Pig Liver Phosphofructokinase: Asymmetry Properties, Proof of Rapid Association–Dissociation Equilibria, and Effect of Temperature and Protein Concentration on the Equilibria†

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ABSTRACT: Sedimentation coefficient determinations show that, under comparable conditions, pig liver phosphofructokinase (PL-PFK) is larger than any previously studied phosphofructokinase; the molecular weight of PL-PFK at 4 °C at a protein concentration of 5 mg/mL is estimated to be greater than 10 000 000. Furthermore, the enzyme is very asymmetric as indicated by a strong concentration dependence of the sedimentation coefficient data and by a minimum intrinsic viscosity value at 20.0 °C of 30.8 cm^3/g. Kinetic and reversibility tests show that the enzyme exists as a temperature-dependent, concentration-dependent equilibrium mixture of polymeric forms. Dissociation (partial) is favored by either higher temperatures or lower protein concentrations. PL-PFK sediments as a *single* peak ($s^0_{20,w}$ = 104 S) at both 4 and 23 °C, at concentrations above 3 and 9 mg/mL, respectively. Below 9 mg/mL, the enzyme is more dissociated at 23 than at 4 °C. At 35 °C, PL-PFK initially exhibits a single peak, but in the latter stages of sedimentation the single peak partially evolves into a *biomodal* (54 and 58 S) peak. The data for the predominant peak at 35 °C extrapolates to a value of $s^0_{20,w}$ = 60 S at zero protein concentration. At all three temperatures studied, the sedimentation coefficient of the enzyme shows a drastic drop as the concentration decreases below about 0.5 mg/mL, suggesting very extensive dissociation into much smaller subunits. Some patterns exhibit a low-molecular-weight material which barely sediments away from the meniscus of the cell. Exposure of the enzyme to either 23 or 35 °C for too long produces an irreversible modification to a 60S form which does not reassociate to the 104S form upon being cooled to 4 °C.

In mammals, control of liver phosphofructokinase (PFK)[1] is necessary for coordination of utilization of glucose through glycolysis and synthesis of glucose through gluconeogenesis. In addition, these reactions must be coordinated with the overall needs for, and supplies of, carbohydrates from all sources, including amino acids, Krebs cycle metabolites, fatty acids, etc. In this respect, the mammalian liver PFKs differ from their widely studied muscle counterparts, which, for the most part, are turned on or off only in response to needs for energy through degradation of glucose. This difference in complexity of functions raises the possibility of differences in structures and control properties between mammalian muscle and liver phosphofructokinases.

Although procedures have recently been published for purification of phosphofructokinases from various liver sources, including pig (Massey and Deal, 1973, 1975), rat (Dunaway and Weber, 1974; Brand and Soling, 1974), rabbit (Ramaiah and Tejwani, 1970; Kemp, 1971, 1975; Massey and Deal, 1975), sheep (Brock, 1969), and chicken (Kono and Uyeda, 1971, 1973), no detailed structural studies have been carried out on a phosphofructokinase from a liver source except for the studies reported here and limited studies on the avian liver enzyme (Kono and Uyeda, 1971, 1973). A preliminary survey (Massey and Deal, 1973, 1975) of the physical and catalytic properties of pig liver phosphofructokinase had suggested that it differed from other phosphofructokinases.

This paper extends those studies and presents the first detailed structural analysis of a phosphofructokinase from mammalian liver. The results show that among the phosphofructokinases studied thus far, the pig liver enzyme, under comparable conditions, is unique with respect to its large size ($s^0_{20,w}$ = 104 S; molecular weight greater than 10 000 000) and the fact that it exhibits a *single* peak in sedimentation velocity experiments. In addition, the first viscosity studies reported on any phosphofructokinase have led to the important discovery that pig liver phosphofructokinase is highly asymmetric.

Materials and Methods

Enzyme Preparation and Assay. The techniques were those described previously (Massey and Deal, 1975, 1973) except for the following modifications: (1) when as many as three or four livers were used, the purification schedule was extended to 2 or 3 days and the first day ended at the completion of step 2; (2) after completion of step 4 (washing), the enzyme was always kept at 4 °C (not raised to room temperature for solubilization); and (3) in step 5 extraction and reprecipitation were carried out in the presence of 100 mM dithioerythritol or 100 mM dithiothreitol instead of 50 mM mercaptoethanol. Phosphofructokinase was stored at 4 °C in buffer A-D (see buffers); it was solubilized in buffer B-D (I) for use in experiments.

Buffers. There was a slight change from the original buffers (Massey and Deal, 1973, 1975): (1) stronger reducing agents, either dithiothreitol or dithioerythritol, at a higher concentration were used, and (2) for these physical studies the solubilization buffer, buffer B, was changed from Tris to imidazole to avoid the variation of pH which occurs with Tris buffers at different temperatures. These two changes are designated by the letters D and I, respectively. Buffer A-D contains 50 mM

† From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823. *Received August 13, 1976.* This is paper 17 in a series entitled, "Metabolic Control and Structure of Glycolytic Enzymes". Paper 16 is the work of Dagher and Deal (1977). This research was supported in part by grants from the United States Public Health Service (GM-11170 and CA-14017) and the Michigan Agricultural Experiment Station (Hatch 932, Publication No. 7831).

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[1] Abbreviations used are: PFK, phosphofructokinase; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

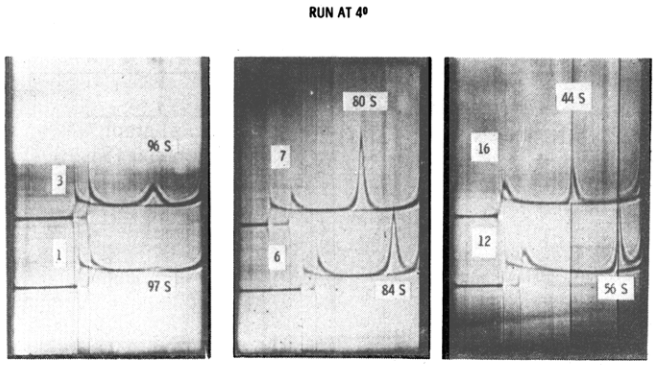

FIGURE 1: Effect of protein concentration on the sedimentation velocity patterns of pig liver phosphofructokinase at 4 °C. Pig liver phosphofructokinase samples at different protein concentrations were prepared by dilution of a stock solution of PFK. The stock solution was prepared by spinning down a sample of enzyme suspension in buffer A. The solvent was 0.05 M imidazole buffer, pH 8.0, containing 110 mM ammonium sulfate and 100 mM dithioerythritol. The numbers at the left of each pattern give the protein concentrations in units of mg/mL and the numbers above or below the peaks are $s_{20,w}$ values. Sedimentation proceeds from left to right. The photographs were taken at a phase plate angle of 60° about 12 min after reaching a speed of 35 600 rpm.

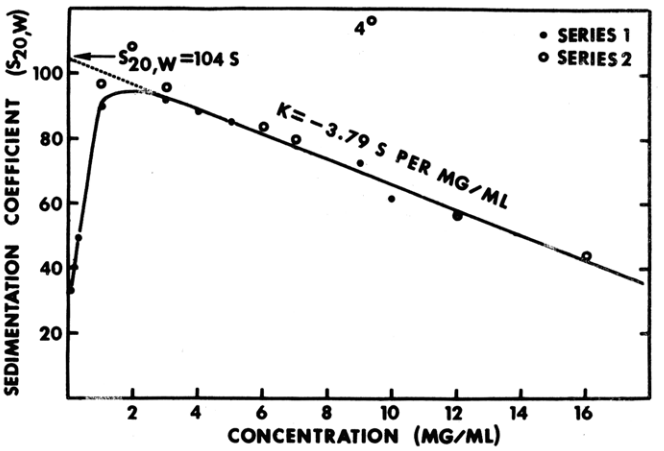

FIGURE 2. Effect of protein concentration on the sedimentation coefficient of phosphofructokinase at 4 °C. The data were calculated from the experiments described in Figure 1 (series 2) and from other data obtained previously (series 1).

Tris-HCl, 100 mM dithioerythritol, 5 mM $MgCl_2$, 0.1 mM FDP, and 0.1 mM ATP at pH 8.0. Buffer B-D is the same as buffer A-D except that it contains, in addition, 110 mM ammonium sulfate. Buffer B-D (I) is the same as buffer B-D except that imidazole (pH 8.0) is used instead of Tris. The buffer for the preparation of the crude homogenate contained 50 mM Tris-HCl, 50 mM β-mercaptoethanol, and 5 mM EDTA (Massey and Deal, 1973, 1975).

Sedimentation Velocity Experiments. Sedimentation velocity experiments were performed in a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics and an RTIC unit. Sedimentation coefficients were calculated from sedimentation velocity experiments in the usual manner (Schachman, 1957). Samples with concentrations of phosphofructokinase below 1 mg/mL were analyzed in 30-mm double-sector cells in the An-E rotor. All other samples were run in standard 12-mm double-sectors cells in the An-D rotor. The $s_{t,w}$ values were corrected to standard conditions ($s_{20,w}$) using the International Critical Tables (Washburn, 1929) for the viscosity of water. Viscosity contributions of buffers and salt were determined at 20 °C using a Ubbelohde viscometer.

Viscosity Experiments. These studies were carried out with a Cannon Ubbelohde semimicro dilution viscometer in a water bath regulated at 20.00 ± 0.02 °C; the flow time for solvent at 20.0 °C was 275.5 s. The solvent was buffer B (Massey and Deal, 1973).

Results

Sedimentation Velocity Patterns and Concentration Dependence of Sedimentation Coefficient of Pig Liver PFK at 4 °C. As shown in Figure 1, the sedimentation velocity patterns of pig liver phosphofructokinase at 4 °C show a single peak over the entire concentration range analyzed (1–16 mg/mL). The peaks are generally broad at the lower protein concentrations, get sharper at higher concentrations, and sediment essentially as *spikes* at the highest concentrations tested (12 and 16 mg/mL). The spikes seem sharper on the trailing edge than on the leading edge. In the picture on the right in Figure 1 a small shoulder of material is moving out from the meniscus. The amount of this material varies somewhat from preparation to preparation. It appears to be a very low-molecular-weight form of PFK not in equilibrium with the bulk of the PFK.

The data above 1.5 mg/mL fit[2] the regression curve $s_{20,w} = s^0_{20,w}(1 - kc) = s^0_{20,w} - Kc$, where $s^0_{20,w} = 104.3 \pm 3.5$ S, $K = 3.79 \pm 0.39$ S per mg/mL, $k = 0.036$ per S per mg/mL, and the units of c are mg/mL (Figure 2). This extraordinarily strong concentration dependence indicates a highly asymmetric molecule. Below 1.5 mg/mL, the values decrease sharply, suggesting extensive dissociation into much smaller particles; sucrose density gradient studies in this range of protein concentration have confirmed these results.

The 104S value corresponds to a molecular weight of at least 10 000 000 (Table I). Since a particle of this size would not show nearly so much diffusion during sedimentation velocity experiments, it appears that the broad single peaks at low (e.g., 3 mg/mL) concentrations (Figure 1) do *not* represent single polymeric species. Rather, the broadening must be due to either concentration-dependent boundary desharpening,[3] association–dissociation, or to heterogeneity.

The fact that the sharpness of the peaks increases with increase in concentration suggests that the broadening is not due to heterogeneity. In addition, the fact that greatly decreased sedimentation coefficients are observed at low concentrations suggests that this is an association–dissociation system. It appears that the rate of equilibrium is rapid, since we do not see resolution of the association–dissociation components. The observation of a single maximum suggests that this must be either: (1) a monomer–dimer mixture (Gilbert, 1955, 1959) or (2) a monomer–n-mer mixture, where n is 3 or more *and where intermediates between monomer and n-mer are present* (Rao and Kegeles, 1958; Bethune and Grillo, 1967; Cann, 1970).

Sedimentation Patterns and Concentration Dependence of the Sedimentation Coefficient of Pig Liver PFK at 23 °C. At

[2] K (S per mg/mL) = $ks^0_{20,w}$, where K is the observed concentration dependence, numerically equal to the slope of the curve, k is the intrinsic concentration dependence parameter, and $s^0_{20,w}$ is the intrinsic sedimentation coefficient obtained by extrapolation to zero protein concentration. K is proportional to the absolute value of $s^0_{20,w}$, but k is not. Hence, k is a better parameter for comparison of concentration dependence of particles of widely different size.

[3] This sharpening or broadening as the concentration is increased or decreased, respectively, seems not to be due to "artificial sharpening or desharpening" often produced by the s vs. C effect; the shape characteristics are not consistent with such an explanation (Fujita, 1962).

TABLE I: Subunit Structure and Sedimentation Coefficients at Various Stages of Association–Dissociation for a Hypothetical Situation in Which All Polymeric Forms Are Globular.

No. of subunits	Mol wt	Stage of assoc	Sedimentation coefficient: From $M^{2/3}$ calc[a] for theor spheres (S)		Sedimentation coefficient: From empirical graph[b] for known proteins (S)
128	10 240 000	8	127		104
				↑↓7	
64	5 120 000	7	80		66
				↑↓6	
32	2 560 000	6	50		43
				↑↓5	
16	1 280 000	5	32		29
				↑↓4	
8	640 000	4	20		19
				↑↓3	
4	320 000	3	12.6		12
				↑↓2	
2	160 000	2	7.9		7.9
				↑↓1	
1	80 000	1	5.0		5.0

[a] This is a maximum value for this molecular weight; this is the value calculated for a sphere with this molecular weight, using the relation $s_i = (M_i/M_1)^{2/3}s_1$, assuming s_1 and M_1 values of 5.0 S and 80 000, respectively. [b] This is the *probable* value if the particle of this molecular weight is a "typical" protein (W. C. Deal and Y. J. Farrar, in preparation).

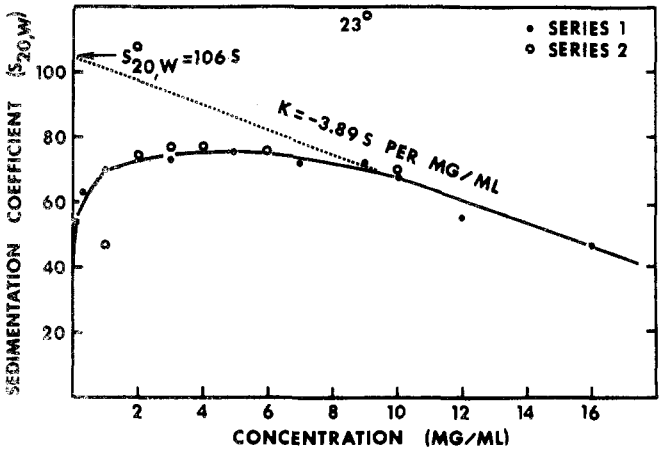

FIGURE 3: Effect of protein concentration on the sedimentation coefficient of phosphofructokinase at 23 °C. The data were calculated from experiments in which the enzyme samples were allowed to stand at 23 °C for 30 min (open circles) or 5 h (filled circles) before running. The series 1 stock solution contained a small amount of slower-moving material, so the study was repeated (series 2) with a new preparation which did not contain this contaminant, to ensure that this did not influence the results; a shorter time of exposure to 23 °C was also used.

23 °C a broad single peak is observed but only the sedimentation-coefficient values in the high-concentration range (9–16 mg/mL) lie on a fairly good straight line (Figure 3). The values for the regression curve for this data above 9 mg/mL are: $s^0_{20,w}$ = 106.3 S ± 9.2 S, K = 3.89 ± 0.76 S per mg/mL, and k = 0.038 per S per mg/mL. All these values are virtually identical to those found at 4 °C.

In the range from 9 mg/mL down to about 1 mg/mL, the sedimentation coefficients dip sharply below the straight line from the high concentration region. Furthermore, in the region *below 1 mg/mL*, the sedimentation coefficient decreases drastically with slight decrease in concentration, suggesting very pronounced dissociation into subunits very much smaller than those in the concentration region above 1 mg/mL.

Since a monomer–dimer equilibrium is one of the possible explanations for the observed single peaks, we analyzed the data in Figure 3 in the concentration region from 1.5 to 9 mg/mL. In this region there appears to be a substantial shift from mostly associated (104 S) material to a dissociated, 60S form.

We tested to see whether this data fit a 60S monomer–104S dimer equilibrium in two ways. First we used the observed S values to calculate α, the fractional concentration of monomer and from that, an equilibrium constant, at the various concentrations. The requirement that the same value be calculated at the different concentrations was not met; the values for the equilibrium constant increased by a factor of 10 as the concentration increased from about 1 to about 6 mg/mL.

Second, we assumed a value of K, the equilibrium constant, and for each of about 200 values of α, we calculated the concentration at which that α value would occur. Using the experimental values of s^0_m = 60.4 S (see next section), s^0_p = 104.3 S, k_m = 0.015 per S per mg/mL, k_p = 0.036 per S per mg/mL, we tested polymerizing systems involving as higher polymer dimer, or trimer, or tetramer to see if they yielded theoretical curves close to the experimental points (Figure 4). As shown in Figure 4B, the curve for the monomer–trimer system fits better than that for the monomer–dimer system, but both fit rather poorly in the intermediate concentration region. The theoretical curves peak at lower concentrations and have less concentration dependence at higher concentrations.

The experimental data could be fit almost perfectly by a curve calculated from different values of the constants, namely, s^0_m = 68 S, s^0_p = 127 S, k_m = 0.015 per S per mg/mL, k_p = 0.045 per S per mg/mL, and using a monomer–trimer system with an equilibrium constant of 15 mg/mL (Figure 4A). Without attaching any special physical significance to the fact, it should be noted that there was a very narrow range of values for each of the variables which would give this fit over the whole range. Values of n = 3 and n = 4 gave approximately equally good fit, with slightly different values of the other parameters, but values of n = 2 did not give as good a fit. It should be noted that the data in the region approaching and above 10 mg/mL are subject to very large experimental errors, because the fractional dissociation value is calculated from a

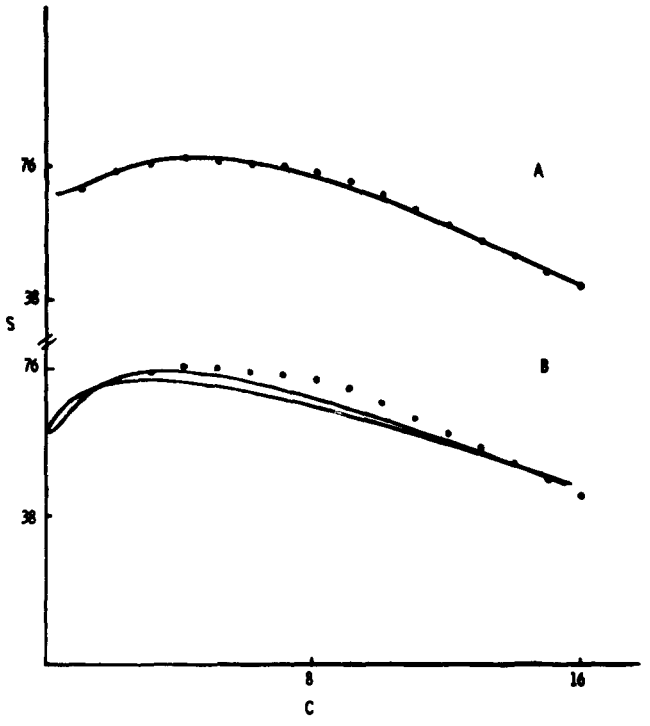

FIGURE 4: Comparison of coincidence of theoretical curves of s vs. C with experimentally determined points. The points are from experimental data obtained at 23 °C. (A) The curve is for a monomer-trimer equilibrium in which all the various parameters were arbitrarily chosen to give the best fit. Although arbitrary, only a very limited range of values for each parameter would give this fit, and the values chosen differ no more than 20% from the experimentally determined parameters. The values required to give this fit are: $n = 3$, $K = 15$ mg/mL, $s^0_m = 68$ S, $s^0_p = 127$ S, $k_m = 0.015$ per S per mg/mL, $k_p = 0.045$ per S per mg/mL. (B) The upper curve is for a monomer-n-mer equilibrium where $n = 3$ and K, the dissociation equilibrium constant, is 2.0 mg/mL. The lower curve is for $n = 2$ and $K = 2.0$ mg/mL. These curves were calculated using experimentally determined parameters of s^0_m, s^0_p, k_m, k_p from the data in Figures 2 and 5. The values for s were calculated using the expression $S = \alpha s_m + (1 - \alpha)s_p$, where $\alpha = c_m/C$, $(1 - \alpha) = c_p/C$, where m stands for monomer and p stands for polymer, $s^c_m = s^0_m [1 - k_m c_m - 0.5 (k_m + k_p)/C_p]$, $s^c_p = s^0_p [1 - k_p c_p - 0.5(k_m + k_p)/c_m]$ and the values $s^0_m = 60.4$ S, $s^0_p = 104.3$ S, $k_m = 0.015$ per S per mg/mL, $k_p = 0.038$ per S per mg/mL. About 200 points were calculated on each line; for each point a value of α was assumed and C was calculated from the equation $K = (\alpha)^n C^{n-1}/(1 - \alpha)$.

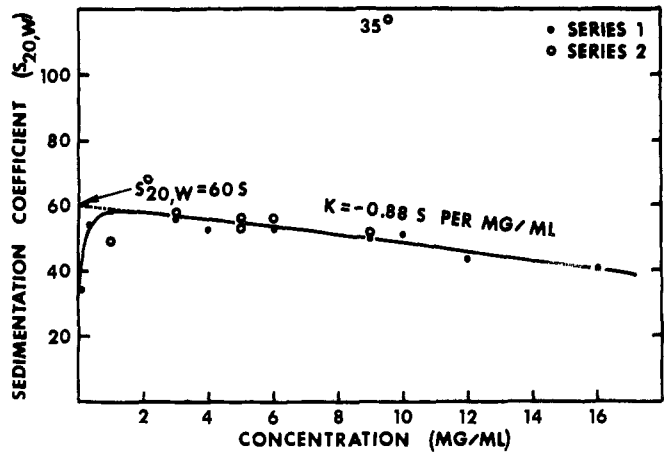

FIGURE 5. Effect of protein concentration on the sedimentation coefficient of phosphofructokinase at 35 °C. The data were calculated from experiments in which the enzyme samples were allowed to stand at 35 °C for 30 min (series 2) before sedimentation began, and from a similar series of experiments with enzyme preincubated for 5 h (series 1) before sedimentation began. Series 2 was run to ensure that the modification subsequently discovered in the samples in series 1 did not influence the results.

fraction whose numerator and denominator go to 0 and become negative as c passes through 10 mg/mL. This is because the curves of s vs. c for the 60S monomer and the 104S polymer cross in that region and s_p becomes less than s_m at concentrations greater than that; this is a result of the very pronounced concentration dependence of the 104S polymer. The overall conclusion is that for this system, as for most others, both molecular weight data and sedimentation coefficient data are necessary to define the equilibria involved; work is in progress on this problem.

Sedimentation Velocity Patterns and Concentration Dependence of the Sedimentation Coefficient of Pig Liver PFK at 35 °C. The sedimentation velocity patterns of pig liver PFK at 35 °C exhibit a single main peak, at least in the initial stages; under some conditions, a slight bimodality is observed in the later stages of sedimentation. The sedimentation coefficients in the concentration range of 1–16 mg/mL are markedly lower at 35 °C than at 4 or 23 °C, and the concentration dependence of the sedimentation coefficient is considerably less. In the protein concentration region from 1 to 16 mg/mL, samples exposed to 35 °C for 30 min (Figure 5, series 2) fit a regression curve with $s^0_{20,w} = 60.4 \pm 1.6$ S, $K = 0.88 \pm 0.26$ S per mg/mL, and $k = 0.015$ per S per mg/mL, while samples exposed to 35 °C for 5 h (Figure 5, series 1) fit values of $s^0_{20,w} = 59.5$ S $\pm$ 6.7 S, $K = 0.79 \pm 0.08$ S per mg/mL, and $k = 0.013$ per S per mg/mL.

Both sets of data lie on the same curve (Figure 5) and the Schlieren patterns from both are indistinguishable, except for some dissociation into smaller products by the 5-h samples at lower protein concentrations. Other studies (see later section) showed that the samples exposed to 35 °C for 30 min retained their ability to reversibly associate to the higher polymeric form upon cooling to 4 °C; however, approximately half of the material exposed to 35 °C for 5 h was irreversibly modified so that it did not associate to the higher polymeric form upon cooling to 4 °C.

The fact that the data for the 30-min samples (and the 5-h samples) lie on a straight line over such a wide region of concentration raises the possibility that essentially one polymeric species is present in this region; the value of 60 S corresponds to a molecular weight near 5×10^6 (Table I).

Kinetic and Reversibility Tests for Equilibrium and for Rapid Equilibrium. Samples taken from 4 °C and exposed to 23 °C for times ranging from 15 to 180 min appeared to reach equilibrium within 15 min at 23 °C. There were no differences with time in sedimentation patterns or in sedimentation coefficient values. Furthermore, the partial dissociation occurring upon transfer from 4 to 23 °C was completely reversible and, up to about 3 h, independent of the time of exposure at 23 °C; beyond 5 h at 23 °C, irreversible modification was evident.

The results at 35 °C were a little more complex. There was only one significant change with time at 35 °C in the time range of 5 to 30 min. With the 5-min sample the peak is slightly bimodal and the right mode is slightly higher, whereas with the 30-min sample the peak is still bimodal but the left mode is slightly higher. This indicates a small shift of some faster-moving material to slower-moving material.

The partial dissociation upon going from 4 to 35 °C is also virtually completely reversible if the time at 35 °C is limited to 5–30 min (Figure 6). With 5-min exposure (Figure 6, upper), the amount of irreversibly modified 58S enzyme is almost undetectable (<2%); however, 5–8% of irreversibly modified 57S enzyme is observed in the 30-min sample. Longer

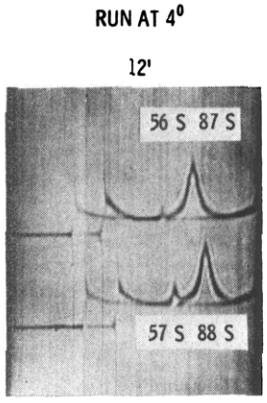

FIGURE 6: Test for reversibility (4 to 35 to 4 °C) and ability to distinguish irreversibly modified enzyme and unmodified enzyme upon reversal from 35 to 4 °C. Four enzyme samples (6 mg/mL) were prepared at 4 °C. Duplicate samples in two groups were preincubated at 35 °C for either 5 or 30 min. After the incubation, the first sample in each group was centrifuged at 35 °C (not shown) and the second sample from that group was incubated at 4 °C for 12 h and centrifuged at 4 °C, yielding the patterns shown. The *upper* schlieren pattern shows the sample incubated at 35 °C for *5 min* and the *lower* pattern shows the sample incubated at 35 °C for *30 min*, before the reversal to 4 °C. Note in the lower pattern the increase in the 57S material resulting from the longer exposure to 35 °C; this represents irreversibly modified enzyme which is easily distinguished from the 87S unmodified enzyme.

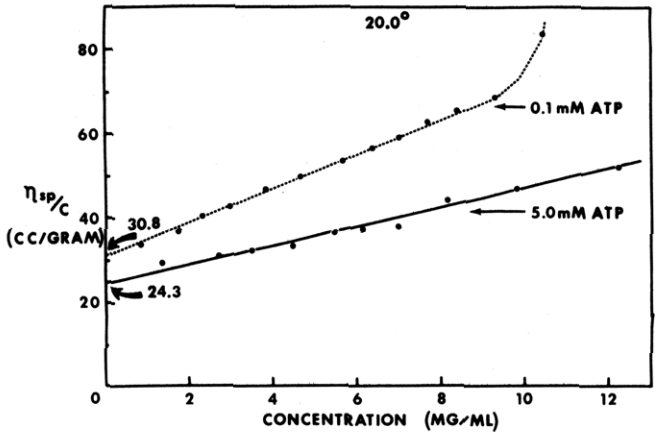

FIGURE 7: Concentration dependence of reduced viscosity of pig liver PFK at 20.0 °C. The experiments for the curve labeled 0.1 mM ATP used solutions containing buffer B (Massey and Deal, 1973): 50 mM Tris, pH 8.0; 110 mM ammonium sulfate; 0.1 mM ATP; 0.1 mM FDP; and 50 mM mercaptoethanol. The experiments for the other curve used solutions containing, in addition, 5 mM ATP, which previous studies have shown to disaggregate the enzyme.

periods of exposure to 35 °C result in proportionately greater conversion to irreversibly modified enzyme. The use of higher protein concentrations does not diminish the irreversible modification. On the contrary, the *absolute* amount of irreversibly modified enzyme produced seems to be *directly related* to the protein concentration. Also, there is no direct correlation between the areas under the slower curve in the bimodal peaks at 35 °C and the amount of irreversible 57S material found in sedimentation velocity experiments with samples cooled to 4 °C. The 60S *irreversibly* modified enzyme is indistinguishable from the 60S *reversible* (unmodified) enzyme in velocity experiments at 35 °C.

Viscosity Studies: Establishment That PL-PFK Is Very Asymmetric. Viscosity studies were carried out at 20 °C, where a concentration-dependent equilibrium seems to shift from the 100S form at high concentration to increasing amounts of the 60S form as the concentration is decreased below about 9 mg/mL (Figure 3). The low-concentration form (60S) is very asymmetric, as shown by the value of 30.8 cm^3/g for the intrinsic viscosity extrapolated from the low-concentration data (Figure 7). In contrast, most globular proteins have intrinsic viscosity values near 3–4 cm^3/g (Schachman, 1963). The high-concentration form (104 S) is even more asymmetric. Reduced viscosity values of 69, 84, and 470 cm^3/g were measured at concentrations of 9.2, 10.4, and 20 mg/mL, respectively. The results at 20 mg/mL (not shown) were especially striking; the flow time for the sample was more than 2800 s, in comparison to a flow time of 276 s for the solvent.

As expected (Massey and Deal, 1973; Trujillo, J. and Deal, W. C., unpublished results), ATP decreases the viscosity of pig liver PFK. Further studies are under way to determine whether this is a direct effect on shape, or the indirect result of ATP-induced dissociation into particles which are more asymmetric.

The steep slope shown by the data in Figure 7 could be due to nonideality effects or due to a shift in equilibrium to a more asymmetric, associated polymeric form, or both; it is not possible to distinguish between the possibilities.

It should be noted that estimates of asymmetry based on the intrinsic viscosity values measured in this study are minimum values; if orientation of these asymmetric molecules occurred during flow in our viscometer the resulting solutions would have less resistance to movement, and, hence, lower measured viscosities, than would be obtained in a viscometer with a very low shear rate.

Temperature Dependence of the Catalytic Rate. The Arrhenius plot shown in Figure 8 indicates that, at 24 °C, the temperature dependence of the rate of catalysis undergoes a sharp change from 21.2 to 11.1 cal/(mol deg). However, these experiments, which were carried out at a protein concentration of about 0.55 μg/mL, cannot be directly compared with the previously described physical analyses, which were carried out with protein concentrations above 0.3 mg/mL.

Discussion

Unique Features of Pig Liver Phosphofructokinase: Large Size, Single Main Peak System, Rapid Equilibrium, and Asymmetry. These studies quantitatively establish that under comparable conditions the phosphofructokinase from pig liver is larger than any previously reported phosphofructokinase. In the higher concentration ranges, PL-PFK has sedimentation coefficient values of $s^0_{20,w}$ = 104 S at 4 and 23 °C and $s^0_{20,w}$ = 60 S at 35 °C. The 104S value would correspond to a molecular weight of about 10 000 000 if PL-PFK possessed typical globular symmetry (Table I), but since it is quite asymmetric, the 104S value must reflect a much larger molecular weight. The largest sedimentation coefficient values previously reported for native phosphofructokinases are 54 S for the sheep heart enzyme (Mansour, 1972; Mansour et al., 1966) and 80 S for the human erythrocyte enzyme (Tarui et al., 1972). However, the 80S value was judged by these workers to be a property only of the purified enzyme and not of the original native enzyme (Tarui et al., 1972); on the other hand, several different laboratories have estimated molecular weight values in the range of 6 000 000 for native erythrocyte phosphofructokinases, based on gel-filtration studies (Layzer et al., 1969; Zimmerman et al., 1973). Recent reports from studies with the sheep heart enzyme indicated a 7S, 30S bimodal boundary with a 19S shoulder on the 30S peak (Brennan et al., 1974).

These studies also show that PL-PFK differs from virtually all other mammalian phosphofructokinases in that it exhibits a unimodal main peak in sedimentation velocity patterns at 4 and 23 °C. (At 35 °C, most patterns show a unimodal peak (58 S) but there is a barely perceptible hint of a shoulder (54S) under certain conditions.) In contrast, the rabbit skeletal muscle phosphofructokinase generally exhibits a *trimodal* boundary (13, 18, 30 S) and the sheep heart enzyme exhibits a *bimodal* boundary (7, 30 S), with an indication of a 19S shoulder on the 30S peak. It should be noted, however, that more symmetrical, although not unimodal, peaks have been observed with the rabbit skeletal muscle enzyme (Parmeggiani et al., 1966) and the sheep heart enzyme (Mansour, 1972; Mansour et al., 1966). The erythrocyte phosphofructokinases generally exhibit multiple peaks on gel-filtration columns (Zimmerman et al., 1973) and would also be expected to do so in sedimentation velocity experiments, which have even greater resolving power. An avian liver (chicken) phosphofructokinase exhibits a 28S peak typically, but also, under appropriate conditions, may indicate peaks of 5 and 21 S (Kono et al., 1973); avian liver phosphofructokinases differ from most mammalian liver phosphofructokinases (except sheep) in being cold inactivated (Kono et al., 1973).

PL-PFK is shown by these studies to be in rapid equilibrium under all conditions studied. The erythrocyte phosphofructokinase has also been reported (Zimmerman et al., 1973) to be a rapidly equilibrating system in gel-filtration studies, although, as mentioned earlier, it yields multimodal patterns. There are conflicting results on the question of whether all of the rabbit skeletal muscle PFK is in rapid equilibrium (Leonard and Walker, 1972; Aaronson and Frieden, 1972).

Another difference between pig liver PFK and rabbit skeletal muscle PFK is that the state of association of PL-PFK is very temperature dependent, while the sedimentation velocity patterns of the rabbit muscle enzyme are reported to be essentially unchanged at pH 8.0 upon variation of the temperature from 4 to 22 °C (Aaronson and Frieden, 1972).

One major discovery of these studies is the marked asymmetry of PL-PFK, as shown by the viscosity studies and the concentration dependence of the sedimentation coefficients. The shape properties of other phosphofructokinases had not previously been studied, except indirectly in gel-filtration studies on rabbit skeletal muscle PFK (Pavelich and Hammes, 1973; Lad et al., 1973). These studies did not indicate any unusual asymmetry. Recent studies from this laboratory (Johnson et al., 1976) have extended these results and shown that pig kidney PFK is also very asymmetric but that rabbit muscle PFK exhibits comparatively little asymmetry.

Application of Gilbert Theory. Only a single peak is observed in the experiments in the higher concentration range at 4 and 23 °C. Gilbert theory (Gilbert, 1959) predicts that *only* a *monomer–dimer* equilibrium can give rise to a boundary with a *single* maxima and, conversely, that a monomer–trimer or monomer–tetramer or other such equilibrium must give rise to *multiple* maxima. However, it has been shown (Rao and Kegeles, 1958; Bethune and Grillo, 1967) that, depending on the equilibrium constant and the protein concentration, a *single* peak can be predicted for a monomer–higher polymer association system in rapid equilibrium, *if intermediates* are present (also, see Cann, 1970). Hence, with this extension, Gilbert theory predicts that this single peak system is either: (1) a monomer–dimer system or (2) a monomer–*n*-mer system with *n* greater than two and with intermediates present. We have previously mentioned under Results that the monomer–dimer model gives only a fair fit and that the fit is not good enough

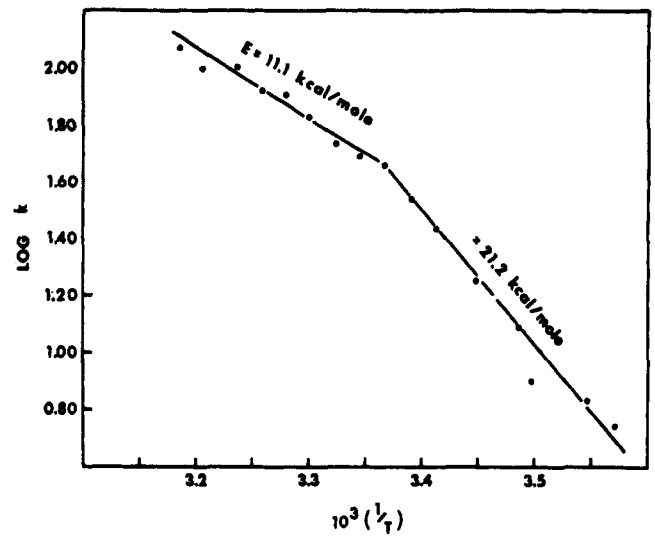

FIGURE 8: Effect of temperature on the activity of phosphofructokinase. Phosphofructokinase was assayed, as described under Methods, over a temperature range of 7 to 41 °C. The plot shows the log of the velocity (V) in arbitrary units vs. the reciprocal of the absolute temperature.

to definitively establish this model for the equilibrium. Gilbert theory, in its simplest form, assumes strong associations and constant sedimentation velocities. Gilbert (1963) has shown that for sufficiently weak association, interplay between the strength of association and hydrodynamic retardation of sedimentation could prevent bimodality from developing in a case where it would ordinarily. For substances with strong dependence of sedimentation coefficient upon concentration, such as the 104S material, sedimentation velocity patterns with unimodal boundaries might occur, even though the association system might involve a monomer and a polymer higher than a dimer. The protein, β-lactoglobulin A, is an example of such a combination; it exhibits a single peak over a wide range of protein concentration, even though it exists as a monomer–tetramer equilibrium mixture (Cann, 1970).

The discussion up to this point has ignored the low-molecular-weight material at the meniscus because Gilbert theory predicts that for a system in rapid equilibrium the curve between maxima will never come down completely to the baseline. However, the curve between the main peak and the low-molecular-weight material definitely does come down to the baseline (Figures 1 and 3), in contradiction to the predictions of Gilbert theory if this low-molecular weight material were in equilibrium. However, the myosin rapid equilibrium system is known to be an exception to this prediction (Josephs and Harrington, 1967).

Although the s vs. c data at higher concentrations at 4 and 23 °C fall on a straight line, suggesting the presence of a single-type polymer, we cannot definitely exclude the possibility that the straight lines are fortuitous and that the enzyme is an open-polymerizing system; i.e., that it undergoes progressive association to higher and higher molecular weight forms as the concentration is increased. If this is so, the associated products must become more and more asymmetric, since the apparent sedimentation coefficient decreases, rather than increases, which it normally would with an increase in molecular weight.

Studies are currently under way to obtain the molecular weight data necessary to distinguish between the possibilities raised above and to answer the questions regarding the types of equilibria and types of polymers involved in the PL-PFK association–dissociation reactions.

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