formational changes, whereas that associated with ATP and CTP binding probably involves a stepwise change in the conformation of the subunits. A detailed molecular picture of the control processes remains to be specified as a better understanding of the structure of the enzyme emerges.

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Aggregation of Rabbit Muscle Phosphofructokinase†

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ABSTRACT: Rabbit skeletal muscle phosphofructokinase has been found to consist of polypeptide chains of essentially identical molecular weight. This molecular weight was found to be 75,000 (±10,000) by acrylamide gel electrophoresis in sodium dodecyl sulfate and 85,000 (±10,000) by gel chromatography in 6 and 7 M guanidine-HCl–0.1 M β-mercaptoethanol. The aggregation state of the enzyme was studied by frontal gel chromatography in 0.1 M phosphate buffer over the pH range 6.0–8.0 and the protein concentration range 0.001–1.0 mg/ml. The results obtained indicate that a stable tetramer with a Stokes' radius of 67 Å and a molecular weight of approximately 320,000 exists over an appreciable concentration range at pH 8.0. This tetrameric aggregate can be broken down into smaller species by lowering either the protein concentration or pH. The data at concentrations less than 0.2 mg/ml can be described by a mechanism involving a pH-dependent equilibrium between a dimer and tetramer, and information about the shape of the aggregates has been inferred. The enzymatic activity appears to be dependent on the aggregation state of the enzyme, with aggregates smaller than the tetramer not possessing appreciable enzymatic activity.

Rabbit skeletal muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) exhibits kinetic properties in the physiological pH range which are indicative of an allosteric enzyme (Hofer and Pette, 1968b). Sigmoidal initial velocity–substrate concentration isotherms are observed, and the enzymatic reaction is susceptible to activation and inhibition by a number of nonsubstrate effectors (Passonneau and Lowry, 1962). Because of these properties and its strategic location in the metabolic process, phosphofructokinase is considered to be of primary importance in the regulation of glycolysis.

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The enzyme is known to exist in a number of interconvertible polymeric forms (Ling *et al.*, 1965; Mansour, 1965; Parmeggiani *et al.*, 1966). In phosphate buffer, at concentrations greater than 1 mg/ml, fully active aggregates having molecular weights of approximately 320,000–360,000 and larger have been observed in ultracentrifuge studies, and the concentration and pH dependence of the aggregation have been determined (Aaronson and Frieden, 1972; Leonard and Walker, 1972). An inactive aggregate having a molecular weight of approximately 160,000–180,000 also has been found at pH 6.0 (Aaronson and Frieden, 1972). Since a knowledge of the aggregation state of the enzyme is essential for an understanding of the kinetic and regulatory properties of the enzyme, a study of its polymerization at protein concentrations below 1 mg/ml in phosphate buffer has been conducted using gel chromatography. The results obtained indicate that extensive dissociation of the 320,000–360,000 molecular weight aggregate occurs at low pH and protein concentrations, and that smaller aggregates do not have appreciable enzymatic activity. The enzyme also was shown to consist of polypeptide chains having a molecular weight of approximately 80,000 by acrylamide gel electrophoresis in sodium dodecyl sulfate and by gel chromatography in guanidine-HCl.

Experimental Section

Materials. The sodium dodecyl sulfate, purchased from Schwarz BioResearch, was recrystallized once from absolute ethanol before use. Guanidine hydrochloride (Grade I), purchased from Sigma Chemical, was used without further purification. Other chemicals were reagent grade or the highest purity commercially available. The proteins aldolase (rabbit muscle), α-glycerophosphate dehydrogenase–triose phosphate isomerase (rabbit muscle), albumin (bovine), β-galactosidase (*Escherichia coli*), L-glutamic dehydrogenase (bovine liver, Type I), and phosphorylase *a* (rabbit muscle) were purchased from Sigma Chemical; β-lactoglobulin (bovine milk) was purchased from Pentex; and aspartate transcarbamylase (*E. coli*) was a gift from Mr. Steven Matsumoto. Deionized distilled 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 dipotassium phosphate–1.0 mM EDTA, and dialyzed against this buffer to provide stock solutions of 10–13 mg/ml. The ratio of the absorbance at 280 nm to that at 260 nm for the purified enzyme varied from 1.58 to 1.64. The enzyme concentration was determined from the 280-nm absorbance using an extinction coefficient of 1.02 ml/(mg cm) (Parmeggianti *et al.*, 1966). Activity was measured using a coupled assay system at pH 8.0 (Ling *et al.*, 1966). The specific activity at 28° varied from 140 to 160 units per mg (*cf.* Ling *et al.* for the definition of a unit). When the enzyme was assayed at room temperature (~23°), values of 105–120 units/mg were obtained.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. The sodium dodecyl sulfate gel electrophoresis was carried out as described by Weber and Osborn (1969), except that a Tris-Cl buffer system was used and a dilute spacer gel was employed to ensure sharp protein bands. The separatory gel was prepared by adding 0.15 ml of 0.1-g/ml ammonium persulfate to a solution containing 6.75 ml of acrylamide stock solution (30 g of acrylamide and variable amounts of methylenebisacrylamide made up to100 ml with water), 7.5 ml of 1.0 M Tris-Cl at pH 8.8, 0.01 ml of tetramethylenediamine, 5.5 ml of water, and 0.10 ml of 20% sodium dodecyl sulfate. The spacer gel was prepared by adding 0.025 ml of 0.1-g/ml ammonium persulfate to a solution containing 0.5 ml of acrylamide stock solution (0.8 g of methylenebisacrylamide/100 ml), 1.25 ml of 0.5 M Tris-Cl at pH 6.9, 0.003 ml of tetramethylenediamine, 3.75 ml of water, and 0.025 ml of 20% sodium dodecyl sulfate. The glass gel tubes were 13 cm in length with a 0.5-cm i.d. The separatory gels were normally 10 cm in length and the spacer gels 0.7 cm.

The proteins were denatured by heating a solution containing 0.1–0.5 mg/ml of protein, 0.1 M β-mercaptoethanol, and variable amounts of sodium dodecyl sulfate at 95–100° for 10 min. The solution was cooled and 0.3 ml were combined with 0.025 ml of 1 M β-mercaptoethanol, 0.10 ml of 0.5 M Tris-Cl at pH 6.9, 0.025 ml of 0.05% Chlorophenol Red, and two drops of glycerol. The solution was allowed to stand at room temperature for 2 hr before 0.01–0.1 ml was layered on the spacer gel. Electrophoresis was conducted at 2.5 mA/tube. The electrophoresis buffer was 0.025 M Tris, 0.19 M glycine, and 0.1% sodium dodecyl sulfate by weight. The staining and destaining of the gels as well as the measurement of protein mobilities were carried out as described by Weber and Osborn (1969).

Guanidine-HCl Gel Chromatography. Proteins were denatured in 6.0 or 7.0 M guanidine-HCl–0.1 M β-mercaptoethanol and chromatographed essentially as described by Fish *et al.* (1969). Phosphofructokinase was denatured by adding solid guanidine-HCl and β-mercaptoethanol to enzyme solutions. In calculating the amounts of reagents to be added, it was assumed that both 6.0 and 7.0 M guanidine-HCl solutions have a density of 1.2 g/cm^3. Bio-Gel A-15m (Bio-Rad Laboratories, lot 97813) was packed to a height of 62 cm in a 1.5-cm i.d. column. A flow rate of 6 ml/hr was maintained by positioning a Mariotte flask at a 30-cm pressure differential. Effluent was collected in 1-g fractions and analyzed using trichloroacetic acid. The void volume of the column was periodically measured with Blue Dextran 2000.

Gel Chromatography of Phosphofructokinase in Phosphate Buffer. A number of agarose chromatography columns were used to study the aggregation of phosphofructokinase in 0.1 M dipotassium phosphate–1.0 mM EDTA buffer. Three of the columns employed were made from Bio-Gel A-5m (Bio-Rad Laboratories, lots 97384 and 98783) and a fourth column was made from A-1.5m resin (Lot 100543). The columns had an inner diameter of 1.5 cm and a height of about 50 cm. The top and bottom of each column contained 0.8 cm plugs of Sephadex G-25 to ensure stable agarose interfaces. Buffer changes were accomplished by passing two bed volumes of buffer through a column.

The frontal elution procedure (Winzor, 1969) was employed for the chromatography of all protein solutions. An amount of stock protein solution was diluted to the desired concentration with buffer and the solution was allowed to stand at 5° for 30–90 min. The column was drained to the top of the G-25 plug, the protein solution applied and the elution begun. The amount of protein solution applied to the column varied from 50 to 70 ml. A Mariotte flask was employed to insure a constant flow rate during the elution. Flow rates of 12–21 ml/hr were used. All elutions were conducted in a cold room at 5°. The effluent was collected in 1- to 2-ml fractions, the exact amount being determined by weight. Normally the fractions were analyzed by reading their absorbance at 280 or 225 nm. When phosphofructokinase solutions of less than 0.01 mg/ml were chromatographed, the fractions were

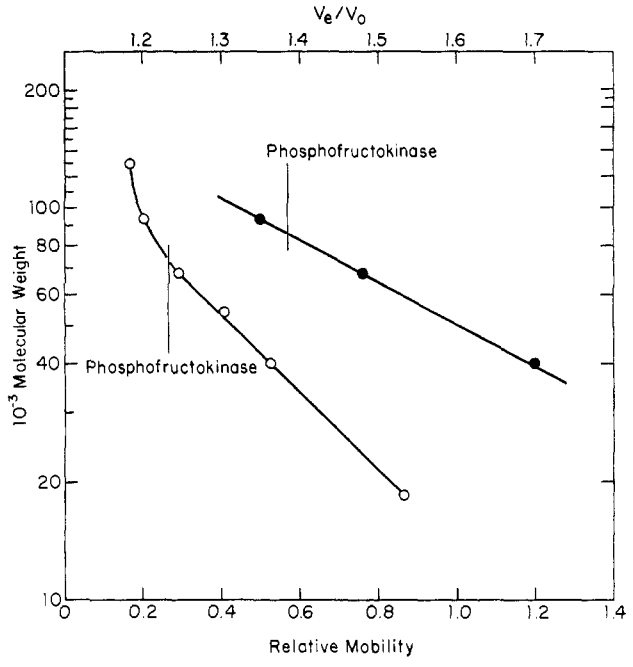

FIGURE 1: Representative data used for the determination of the molecular weight of phosphofructokinase under denaturing conditions. The open circles and relative mobility abscissa are for sodium dodecyl sulfate acrylamide gel electrophoresis; the filled circles and the abscissa indicating the ratio of the elution volume to the void volume, V_e/V_0, are for agarose chromatography in 7 M guanidine-HCl–0.1 M β-mercaptoethanol. The standard points in order of increasing molecular weight are β-mercaptoethanol, aldolase, glutamic dehydrogenase, albumin, phosphorylase *a*, and β-galactosidase. See text for experimental details.

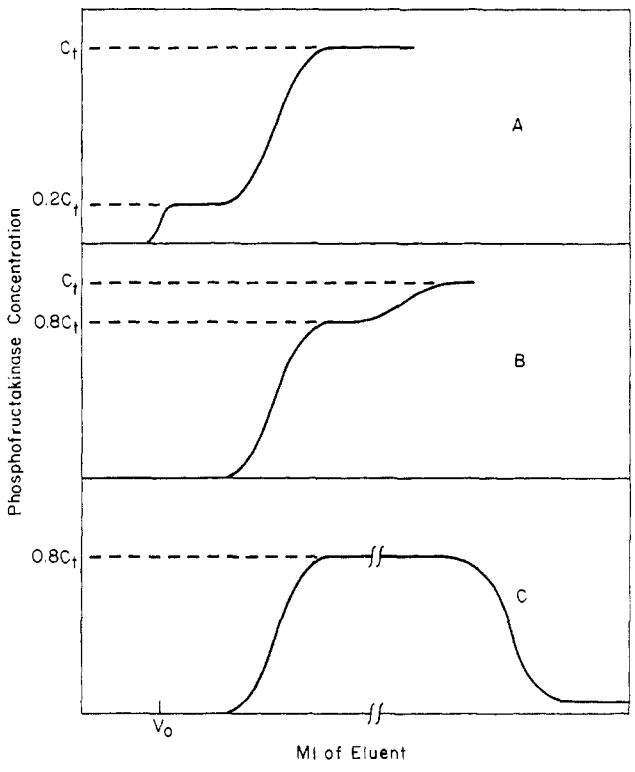

FIGURE 2: Schematic illustrations of the types of phosphofructokinase chromatographic elution profiles observed in 0.1 M dipotassium phosphate buffer, where C_t is the concentration of protein solution applied to the agarose column and V_0 is the void volume of the column.

analyzed using the pH 8.0 coupled assay (Ling *et al.*, 1966) at room temperature.

The protein elution volume, V_e, is taken as the centroid of the advancing elution front (*cf.* Winzor, 1969). The distribution constant is defined as

$$K_d = \frac{V_e - V_0}{V_t - V_0} \quad (1)$$

where V_0 is the zonal elution volume of Blue Dextran 2000 and V_t is the zonal elution volume of sucrose minus one-half the volume of the G-25 plugs (*i.e.*, the elution volume of a substance excluded by G-25 but completely included by agarose).

The agarose portion of the columns contracted slightly with time (0.5–2 cm in 6 weeks). Periodic chromatography of the standard proteins was used to correct for this. Also it was noted that after about 2 months the top portion of the agarose (1–2 cm) developed a brown tint. This did not affect the elution volume of any protein; however, Blue Dextran 2000 adhered to this altered agarose. When this browning was observed the column was discarded.

Results

Polypeptide Chain Molecular Weight. The results of a typical sodium dodecyl sulfate polyacrylamide gel electrophoresis experiment are shown in Figure 1. In this experiment the proteins were denatured in 2% sodium dodecyl sulfate while the acrylamide stock solution contained 0.4 g of methylenebisacrylamide/100 ml. Only a single protein band was observed with phosphofructokinase, the molecular weight of the polypeptide chain being 75,000. Within a variation of ±5000, identical results were obtained when the proteins were denatured in 1 or 3% sodium dodecyl sulfate and when half as much methylenebisacrylamide was used.

The results of an agarose chromatography experiment in 7.0 M guanidine-HCl–0.1 M β-mercaptoethanol also are shown in Figure 1. The elution of phosphofructokinase showed only a single symmetrical peak. The elution volume indicates a polypeptide chain molecular weight of 85,000 ± 10,000. An identical result was obtained in 6.0 M guanidine-HCl–0.1 M β-mercaptoethanol.

Phosphofructokinase Aggregation in Potassium Phosphate Buffer. The agarose columns used to study the aggregation of phosphofructokinase were standardized by frontal chromatography (Winzor, 1969) of five well-characterized proteins. Solutions of 0.1–0.3 mg/ml of protein in 0.1 M dipotassium phosphate buffer at pH 7–8 were chromatographed at 5°. The data were plotted as $(-\ln K_d)^{1/2}$ *vs.* R_S (Laurent and Killander, 1964), where K_d is the observed distribution constant (eq 1) and R_S is the Stokes' radius of the protein (*i.e.*, its effective hydrodynamic radius in solution). The values of R_S were calculated according to eq 2 or 3 (Winzor, 1969).

$$R_s = \frac{kT}{6\pi\eta D} \quad (2)$$

$$R_s = (f/f_0)\left[\frac{3\bar{v}M}{4\pi N}\right]^{1/3} \quad (3)$$

Here k is Boltzmann's constant, N is Avagadro's number, T is the absolute temperature, η is the solvent viscosity, D is the

TABLE I: Correlation of Specific Activity and Stokes' Radii for Phosphofructokinase.[a]

mg/ml	pH	R_S (Å)	Units/mg[b]
0.146	7.98	66.0	100
0.152	7.60	67.8	100
0.152	7.22	63.6	120
0.030	7.98	62.9	130
0.011	8.00	61.4	100
0.0033	8.00	59.5	75
0.0027	8.00	56.6	69
0.146	7.01	54.3	55
0.0011	8.00	54.0	65
0.153	6.94	52.0	75
0.0009	8.00	49.6	68
0.142	6.05	(44)[c]	2
0.152	6.60	(39)[c]	26
0.143	6.35	(43)[c]	6

[a] 0.1 M dipotassium phosphate–1 mM EDTA. [b] Measured at 23°. [c] Not reproducible.

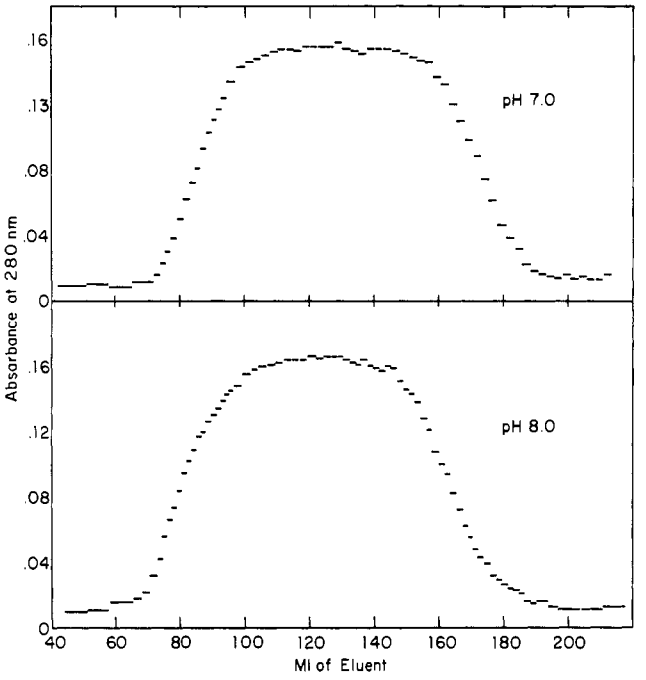

FIGURE 3: Elution profiles of phosphofructokinase from A-5m agarose at 5° in 0.1 M dipotassium phosphate–1.0 mM EDTA. Top profile, pH 7.0; bottom profile, pH 8.0.

diffusion constant of the macromolecule, M its molecular weight, $\bar{v}$ its partial specific volume and f/f_0 its frictional coefficient ratio. The pertinent data used for the various proteins are: β-lactoglobulin, $D = 7.82 \times 10^{-7}$ cm²/sec (Tanford, 1961), $R_S = 27.4$ Å; albumin, $D = 5.94 \times 10^{-7}$ cm²/sec (Tanford, 1961), $R_S = 36.2$ Å; aldolase, $D = 4.63 \times 10^{-7}$ cm²/sec (Taylor *et al.*, 1948), $R_S = 46.3$ Å; aspartate transcarbamylase, $f/f_0 = 1.30$, $M = 305{,}000$, $v = 0.730$ cm³/g (Rosenbusch and Weber, 1971), $R_S = 57.9$ Å; thyroglobulin, $D = 2.49 \times 10^{-7}$ cm²/sec (Edelhoch, 1960), $R_S = 86.1$ Å. (The diffusion constants were all obtained by extrapolation to zero protein concentration in water at 20°.) The $(-\ln K_d)^{1/2}$ *vs.* R_S plots showed random scatter of the data points about straight lines determined by least-squares analyses. The K_d values were reproducible to within ±0.01. No data points varied from the best-fit straight line by more than twice this experimental uncertainty.

The elution profiles of phosphofructokinase in 0.1 M dipotassium phosphate–1.0 M EDTA indicate that approximately 20% of the protein exists in a highly aggregated, largely inactive form. The three types of elution profiles observed are illustrated in Figure 2, where C_t is the concentration of the protein solution applied to the column. For elution profiles of the type illustrated in Figure 2A, 20% of the enzyme eluted in the void volume. The material in the first plateau region had a specific activity which was only 25% of that in the final plateau region. Most often the material eluted behind the major profile in a highly spread profile (Figure 2B) or as an extended low concentration tail (Figure 2C). With these types of profile, the phosphofructokinase in the plateau region had the same activity per milliliter as did the solution originally applied to the column. The large variation in the elution position of the less-active enzyme probably reflects a variation in solubility of the denatured protein. The elution position of the less-active enzyme depends not only on the elution conditions, but also on the particular enzyme preparation used. However, the type of overall profile observed had no effect on the elution volume of the high activity enzyme. Therefore it can be concluded that the two states of the enzyme are noninteracting.

The R_S values presented below refer only to the high activity enzyme. The specific activity of the phosphofructokinase preparations undergo a slow decrease with aging, but this has no effect on the elution position of the high activity enzyme or on the amount of material comprising its elution profile.

Often a portion of the chromatography solution was stored at 5° and periodically assayed. Within 30 min the activity reached a characteristic value (see Table I) and then maintained this value with only a slight decrease (10–20%) during the time required for chromatography (5–8 hr). This indicates that the phosphofructokinase solutions were at equilibrium when applied to the column and were stable during elution. There was no indication that the agarose affected the enzymatic activity.

Full elution profiles of the enzyme at a concentration of 0.15 mg/ml and pH values of 7 and 8 are shown in Figure 3. The specific activity of the enzyme was found to be constant throughout the advancing and plateau regions of these profiles. These profiles are reasonably symmetric; the slight differences in boundary spreading observed for the advancing and trailing profiles can be ascribed to the inactive enzyme that elutes with the trailing profile. The advancing profiles normally were found to be somewhat less sharp than that observed for the nonaggregating enzyme aspartate transcarbamylase. These results indicate that the rate of equilibration of the different molecular weight forms of the enzyme may be similar to the chromatographic separation rate. However, since the protein solutions were at equilibrium when applied to the column and since the concentration of the major portion of the active enzyme did not change during elution, the centroid of the advancing profile corresponds to the weight average elution volume of the constant concentration portion of the elution profile. At concentrations greater than 0.2 mg/ml at pH 7.0, the advancing profile was quite broad and showed definite asymmetry. When the concentration was greater than 0.3 mg/ml, the elution profile became "bumpy," suggesting

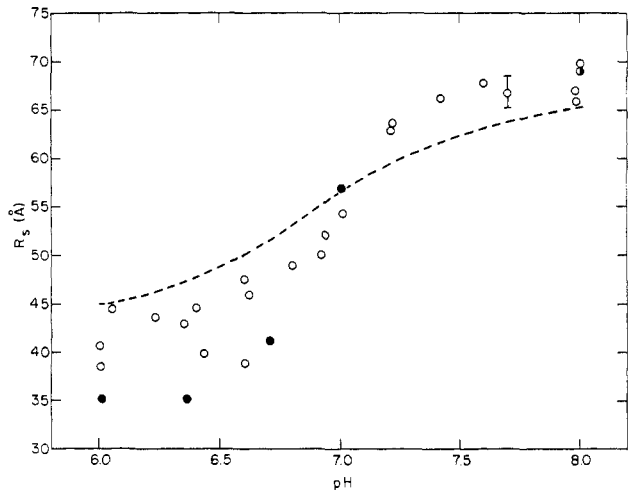

FIGURE 4: Weight-average Stokes' radius, R_S, of phosphofructokinase *vs.* pH in 0.1 M dipotassium phosphate–1.0 mM EDTA at 5°. (○) A-5m agarose columns; (◑) A-1.5m agarose column (a point at pH 6.0 and 27 Å is not shown); (●) A-1.5m agarose column in presence of 1.0 mM dithiothreitol. The dashed line is the calculated fit according to the mechanism of eq 4 and 5.

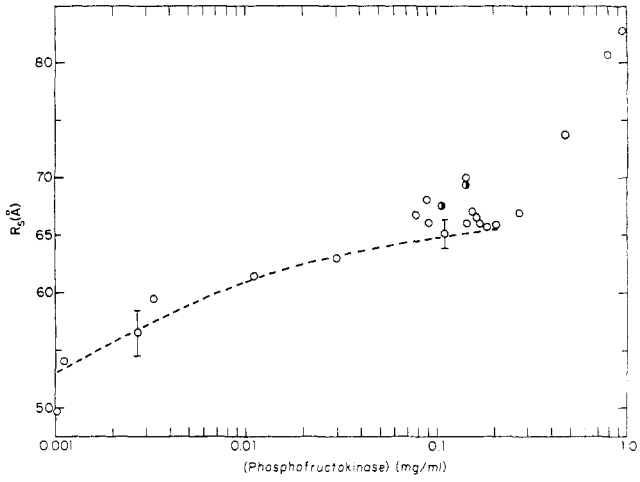

FIGURE 5: Weight-average Stokes' radius, R_S, *vs.* the logarithm of the phosphofructokinase concentration in pH 8.0, 0.1 M dipotassium phosphate–1.0 mM EDTA buffer at 5°. (○) A-5m agarose columns; (◑) A-1.5m agarose columns. The dashed line is the calculated fit according to the mechanism of eq 4 and 5.

the existence of very slowly equilibrating species. A quantitative interpretation of these data was not attempted as an excessive amount of protein would be required to obtain comprehensive data.

The weight-average Stokes' radius of phosphofructokinase at 0.15 mg/ml in 0.1 M dipotassium phosphate–1.0 mM EDTA buffer is plotted in Figure 4 as a function of pH. The protein shows a constant degree of aggregation with Stokes' radius of approximately 67 Å for the pH region 7.5–8.0. As the pH becomes more acidic the protein dissociates attaining an observed average radius of about 40 Å in the pH region 6.0–6.5. The error bars indicate an experimental uncertainty of ±0.01 in K_d. When the Stokes' radius is greater than 48 Å ($\geq\sim$pH 6.8) the data show good reproducibility. The values of R_S show no dependence on the enzyme preparation used, the type of agarose used, or on the presence or absence of dithiothreitol. When the Stokes' radii are below about 48 Å, the data are quite irreproducible, depending on all three of the variables mentioned above. In this irreproducible region, R_S values obtained in the presence of dithiothreitol were larger than those obtained in the absence of this reagent.

The variation in the Stokes' radius as a function of the protein concentration at pH 8.0 is shown in Figure 5. A plateau region at 67 Å is observed from 0.08 to 0.3 mg per ml. The protein associates at higher concentrations and dissociates at low concentrations attaining an average radius of about 52 Å at 0.001 mg/ml. Figure 6 shows the concentration dependence of the Stokes' radius at pH 7.0. Again the protein associates at higher concentrations and dissociates at lower concentrations. At concentrations less than 0.01 mg/ml the protein completely denatured giving elution profiles which were uninterpretable. Data also were collected on the concentration dependence of the Stokes' radius at pH 6.0. The data at 0.15 mg/ml are shown in Figure 4. Using an A-5m Bio-Gel column, Stokes' radii of 44 and 51 Å were determined at 0.4 and 1.2 mg per ml, respectively.

For a number of elutions the enzyme in the plateau region was analyzed for enzymatic activity. Normally the protein fraction at 5° was added directly to the room temperature coupled assay mixture (Ling *et al.*, 1966). However, allowing the protein fraction to stand at room temperature for 60 min before the assay had no effect on the specific activity measured. The data obtained are summarized in Table I. The only correlation that appears is a rough relationship between aggregation state and specific activity. High activity (100–130 units/mg) is observed when the Stokes' radius is greater than about 60 Å, while for Stokes' radii of 50–60 Å the activity is noticeably lower (55–75 units/mg). Very little activity is observed below a Stokes' radius of 45 Å. The scatter in the specific activity values probably arises from a number of sources: the inherent uncertainty of the assay method, the 10–20% loss in activity during elution and the age of the enzyme preparation.

Discussion

The results of sodium dodecyl sulfate gel electrophoresis and gel chromatography in guanidine-HCl at high concentrations of sulfhydryl reagent and variable amounts of denaturing reagents indicate that phosphofructokinase consists of essentially identical polypeptide chains with a molecular weight of approximately 80,000. Since both methods are empirical in nature, the uncertainty in this molecular weight is rather large, probably ±5000–10,000. This conclusion is in good agreement with the recent ultracentrifuge measurements of Leonard and Walker (1972), who obtain a molecular weight of approximately 80,000 in 6 M guanidine-HCl and in 4 mM sodium dodecyl sulfate. This result does not agree with earlier ultracentrifuge measurements of Paetkau *et al.* (1968), who reported much lower molecular weights in guanidine-HCl.

The data in Figures 4–6 indicate the sensitivity of enzyme aggregation to protein concentration and pH. In order to interpret these data on a molecular basis, the measurements of the Stokes' radii must be correlated with molecular weights. An *a priori* correlation between these parameters cannot be made since the necessary frictional coefficient ratios are not known (*cf.* eq 3). However, a comparison with other data indicates that the aggregate with a Stokes' radius of 67 Å is a tetramer, whose molecular weights is 320,000–360,000. Ling *et al.* (1965) have carried out sucrose gradient measurements at 0.2 mg/ml of enzyme in 0.1 M potassium phosphate–1.0 mM potassium phosphate–1.0 mM EDTA (pH 8.0) buffer at 4°,

which corresponds to the conditions utilized in the present work (see Figure 5). They observe a single molecular weight species with a molecular weight of approximately 360,000. Leonard and Walker (1972), utilizing ultracentrifuge measurements, have observed a stable tetrameric species of mol wt 340,000 at pH 10.5. They found a diffusion coefficient for this species of 3.22×10^{-7} cm^2/sec, which corresponds to a Stokes' radius of 67 Å (eq 2), in good agreement with the value reported here. If the molecular weight of the tetramer is assumed to be 320,000, corresponding to a monomer molecular weight of 80,000, the frictional coefficient ratio for the tetramer is 1.49 (eq 3 with $\bar{v}$ taken as 0.73 cm^3/g). For a prolate ellipsoid, this corresponds to an axial ratio of approximately 7 to 9, assuming the relative volume of hydrated water is in the range 0–0.3.

The reproducible Stokes' radii between 48 and 67 Å are best explained in terms of a dimer–tetramer equilibrium. A stable aggregate smaller than a trimer must exist since the weight average Stokes' radius of 51 Å found in this study at pH 6.0 and a protein concentration of 1.2 mg/ml corresponds to a molecular weight of 210,000, as determined under very similar conditions (except for a 5° temperature difference) by Aaronson and Frieden (1972). On the other hand, the existence of a stable monomer seems unlikely since a stable aggregate with a molecular weight less than 160,000 has never been observed in nondenaturing solvents. Also at pH 7.0 the enzyme is extensively denatured at concentrations below 0.01 mg/ml (R_S < 48 Å), suggesting that further dissociation into a less stable species is occurring at lower concentrations. Unfortunately the lack of reproducibility of Stokes' radii below about 48 Å (*cf.* Figure 4) makes it impossible to determine the Stokes' radius of the dimer precisely. The reason for this lack of reproducibility is not entirely clear. However, with a protein concentration of 0.15 mg/ml at pH 4.5 this problem is so severe that the enzyme cannot be eluted from A-1.5m agarose. Furthermore, precipitation of the protein is sometimes observed at low pH. These results suggest that the dissociation of the dimer gives a variety of denatured monomeric species with varying degrees of solubility, the extent of denaturation and solubility being dependent on the particular protein preparation used and on the presence or absence of sulfhydryl reagent. If this explanation is correct, the Stokes' radii given in Figure 4 below pH 6.5 would represent lower bounds to the Stokes' radii of the soluble species present. A molecular weight near that of the dimer was found at pH 6.0 and a concentration near 0.5 mg/ml (Aaronson and Frieden, 1972). In the present work a Stokes' radius of 44 Å was obtained with a protein concentration of 0.4 mg/ml at pH 6.0. In light of these facts, it is reasonable to assume a value of approximately 44 Å for the Stokes' radius of the dimer.

If the dimer molecular weight is assumed to be 160,000, the calculated frictional coefficient ratio for this species is 1.22 (eq 3 with $\bar{v}$ as 0.73 cm^3/g). Again assuming the relative volume of hydrated water varies from 0 to 0.3, an axial ratio of 3–4 can be estimated for a prolate ellipsoid. If this is correct, the formation of the tetramer may be due to end-to-end aggregation of dimers.

Consistent with the above discussion, the aggregation states with Stokes' radii below 67 Å can be represented by the equilibrium

$$2P_2 \rightleftharpoons P_4 + nH^+ \qquad (4)$$

where P_2 is the dimeric species, P_4 is the tetrameric species, and K is the equilibrium constant for the reaction [$K = (P_4)(H^+)^n/(P_2)^2$]. The weight-average Stokes' radius is

$$R_s = \frac{R_2(P_2)M_2 + R_4(P_4)M_4}{(P_2)M_2 + (P_4)M_4} \qquad (5)$$

where R_2 and R_4 are the Stokes' radii of the dimer and tetramer, assumed to be 44 and 67 Å, respectively, M_2 and M_4 are the corresponding molecular weights assumed to be 160,000 and 320,000, and the (P_i) are the molar concentrations of the two species. A reasonable fit to all of the data can be obtained with $K = 4.1 \times 10^{-7}$ M$^{0.8}$ and $n = 1.8$. The dashed lines in Figures 4–6 were calculated using these constants and eq 5. In view of the several assumptions made and the simplicity of the aggregation mechanism, the data are fit reasonably well. The poor fit obtained above about 0.2 mg/ml at pH 7.0 and 8.0 is due to the presence of species larger than the tetramer. This is suggested by the complex elution profiles observed at concentrations greater than 0.2 mg/ml at pH 7.0 and by the fact that the values of R_S do not level off at 67 Å. The rather qualitative fit of the pH dependence of R_S at a constant protein concentration (Figure 4) is probably related to the fact that the concentrations of the various ionic species of phosphate buffer vary with pH; this almost certainly influences the aggregation equilibria. At pH 8.0, the aggregation state of the enzyme is dependent on the concentration of phosphate; however, since the elution profiles at low phosphate concentrations are extremely broad, a detailed investigation of this phenomenon was not attempted. In any event, the assumed pH dependence of the aggregation equilibrium is very probably an oversimplification so that a simple interpretation of the value of n is not appropriate. The analysis presented does have the virtue of representing a considerable amount of data very simply in a semiquantitative manner. A more detailed, and necessarily more complex, analysis is not warranted at this time.

An ultracentrifuge study of the aggregation of phosphofructokinase at higher concentrations than those used in this work has been carried out by Aaronson and Frieden (1972). Their results are complementary to those reported here: at pH 8.0, tetrameric and higher aggregation states were found, while at pH 6.0 a dimeric species was found. In the presence

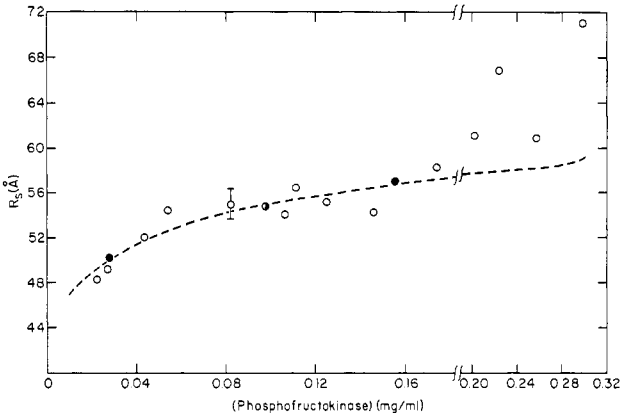

FIGURE 6: Weight-average Stokes' radius, R_S, *vs.* the phosphofructokinase concentration in pH 7.0, 0.1 M dipotassium phosphate–1.0 mM EDTA at 5°. A change in the concentration scale occurs at 0.2 mg/ml. (○) A-5m agarose columns; (◑) A-1.5m agarose column; (●) A-1.5m agarose column in presence of 1.0 mM dithiothreitol. The dashed line is the calculated fit according to the mechanism of eq 4 and 5.

of adenosine diphosphate, the molecular weight of the heart muscle enzyme has a qualitatively similar pH dependence (Mansour, 1965).

The correlation between aggregation state and activity found in Table I is only semiquantitative. Nevertheless, these results are consistent with the idea that the enzymic activity of the dimer is much less than that of the tetramer (*cf.* Hofer and Pette, 1968a; Hofer, 1971). The dimer obtained under a variety of conditions has not been found to have appreciable enzymatic activity (Aaronson and Frieden, 1972; Paetkau and Lardy, 1967). A quantitative correlation of the data would have to take into account the intrinsic pH dependence of the enzymatic reaction itself, as well as the enzymatic activity of the individual aggregation states. The implicit assumption has been made in this discussion that the aggregation state of the enzyme is not altered when it is diluted into the assay mixture. This is qualitatively consistent with the known hysteretic behavior of the enzyme (Frieden, 1970) and our own observations, but has not been rigorously proven.

The principal conclusions to be derived from this work are that at low protein concentrations over the pH range 6–8 an equilibrium between dimer and tetramer exists, with the tetramer possessing considerably more enzymic activity than the dimer. This must be taken into account in interpreting kinetic measurements and in assessing the regulatory processes associated with this enzyme. In addition, the protomer molecular weight has been found to be approximately 80,000, and information has been inferred about the shape of the tetrameric and dimeric aggregates.

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