerization or that the binding of ATP to sites other than the inhibitory sites stabilizes the tetramer under the incubation conditions.

The correlation of enzyme specific activity with Stokes' radius is approximately the same whether the enzyme is assayed at pH 7 or 8 or when the initial velocities are obtained at a concentration of fructose 6-phosphate such that the enzyme is saturated or half-saturated. Thus the assays all apparently reflect the same aggregation state, namely that in the incubation mixture, and the primary correlation is between the maximal velocity and aggregation state.

As expected from the above discussion, phosphofructokinase undergoes parallel decreases in activity and molecular weight following dilution from conditions which stabilize the tetramer and higher aggregates to conditions where aggregates smaller than the tetramer are found (*cf.* Figures 4 and 5). A quantitative correlation between the specific light-scattering intensity and activity changes is found (Figure 6), but the kinetics cannot be quantitatively interpreted since the exact nature of the aggregation states is not known. This depolymerization is relatively slow, having a reaction half-time of about 1.5 hr at 5°. The apparent first-order rate constant is somewhat dependent on the final protein concentration. A similar correlation between specific light-scattering intensity and specific activity due to changes in pH has been reported by Paetkau and Lardy (1967). In contrast, depolymerization of the tetramer to small aggregates (weight-average Stokes' radius of 37 Å) is very rapid in the presence of citrate with a reaction half-time at 5° of less than 1 min (Figure 7). Light-scattering measurements also indicate that the rate of depolymerization of higher aggregates to the tetramer is relatively rapid, with a reaction half-time of about 10 min at pH 8, 5°. A parallel loss in specific activity is not observed confirming that the tetramer and higher aggregates have essentially the same specific activity (Paetkau and Lardy, 1967; Aaronson and Frieden, 1972; Pavelich and Hammes, 1973).

Addition of fructose 1,6-bisphosphate (final concentration, 5 mM) to diluted solutions of phosphofructokinase causes a relatively rapid increase in activity and molecular weight with a reaction half-time less than 5 min at 5° (Figure 5). The aggregation is reversible for up to 6 hr at a relatively high concentration of dithiothreitol (5 mM). Polymerization in the presence of inhibitory concentrations of citrate proceeds at a similar rate, but is less reversible even in the presence of 5 mM dithiothreitol (Figure 7). Apparently the enzyme sulfhydryl groups must be in a reduced state for polymerization to the tetramer. Reduced thiols previously have been reported to influence the molecular weight of the enzyme (Paetkau and Lardy, 1967). The final equilibrium molecular weight and specific activity are constant in the presence or absence of dithiothreitol for at least 1 day, suggesting some very unusual aggregation equilibria are occurring.

The assumption that the steady-state assay reflects the aggregation state of the enzyme in the incubation mixture of enzyme (at 0.15 mg/ml) and ligand can now be defended more rigorously. The half-time for depolymerization of the tetramer on dilution is so long (about 1.5 hr) that the tetramer cannot significantly dissociate during the assay procedure. On the other hand, polymerization to the tetramer in the presence of activators has a half-time of a few minutes at a protein concentration of 0.15 mg/ml. Since polymerization is protein concentration dependent, its half-time must be significantly longer under steady-state assay conditions (0.1 μg/ml), and it can be reasonably extrapolated that polymerization will not significantly influence the steady-state assay. Because of the slowness in response of the steady-state assay to effector molecules, phosphofructokinase has been postulated to belong to a class of enzymes termed "hysteretic" (Frieden, 1970). However, in the case of phosphofructokinase this is unlikely to be physiologically relevant. The physiological concentration of the enzyme is of the order of magnitude of a few tenths of a milligram per milliliter (calculated from the data of Parmeggiani *et al.* (1966) according to the method of Srere, 1967). The results reported here indicate that in the presence of the activator fructose 1,6-bisphosphate and the inhibitor citrate, polymerization and depolymerization have characteristic half-times of a few minutes at pH 7 and 5°. At higher temperatures, the rate processes would be even faster. Therefore, under physiological conditions any regulation by polymerization–depolymerization reactions probably is quite rapid, with characteristic half-times of 1 min or less.

Aggregation phenomena may account for a discrepancy in the literature, namely the binding of effectors and substrates to the enzyme is associated with hyperbolic binding isotherms (with the exception of ATP; Kemp and Krebs, 1967; Kemp, 1969) while sigmoidal steady-state velocity isotherms are observed (*cf.* Hofer and Pette, 1968). The equilibrium binding experiments have been done under conditions where the enzyme is a tetramer or larger, while the steady-state experiments are done under conditions where aggretates smaller than the tetramer may exist. Preferential binding to particular enzyme aggregates can produce sigmoidal binding and kinetic isotherms (*cf.* Nichol *et al.*, 1967). Binding experiments are currently being done under conditions where enzyme species smaller than the tetramer are present in the absence of ligands. The binding of fructose 6-phosphate to sheep heart phosphofructokinase has been found to be dependent on the protein concentration, as anticipated for an aggregating system (Lorenson and Mansour, 1969).

All available data are consistent with a model where the depolymerization of higher aggregates to the tetramer is relatively rapid and is pH and protein concentration dependent. Depolymerization of the tetramer to lower aggregates is also pH and protein concentration dependent; at pH 7.0 it is slow in the absence of citrate, but is quite rapid in the presence of citrate. Polymerization of lower aggregates is rapid in the presence of activators (*e.g.*, fructose 1,6-bisphosphate) at physiological enzyme concentrations of tenths of a milligram per milliliter. The results reported here clearly establish that the specific activity of rabbit muscle phosphofructokinase is altered by changes in aggregation state induced by allosteric ligands. They do not establish the detailed pathway for the alteration of specific activity. In particular it is not clear whether aggregation equilibria or conformational equilibria are the primary causes of changes in specific activity. Finally, it cannot be proven that polymerization–depolymerization is an important physiological mode of regulation; the enzyme and ligand concentrations are certainly appropriate for this to be feasible. However, aggregation equilibria may not be the only mode of regulation. They have not yet been shown to account for the sigmoidal initial velocity isotherms which have been observed (Hofer and Pette, 1968) and probably do not account for inhibition of the reaction by MgATP.

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Threonine-Sensitive Aspartokinase from *Escherichia coli*. Magnetic Resonance and Binding Studies†

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ABSTRACT: The interaction of manganese and manganese–ATP with the threonine-sensitive aspartokinase–homoserine dehydrogenase complex of *E. coli* K 12, Tir-8 has been investigated by nuclear magnetic resonance and direct binding techniques. An enhancement in the proton relaxation rate of 14.1 was found for the binary metal–enzyme complex. This enhancement was reduced to 10.1 in the presence of threonine with no significant change in the affinity of the enzyme for manganese. The ternary complex of aspartokinase with metal–ATP had an enhancement of 10.2. Threonine did not change the enhancement but increased the dissociation constant for Mn–ATP from 1 to 2.8 mM. We have found from kinetic studies apparent competitive inhibition between threonine and Mn–ATP. The binding studies indicate that there are four sites for manganese and ATP and are consistent with four sites for Mn–ATP. Manganese–ATP appears to bind the aspartokinase as a metal-bridge complex. A change at the Mn binding site occurs upon addition of threonine which is reflected by the relaxation rate of water at the active site but the cause of this change in enhancement has not been determined.

Threonine-sensitive aspartokinase of *Escherichia coli* is part of an enzyme complex consisting of aspartokinase I–homoserine dehydrogenase I (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4; L-homoserine:NADP oxidoreductase, EC 1.1.1.3) which catalyzes the first and third steps in the sequence of reactions leading to the biosynthesis of threonine from aspartate. It has recently been shown that the catalytically active form of aspartokinase–homoserine dehydrogenase consists of four identical subunits (Falcoz-Kelly *et al.*, 1972; Wampler, 1972) and that the two activities reside on opposite ends of each polypeptide chain (Véron *et al.*, 1972). Binding studies have indicated four sites for NADPH (Janin *et al.*, 1969; Falcoz-Kelly *et al.*, 1972) and six to eight sites for threonine (Janin and Cohen, 1960; Takahashi and Westhead, 1971). It has been assumed (Heck, 1972) that the enzyme has four binding sites for aspartate and ATP. In the present report we will show data indicating four binding sites for manganese and ATP and also data which is consistent with four manganese–ATP sites.

Both enzyme activities are subject to inhibition by threonine. For homoserine dehydrogenase the inhibition by threonine is noncompetitive with respect to NADPH and aspartic semialdehyde (Patte *et al.*, 1963). For aspartokinase, Stadtman *et al.* (1961) have found competitive inhibition between threonine and aspartate, but the competitive nature of the inhibition has been questioned by Wampler and Westhead (1968). Wampler and Westhead (1968) have also found that the curve of per cent inhibition *vs.* threonine concentration is shifted toward higher threonine concentrations as ATP is increased, indicating antagonism between ATP and threonine. Our kinetic studies show threonine is competitive with ATP at saturating aspartate.

It has been postulated that control of both enzyme activities is produced by a threonine-induced change from a relaxed configuration of the enzyme to a tight configuration

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