Structural Properties of an Active Form of Rabbit Muscle Phosphofructokinase*

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The quaternary structure of an active form of rabbit muscle phosphofructokinase was studied by sedimentation and electron microscopy. Active enzyme centrifugation studies at pH 7.0 and 23 ± 1°C showed that phosphofructokinase sediments as a single component with a sedimentation coefficient of 12.2 ± 0.5 S. Identical results were obtained in two assay and three solvent systems. Boundary sedimentation studies of phosphofructokinase in the presence of 1.0 mM fructose 6-phosphate, 0.1 mM adenylyl imidodiphosphate at pH 7.0 and 23 ± 1°C were performed. The results showed that the sedimentation coefficient of phosphofructokinase remains constant within the range of protein concentration studied and assumes a value of 12.4 S. The molecular weights of the subunit and the 12.4 S component were measured by sedimentation equilibrium yielding values of 83,000 and 330,000 for the monomeric and polymeric species, respectively. It is, therefore, concluded that the active form of phosphofructokinase is indeed the tetrameric species.

The structure of the phosphofructokinase tetramer was also studied by electron microscopy of negatively stained specimens. Particles identified as tetramers measured approximately 9 nm in diameter by 14 nm in length. The observed size and shape are consistent with the hydrodynamic measurements. Structural features within the tetramer were interpreted as due to the four individual subunits, each one approximately 4 X 6 X 6 nm in size, arranged with D5 symmetry.

A knowledge of the structure and hydrodynamic properties of an enzyme molecule under conditions where it is fully active is important for understanding the enzymatic reaction at the molecular level. In an effort to acquire such a knowledge on rabbit muscle phosphofructokinase (ATP: D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11), tetrameric and octameric forms of the enzyme were obtained by chemical crosslinking (Paradies, 1979). The isolated tetramers and octamers were studied with hydrodynamic and small angle x-ray-scattering techniques, and it was reported that the protomers assume different gross conformations in the presence of various ligands. Since native phosphofructokinase undergoes reversible association-dissociation, the structural changes observed in these studies might be partly or wholly due to the physical constraints of the cross-links or an effect of chemical modification. This possibility was not tested.

In a study of the active form of phosphofructokinase, Hesterberg and Lee (1980) reported that the enzyme sediments as a single component at pH 8.55 with a \( \bar{s}_{0.1} \) value of 12.4, as monitored by the technique of active enzyme centrifugation. The low concentration of enzyme utilized by this technique makes it possible in theory to correlate the physical state of the enzyme with its kinetic properties. Although it is possible to obtain accurate values for sedimentation and diffusion coefficients from active enzyme centrifugation (Cohen et al., 1967; Claverie, 1980), it requires extensive and complicated computations. In general practice, the approximation approach, in which sedimentation and diffusion of products are not considered, is usually adopted and only the sedimentation coefficient is reported. The sedimentation coefficient alone is not sufficient, however, to determine the structural features of an oligomeric enzyme or an enzyme such as phosphofructokinase which undergoes association-dissociation (Ling et al., 1965; Parmeggiani et al., 1966; Aaronson and Frieden, 1972; Pavelich and Hammes, 1973; Lad and Hammes, 1974; Leonard and Walker, 1972). Further complicating the analysis is the fact that the aggregation state of phosphofructokinase is dependent on the presence of ligands (Pavelich and Hammes, 1973; Parr and Hammes, 1973; Hill and Hammes, 1975; Lad et al., 1973).

The present study was initiated to employ other physical measurements in addition to active enzyme centrifugation with the goal of characterizing the structure and hydrodynamic properties of the active form of phosphofructokinase. These measurements include sedimentation velocity, sedimentation equilibrium, and electron microscopy in the presence of substrate and substrate analogues. These measurements were made at pH 7.0 in order to relate the results to the extensive kinetic studies reported in the literature (Frieden et al., 1976; Pettigrew and Frieden, 1978; Goldhammer and Hammes, 1978). In addition, phosphofructokinase appears to exhibit enhanced allosteric properties at pH 7 than that at pH 8.5 and the lower pH is closer to physiological conditions.

MATERIALS AND METHODS
Aldolase, triose phosphate isomerase-glycerol 3-phosphate dehydrogenase, and AMP-PNP\textsuperscript{1} were obtained from Boehringer-Mannheim.

The abbreviations used are: AMP-PNP, adenylyl-imidodiphosphate; SDS, sodium dodecyl sulfate; GdnHCl, guanidine hydrochloride.

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Dedicated to the memory of the late Professor Merton F. Utter.

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by the flotation technique (Valentine, 1969). ATP, dithiothreitol, NADH, DzO, p-nitrophenol, and fructose-6-P were obtained from Sigma Chemical Co. Glycerol and sucrose were purchased from Fisher Scientific Co. and Schwarz/Mann, respectively. All commercially obtained enzymes and chemicals were used without further purification.

Rabbit muscle phosphofructokinase was isolated and purified by previously published procedure (Hesterberg and Lee, 1980). The sedimentation coefficient of phosphofructokinase was determined spectrophotometrically at 280 nm with an absorbivity value of 1.07 liter/g cm (Hesterberg and Lee, 1980).

**Enzymatic activity of phosphofructokinase at pH 7.00 was monitored by two assay systems, namely, a modified coupled enzyme system (Rascher, 1947) and a pH-dependent dye-linked system (Shill et al., 1974). The standard coupled enzyme system contained 25 mM Tris-carbonate, 1.0 mM EDTA, 3.4 mM (NH₄)₂SO₄, 6.0 mM MgCl₂, 0.1 mM dithiothreitol, 1 mM ATP, 1 mM fructose-6-P, 0.16 mM NADH, 1.25 units/ml of aldolase, 2 units/ml of mixed enzymes glycerol-3-phosphate dehydrogenase-triose phosphate isomerase with pH adjusted to 7.0 ± 0.05. The coupling enzymes were eluted from a Sephadex G-25 desalting column (0.9 × 4 cm) equilibrated with 25 mM Tris/CO₃, 1 mM EDTA, 6 mM MgCl₂, and 3 mM (NH₄)₂SO₄ at pH 7.00 (TEMA buffer). The reaction was initiated by the addition of 0.1 μl of phosphofructokinase to a volume of 1.45 ml of previously mixed assay mixture and monitored spectrophotometrically at 400 nm.

Active enzyme centrifugation and all subsequent data analyses were carried out using previously published procedures (Hesterberg and Lee, 1980). The values for the apparent partial specific volume of the enzyme in the presence of various solvents were adopted from an earlier report assuming that these values are not pH-dependent (Hesterberg and Lee, 1980).

Sedimentation velocity experiments were conducted at 23 °C, 90,000 rpm or 52,000 rpm with an AN-D or AN-F rotor, respectively, in a Spinco Model E analytical ultracentrifuge equipped with an electronic speed control, RTIC temperature control, and a photoelectric scanner. For all experiments, Kel-F-coated aluminum double-sector centerpieces with sapphire windows were used. The scanner was used to trace the protein boundary during sedimentation at 280 nm or 277.5 nm.

The subunit molecular weight of phosphofructokinase was determined by the high speed determination equilibrium method of Yphantis (1964). The sedimentation experiments were conducted at 20 °C and 20,000 rpm in 6 mM GdnHCl, 0.1% (v/v) β-mercaptoethanol and the interference patterns were recorded on Kodak IGG spectroscopic plates and a Dr. Matic microcomparator. Simultaneously, the molecular weight of phosphofructokinase in the presence of 1 mM fructose-6-P and 0.1 mM AMP-PNP was determined at 23 °C and 12,000 rpm in TEMA buffer. Under these conditions, the concentration gradient was monitored by the photoelectric scanner at 280 nm. The apparent partial specific volume of phosphofructokinase in native condition is 0.790 (Hesterberg and Lee, 1980), whereas in 6 mM GdnHCl it was calculated from amino acid composition (Parmeggiani et al., 1965) using the procedure of Lee and Timasheff (1979).

The molecular weight of denatured phosphofructokinase was also measured by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis using the procedure of Laemmli (1970). Slab gels (20 × 14 × 0.1 cm) of 8.5% acrylamide in 0.375 M Tris-HCl, 0.1% (w/v) SDS at pH 8.8 were employed with a 2-cm stacking gel of 6% acrylamide with 0.0675 M Tris-HCl, 0.1% (w/v) SDS at pH 6.8. All proteins were stained in 0.1% (w/v) Coomassie Blue G-250 in methanol:H₂O:acetic acid (5:5:1, v/v/v) and destained by soaking in methanol:H₂O:acetic acid (3:7:1, v/v/v). The gel was then dried on a sheet of Whatman No. 3MM chromatography paper.

Negatively stained specimens were prepared at room temperature by the negative staining technique (Valentine et al., 1968; Shill et al., 1974). The sedimentation experiments of phosphofructokinase were carried out using previously published procedures (Hesterberg and Lee, 1980). The stability of phosphofructokinase in 0.1 mM dithiothreitol/TEMA buffer at 23 °C was tested using previously published procedures (Hesterberg and Lee, 1980). The results showed that phosphofructokinase retains greater than 90% of its initial activity for a minimum of 8 h. Furthermore, the presence of 1 mM fructose-6-P and 0.1 mM AMP-PNP stabilized the enzyme such that no loss of activity was observed for 12 h, and the enzyme retained more than 80% of its activity for 24 h. Phosphofructokinase is, therefore, quite stable in the TEMA buffer and all experiments in this study were carried out in such a way that the enzyme retained at least 80% of its activity at the conclusion of the experiment.

Steady state kinetic measurements of phosphofructokinase in TEMA buffer gave values for K₅₅ of 65 μM and 75 μM for ATP and fructose-6-P, respectively. These results show that the buffer components did not alter the apparent affinity of phosphofructokinase for substrates. Furthermore, the presence of n-p-nitrophenol, the dye used in the pH-dependent, dye-linked assay system, had no observable effects on the kinetic behavior of phosphofructokinase. It may, therefore, be concluded that neither the buffer nor the dye employed influence the basic kinetic behavior of the enzyme.

**Active Form of the Enzyme**—The enzymic active form of phosphofructokinase was monitored by active enzyme centrifugation using both the coupled enzyme and pH-dependent, dye-linked systems. The sedimenting band of phosphofructokinase was stabilized by three solvent systems: 4% (v/v) or 10% (v/v) glycerol, 50% (v/v) D₂O, and 10% (w/v) sucrose. Under all conditions the scanner tracings indicate the presence of one component. The difference curves constructed from the scanner tracings are apparently symmetrical with no consistent skewing, an observation which indicates the presence of a single homogenous active species. Linear plots of ln radius versus time were obtained regardless of whether the radius was calculated from the midpoint of the boundaries or from the peak of the difference curve. The linearity of these plots for data collected throughout the entire duration of the experiment.

**RESULTS**

**Basic Properties of Phosphofructokinase in Tris/carbonate Buffer**—The Tris/carbonte buffer system employed in this study was chosen so as to eliminate the possibility of a specific buffer effect with phosphate such as that reported by Hofer and Pette (1968). Most reports, however, indicate that phosphate is used to stabilize the enzyme (Leonard and Walker, 1972; Pavlik and Hammes, 1973; Blokhamp and Lardy, 1973; Paradies and Vettermann, 1976; Pettigrew and Frieden, 1979; Liou and Anderson, 1980). It was, therefore, essential to monitor the kinetic and physical properties of the enzyme in the Tris/carbonte buffer.

**TABLE I**

| Assay system | Solvent | S₀₁₀₀₀° " |
|--------------|---------|-----------|
| Coupled enzyme | 4% (v/v) Glycerol | 12.2 ± 9.4 |
| Coupled enzyme | 10% (v/v) Glycerol | 12.3 ± 0.4 |
| Coupled enzyme | 50% (v/v) D₂O | 11.7 ± 0.3 |
| pH-Dye | 10% (v/v) Glycerol | 12.5 ± 0.8 |

*Average values and maximum deviations of at least four duplicate experiments under each set of conditions.*
experiment indicates that no detectable denaturation or dissociation of phosphofructokinase occurred during the experiment.

The effect of solvent systems on the sedimentation behavior of phosphofructokinase was tested and the results are summarized in Table I. These results, which are corrected for preferential solvent-protein interactions, show that the solvents employed do not influence active form of the enzyme. The amount of enzymic activity in the sedimenting phosphofructokinase band was compared to the activity of the enzyme under steady state conditions using previously published procedures (Hesterberg and Lee, 1980). Within an experimental error of ±10%, the enzymic activity, observed as a sedimenting species of $s_{20,w} = 12.2 \pm 0.5$ S, accounts for all of its steady state kinetic activity. The stability of phosphofructokinase during an active enzyme centrifugation experiment was tested. The total enzymic activity of the sedimenting band was a linear function of the time of centrifugation, indicating no inactivation of the enzyme has occurred during the experiment.

The relation between $s_{20,w}$ and substrate concentration was tested using the procedure published previously (Hesterberg and Lee, 1980). The value for $s_{20,w}$ was shown not to vary with substrate concentration within a range of 1 to 5 mM, nor did it change with higher concentration of coupling enzymes. These results indicate that, under the present experimental conditions, both substrates and coupling enzymes were in excess, conditions that must be fulfilled in active enzyme centrifugation experiments.

Active enzyme centrifugation was also conducted in the pH-dependent, dye-linked assay system. The only observable active form sedimented as a homogeneous species with an $s_{20,w} = 12.2 \pm 0.5$ S. There was no observable dependence of $s_{20,w}$ on protein concentrations ranging from 0.5 to 25 μg/ml.

A crude preparation of the enzyme, after the initial (NH$_4$)$_2$SO$_4$ precipitation step, was subjected to active enzyme centrifugation analysis. The results are in good agreement with those observed for the highly purified enzyme, namely, the enzyme sediments as a single active component with an $s_{20,w}$ of 12.0 ± 0.4 S.

**Self-association of Phosphofructokinase**—The sedimentation behavior of phosphofructokinase, in the presence and absence of 1 mM fructose-6-P, 0.1 mM AMP-PNP, is shown in Fig. 1 as a function of phosphofructokinase concentration. Within the concentration range studied there was no detectable change in the sedimentation behavior of phosphofructokinase. It sediments as a single component with a $s_{20,w} = 12.4$ S. This observation is in sharp contrast to the behavior of phosphofructokinase in the absence of substrates at pH 7.0 and 23 °C, as shown by the open circles in Fig. 1. In this case there was a pronounced decrease in the value of $s_{20,w}$ at low protein concentrations, which indicates that phosphofructokinase dissociated under those conditions. One may then conclude that the self-association of phosphofructokinase is enhanced in the presence of both substrates and that the sedimentation coefficient determined by boundary sedimentation velocity is in good agreement with that determined by active enzyme centrifugation.

**Molecular Weights of Phosphofructokinase**—Sedimentation equilibrium experiments were conducted to monitor the molecular weight of phosphofructokinase at loading concentrations of 50, 100, and 150 μg/ml. There is a slight concentration dependence in the apparent weight average molecular weight with an extrapolated value of 330,000 at infinite dilution, as shown in Fig. 2. Since there is overlapping of the apparent molecular weights regardless of the initial loading concentrations, it may be concluded that the system is in equilibrium and that the small increase in molecular weight with increasing protein concentration reflects a further aggregation of phosphofructokinase tetramers.

Reduced and denatured phosphofructokinase was subjected to electrophoresis in a polyacrylamide gel containing 0.1% SDS. The results indicate a molecular weight of 84,000 ± 1,000 for the phosphofructokinase subunits, consistent with reported values ranging from 73,000 to 85,000 (Scopes and Penny, 1970; Leonard and Walker, 1972, Goldhammer and Paradies, 1979).

These results were further checked by equilibrium sedimentation studies in the presence of 6 M GdnHCl and 0.1% β-mercaptoethanol. Linear plots of ln(f) versus r$^2$ were obtained for data points extended to the bottom of the cell. There was no difference between the weight- and number-average molecular weights, as shown in Fig. 3, indicating that the phosphofructokinase sample is monodisperse. A value of 81,000 was obtained for the molecular weight at infinite dilution.

Electron Microscopy of Phosphofructokinase—Negatively stained specimens showed a fairly homogeneous spread of discrete particles, which were interpreted to be phosphofructokinase tetramers. In almost all images, the phosphofructokinase tetramer appears as an approximately rectangular structure measuring 9 × 14 nm as shown in Fig. 4a. This suggests that the particle is roughly cylindrical, measuring 9
nm in diameter by 14 nm long, with the long axis almost always extended on the carbon film. The images show a prominent cleft perpendicular to the long axis, which divides the particle in half. The substructure within each half varies, from particle to particle. This variation can be interpreted to reflect a change in the orientation of the particle about the long axis, so that the different characteristic images represent views of the particle at different angles.

In one view, as shown in Fig. 4b, one of the halves displays a prominent longitudinal cleft, dividing it into halves again. When the bottom half shows this longitudinal cleft, the top half generally shows a trapezoidal shape, tapering upward, but without visible internal structure.

These observations can be simply interpreted in terms of subunit structure. The elongated particle (9 × 14 nm) is the tetramer. The prominent horizontal cleft, which is seen regardless of the orientation about the axis, demonstrates a division into two dimers. The further division of each dimer into individual subunits can be seen only in particular orientations, namely that of the bottom dimers shown in Fig. 4b. Here, the monomeric subunit shows a profile roughly 4 × 6 nm. The length of the subunit in the third dimension must be less than the 9 nm of the complete tetramer. The trapezoidal shape of the upper dimers suggests a complicated one with 6 nm being a reasonable estimate for the average width of an equivalent rectangular prism in this projection.

The volume occupied by a subunit is given by

$$V = \frac{M \tilde{\rho}}{N_A}$$

where $M$ is the molecular weight, $N_A$ is Avogadro’s number, and $\tilde{\rho}$ is the partial specific volume of phosphofructokinase, 0.730 (Hesterberg and Lee, 1980). For a molecular weight of 83,000, the volume for the subunit of phosphofructokinase is 101 nm$^3$, which fits easily into the 144 nm$^3$ volume of rectangular prism (4 × 6 × 6 nm). This allows sufficient volume for a conical shape with corners and edges of the rectangular prism rounded off to give a shape more consistent with that seen in the micrographs.

Fig. 4c shows several examples of another characteristic view of the particle. Here, one of the dimers shows a central spot, presumably a channel of stain in projection, and the other shows a faint horizontal line of stain. The line is centrally located, extending only about halfway to each edge, and may be a slightly tilted view of the same channel seen in the upper dimer. The most likely interpretation is that the channel may represent a high resolution feature of the individual subunit, such as a cleft or channel, and not a further subdivision of the quaternary structure.

The presence or absence of 1 mM fructose-6-P and 0.1 mM AMP-PNP did not produce any noticeable changes in the images of the tetramer. All specimens showed particles with the same range of structural features, in particular the characteristic views seen in Fig. 4, b and c. The electron micrographs suggest that there is no pronounced conformational change under these conditions.

Smaller, less dense particles were seen in all preparations, but did not present characteristic views that could identify them as monomers or dimers. They appeared to be less numerous than the identified tetramers, but a quantitative assessment is difficult because the smallest particles were of insufficient contrast to be counted reliably. In addition, higher polymers than the tetramer were not observed in these images, consistent with the sedimentation analysis.

**DISCUSSION**

The determination of the hydrodynamic properties of the active species of rabbit muscle phosphofructokinase was made feasible by employing AMP-PNP as a nonhydrolyzable analogue of ATP. It is assumed that AMP-PNP interacts with phosphofructokinase in a manner similar to that of ATP and
in particular would influence the physical properties of the enzyme in a similar manner. It is, therefore, important to examine the validity of these assumptions.

Yount et al. (1971a,b) synthesized AMP-PNP and demonstrated that it is very similar structurally to ATP. Results from steady state kinetic studies show that AMP-PNP is a strong competitive inhibitor of the substrate, ATP (Barzu et al., 1977; Lad et al., 1977). Furthermore, based on results from equilibrium binding studies, Wolfman et al. (1978) reported that the interaction between AMP-PNP and phosphofructokinase can be interpreted in terms of binding to both the catalytic and inhibitory sites, a property characteristic of ATP. There is, therefore, strong evidence to support the assumption that the substrate analogue binds to phosphofructokinase in a manner similar to ATP and it is reasonable to assume that the hydrodynamic properties of the enzyme determined in the presence of AMP-PNP are indicative of these in the presence of ATP.

The active enzyme sediments as a single component with a sedimentation coefficient of 12.4 S. This observation is identical with that at pH 8.5 indicating that the active species is not perturbed by a change in pH from 7.0 to 8.5. Since the value of $s_{20,w}$ determined by active enzyme centrifugation involves the approximation method, which does not take into consideration the diffusion and sedimentation of product, a lower apparent value of $s_{20,w}$ might be expected (Cohen and Claverie, 1975). To date no data have been available on a self-associating system to compare the sedimentation coefficients determined by active enzyme centrifugation and conventional sedimentation boundary experiments. In the present study, by employing AMP-PNP as a nonhydrolyzable analogue for ATP, it was possible to determine the $s_{20,w}$ of phosphofructokinase in the presence of fructose-6-P and AMP-PNP by boundary sedimentation. The results of this study show that at the lowest protein concentration examined, i.e. 10 µg/ml, the sedimentation coefficient of the phosphofructokinase-fructose-6-P-AMP-PNP complex remains at 12.4 S. Within the range of protein concentration examined there is no indication of a concentration dependence of the sedimentation coefficient. One may, therefore, safely assume that the enzyme-substrates complex does not dissociate into smaller oligomeric forms. The extrapolated value of $s_{20,w}$ at infinite dilution is 12.4 S, which is in good agreement with that determined by active enzyme centrifugation. The approximation method is, therefore, shown to be capable of yielding accurate values of sedimentation coefficients.

The results of the present studies show that rabbit muscle phosphofructokinase is composed of subunits with a molecular weight of 83,000 ± 2,000. Since the molecular weight of phosphofructokinase in the presence of fructose-6-P and AMP-PNP determined by sedimentation equilibrium is 330,000, four subunits must aggregate to form the complex. It is, therefore, established unequivocally that the 12.4 S species is indeed the tetrameric form of phosphofructokinase rather than a larger, highly asymmetric aggregate. The apparent increase in molecular weight at higher protein concentrations of greater than 100 µg/ml indicates the presence of a small fraction of aggregates larger than the tetramer. It may imply that these larger aggregates are also active, a conclusion in agreement with the report of Paradis (1979). Having determined the molecular weights of the subunit and the tetrameric form of phosphofructokinase, it is possible then to determine the hydrodynamic properties of the enzyme. The sedimentation coefficients of the monomeric and enzymic active tetrameric forms of phosphofructokinase are 4.95 S and 12.4 S, respectively (Hesterberg, 1980). The axial ratios of these polymeric forms can then be examined by means of the frictional ratio, $f/f_0$, since

$$f = \frac{M(1 - \bar{v}_p)}{f_0} S_{20,w} \eta (162\pi N_{A} M \bar{v}_p + \delta \bar{v}_p)^{1/2}$$

where $M$ is molecular weight of the sedimenting species, $\bar{v}_p$ is partial specific volume and assumes a value of 0.730 for phosphofructokinase (Hesterberg and Lee, 1980), $\eta$ is density of solvent, $\eta$ is viscosity of solvent, $N_{A}$ is Avogadro’s number, $\delta$ is water of hydration in g$_{H_2O}$/g$_{molecule}$ (Edsall, 1953) and $v_i$ is the specific volume of solvent which is equal to the inverse of $\rho$. $\bar{v}_p$ was estimated according to the method of Kuntz (1971) and a value of 0.43 g/g was obtained. This value, however, represents the amount of hydration for a totally unfolded protein molecule with every amino acid residue exposed to the solvent. For a protein molecule in its active state, the internal residues are not accessible to solvent, thus the actual value for $\delta$ must be corrected for the non-accessibility of these residues. The correction factor can be derived from comparing the values of $\delta$ determined by the method of Kuntz (1971), which measures hydration for totally exposed residue, and that determined for a native molecule in its folded state, such as the values reported by Bull and Breese (1968). Having compared the values of $\delta$, for a number of proteins it was found that, after correcting for non-accessibility of internal residues, only 70% of $\delta$ was calculated by the method of Kuntz is associated with the folded protein molecule. The corrected value of $\delta$ for phosphofructokinase is, therefore, 0.3 g/g. Using known values for the parameters expressed in Equation 2, $f/f_0$ was calculated to be 1.23 and 1.24 for the monomeric and tetrameric species, respectively. Knowing that $\delta = 0.3$ g/g, these values of $f/f_0$ correspond to a prolate ellipsoid of resolution with an axial ratio, $a/b$, of approximately 5 (Svedberg and Pedersen, 1940).

Having estimated the hydrodynamic shape of the active form, it is of interest to extract information on the spatial arrangement of subunits from the sedimentation studies. The time-averaged distance between subunits of the tetrameric species of phosphofructokinase is related to its sedimentation coefficient by the theory derived by Kirkwood (1954). According to this theory, if the protein is composed of an arbitrary array of connected identical subunits it is possible to calculate its sedimentation coefficient by

$$S = \frac{M(1 - \bar{v}_p)}{n N_0 \bar{v}_p} \left( \frac{1}{f} - \frac{1}{6 \eta} \sum_{i=1}^{n} \sum_{\gamma} \langle R_{ij} \rangle^{-1} \right)$$

where most of the terms assume the same significance, $n$ is the number of subunits in the array, $f$ is the frictional coefficient of the monomer, and $\langle R_{ij} \rangle$ is the time-averaged distance between the $i$ and $j$ subunits. Andrews and Jeffrey (1980a,b) rearranged Kirkwood’s equation to obtain a ratio of the sedimentation coefficient of an oligomer to that of its protomer so that

$$\frac{S_{20}(\infty)}{S_{20}} = \left[ 1 + \frac{1}{n_0} \sum_{\gamma} \sum_{\gamma} \langle R_{ij} \rangle^{-1} \right]^{-1} \times \left[ 1 + \frac{1}{n_0} \sum_{\gamma} \sum_{\gamma} \langle R_{ij} \rangle^{-1} \right]^{-1}$$

where $n_0$ and $n_1$ are the numbers of subunits in the oligomeric and protomeric forms, respectively. Expressing experimental values of sedimentation coefficient as a ratio, it is then possible to compare with values calculated for various structures to aid in the elucidation of the assembly pattern. The hydrodynamic parameters of phosphofructokinase obtained from the sedi-
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The sedimentation studies are, therefore, used to analyze the quaternary structure of the enzyme. The sedimentation coefficients of the tetrameric and monomeric forms are 12.4 S and 4.95 S, respectively (Hesterberg, 1980; Hesterberg and Lee, 1981). Since the reported error in the sedimentation coefficient for the tetramer is ±0.5 S, the sedimentation ratio assumes values ranging from 2.42 to 2.61. Taking the average value of 2.5 from the graph for the sedimentation ratios of tetrameric assemblies (Fig. 6 of Andrews and Jeffrey, 1980) it may be seen that the above data are consistent with at least three different structures with axial ratios ranging from 1 to 1.5. The model does not distinguish between cyclic and dihedral symmetry; however, it does provide a means to estimate the dimensions of the subunit. To obtain the actual dimensions of phosphofructokinase, the reduced dimensions, as shown in Table IV of Andrews and Jeffrey (1980), are multiplied by the cube root of the molecular volume calculated from Equation 1. The predicted dimensions for the phosphofructokinase tetramer are 13 × 18 × 18 nm.

The arrangement and dimensions of subunits deduced from hydrodynamic parameters agree reasonably well with those observed in the electron micrographs. All of the images of phosphofructokinase in Fig. 4 are consistent with D2 dihedral symmetry, with one of the 2-fold axis coincident with the long axis of the cylindrical tetramer. The alternative possibility is that phosphofructokinase exhibits C2, cyclic, symmetry. This would require that the long axis by a 4-fold axis, which in turn would require the prominent lateral cleft and the apparent 2-fold symmetry perpendicular to the long axis be attributed to features within each subunit, rather than to the interfaces between subunits. Although C2 symmetry cannot be excluded by the results available at present, a model with D2 symmetry is a much simpler and more satisfactory interpretation of the images. A similar model was proposed for porcine liver phosphofructokinase (Foe and Trujillo, 1980). Furthermore, the proposed model is consistent with the observation that essentially all tetrameric proteins of known structure display D2 symmetry (Klots et al., 1975). A model for phosphofructokinase constructed with four identical subunits with D2 symmetry is shown in Fig. 5. Each subunit is approximately 4 × 6 × 6 nm (tapered to a trapezoidal shape) and the tetrameric particle measures 9 nm in diameter by 14 nm in length. Certain features of the model, such as the skewed pointed shape of the subunits and the 30° rotation of one dimer with respect to the other, are speculative but entirely consistent with the phosphofructokinase images, especially those seen in Fig. 4b. The higher resolution features shown in Fig. 4c are not included in this model.

An earlier electron microscopic study by Telford et al. (1975) concluded that the individual subunit was a prolate ellipsoid with axis of 6.7 × 2.5 × 2.5 nm. Dimers consisted of two monomers lying side by side and tetramers were formed from two dimers in a planar end to end array, with total dimensions of 13 × 8 × 2.5 nm. The subunit size proposed is measured to about 2 nm from the electron micrographs, and these dimensions most likely represent the size and shape of the molecules in solution. Concerns about conformational changes or significant shrinkage as the molecules are dried in the negative stain have been expressed (Haschemeyer, 1970; Haschemeyer and Myers, 1972). However, in cases where independent structural information is available, the structures determined by electron microscopy are accurate to at least 2 nm resolution. For example, the structure of the stacked disk aggregate of tobacco mosaic virus as determined by electron microscopy is in excellent agreement with x-ray diffraction data of hydrated specimens (Unwin and Klug, 1974). The length of negatively stained fibrinogen dimers, cross-linked at the end-to-end contact, is exactly twice that of individual molecules (Fowler et al., 1981), and the lengths are consistent e.g. chemical cross-linkage or protein staining. Lacking an exact model to describe a protein molecule in solution one is limited to the approximation of structure corresponding to a prolate or oblate ellipsoid of revolution. Such a structure with smooth and impenetrable surfaces can not adequately represent the protein surfaces which consist of many projections and clefts, resulting in an overestimation of asymmetry of the protein molecule. The apparently high axial ratio of 5 estimated for phosphofructokinase should, therefore, be interpreted as the upper limit of asymmetry and the only significance of that value is that phosphofructokinase does not assume a spherical structure. Furthermore, the estimation of axial ratio is also subjected to the uncertainty of estimating the amount of hydration, δ1. It is conceivable that the actual amount of hydration is greater than the value of 0.3 g/g estimated in this study, thus leading to an estimation of a molecule with lower axial ratio than the reported value of 5.

Treatment of sedimentation data by the procedure proposed by Andrews and Jeffrey, however, represents a simple but useful analysis yielding conclusion in reasonably good agreement with results derived from electron microscopy.

The dimensions of the negatively stained particles can be measured to about 2 nm from the electron micrographs, and these dimensions most likely represent the size and shape of the molecules in solution. Concerns about conformational changes or significant shrinkage as the molecules are dried in the negative stain have been expressed (Haschemeyer, 1970; Haschemeyer and Myers, 1972). However, in cases where independent structural information is available, the structures determined by electron microscopy are accurate to at least 2 nm resolution. For example, the structure of the stacked disk aggregate of tobacco mosaic virus as determined by electron microscopy is in excellent agreement with x-ray diffraction data of hydrated specimens (Unwin and Klug, 1974). The length of negatively stained fibrinogen dimers, cross-linked at the end-to-end contact, is exactly twice that of individual molecules (Fowler et al., 1981), and the lengths are consistent
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with the longitudinal periodicity of fibrin fibers as determined both by x-ray diffraction and electron microscopy (Stryer et al., 1963).

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