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SOME MEASUREMENTS OF THE SHAPE AND HYDRODYNAMIC PROPERTIES OF YEAST PHOSPHOGLYCERATE KINASE (E.C.2.7.2.3)

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Using values obtained for sedimentation and diffusion constants the relative mass of phosphoglycerate kinase was calculated to be 45 800 ± 1700. This value is higher than was previously estimated and the difference is thought to be caused by contamination of earlier crystalline preparations. Using the coordinates from X-ray crystallography it was found possible to calculate a frictional ratio for a linear dumb-bell (1.115) which compared well with the ratio calculated from diffusion (1.114 ± 0.033). Since the calculated ratio for a bent molecule was 1.020 the natural state of the molecule in solution is essentially linear. From the concentration dependence of sedimentation and diffusion was calculated the effective interactive radius which resembles haemoglobin in its relationship to the molecular radius.

1. Introduction

The enzyme phosphoglycerate kinase is constructed from a single protein chain folded backwards and forwards to give an overall shape which resembles a dumb-bell [1,2]. Having two large masses separated by a relatively thin stalk means this molecule is capable of considerable flexing so the overall shape in the crystal could be different from that in solution. Measuring the shape in solution of molecules like phosphoglycerate kinase presents a challenge to workers wishing to use crystallographic data to calculate molecular parameters. The problem in the present case is that the ratio of the semi-major axis to the semi-minor axis is between two and three which means it cannot be considered a sphere, while the size of the waist makes it unconvincing to treat the molecule as a prolate ellipsoid. These problems are amplified when the details of the co-ordinate are examined because the two so-called spheres at each end are really ellipsoids not having identical dimensions, in fact phosphoglycerate kinase cannot be considered as a regular geometrical body. This in itself is not an obstacle because numerical procedures are available which overcome the difficulties and of the methods available we have chosen the one developed by Bloomfield and colleagues [3]. In general these calculations have been tested and found to work with large assemblies of macromolecules [4]; there seem to be fewer tests for smaller macromolecules similar in size and dimensions to phosphoglycerate kinase.

Comparisons of calculated parameters with experimental measurements are only useful if the samples are pure and homogeneous. Some confusion is possible here with phosphoglycerate kinase because there is a suggestion that the protein exists in solution as part of a weak associating system [5]. Thus, the present work has two aims, (1) reexamine the hypothesis that association can occur in solutions of phosphoglycerate kinase and (2) if necessary, adjust conditions to produce a homogeneous protein which can then be studied hydrodynamically.

2. Experimental

2.1. Samples of phosphoglycerate kinase

Three separate samples were examined, two were gifts from Dr. H. Watson (University of Bristol, U.K.)

and one prepared by the method of Scopes [6]. The two samples from Dr. H. Watson were crystals, while the third sample was not; the crystals were separated from the 3M-$(NH_4)_2SO_4$ by centrifuging at low speeds and then dissolved in about 1 cm^3 of phosphate buffer pH 7.0 containing 0.05 M KH_2PO_4, 0.05 M Na_2HPO_4, 0.05 M NaCl, and 1 mM EDTA. For most experiments these samples were equilibrated by either dialysing for more than 18 hours against about hundred-fold volume of buffer, or passing the solution down a 10 cm × 1 cm column of G100 sephadex (Pharmacia Ltd, U.K.) and collecting 0.6 cm^3 samples. Where necessary the solutions were then concentrated using either 'Aquadice II' (polyethylene glycol having a molecular weight >250 000, Calbiochem Corp.) or by ultrafiltration using a Millipore filter (No. PTGC 01310, having a limit of relative mass of 10^4) mounted in a 13 mm cell (Millipore (U.K.) Ltd, London). Results from the two methods of equilibration and concentrating solutes were indistinguishable from one another.

2.2. Ultracentrifuge experiments

Photographic records were collected from runs made using a M.S.E. analytical ultracentrifuge. Samples were examined in a two hole rotor using either a 20 mm or a 10 mm double sector centrepiece in which one sector contained solvent and the other solution. The temperature of the rotor was controlled in the region of 20°C and the temperature was stable within the range ±0.1°C. The absolute measurement of temperature was checked regularly and found to be stable. The results from the schlieren optics were recorded on film and coordinates were measured to the nearest 10^{-3} mm using a Nikon measuring microscope. Between 20 and 40 coordinates were measured over the boundary, with additional measurements made on the solvent base-line. These coordinates were converted using second moments (eq. (1)) for each exposure,

$$\bar{r}^2 = \sum_{i=1}^{n} r^2 (dn/dr)_r dr \Big/ \sum_{i=1}^{n} (dn/dr)_r dr, \qquad (1)$$

where $(dn/dr)_r$ is the deflection of light at radial position r, measured in arbitrary units from the photograph. The measurements of $\ln \bar{r}$ (up to 6 per experiment recorded at 400 second intervals) were fitted against time using a regression analysis to fit first a straight line then a quadratic and the improvement from using the quadratic equation tested by a F-test. Similar fittings were made for measurements made for the velocity of the maxima of the schlieren peaks. From the resulting slopes were calculated sedimentation coefficients; the data in this series of experiments always fitted straight lines.

The estimated sedimentation coefficients were converted to $S_{20,w}$ by allowing for density of solution and viscosity of buffer in the accepted manner [7]. The concentrations of protein were estimated from the denominator of equation (1). The sum was converted to concentrations in w/v through a constant which allowed for factors like schlieren angle, refractive index increment and cell thickness and was determined in separate experiments using sucrose solutions of known concentrations. The concentrations estimated for zero time were similar to those calculated from absorbance at 280 nm using $E^{1\%}_{10mm} = 4.9$ [5]. In a sector shaped cell the concentration of the plateau should decrease with time and follow a simple logarithmic relationship (radial dilution law) [8]. Thus plotting $\ln C$ against time should give a straight line whose slope is $-2\,\omega^2 S$ and for a pure solute this S from the plateau should correspond in every way with the S calculated from sedimentation of the boundary. In mixtures containing mainly one component but contaminated with small quantities of other components having a wide range of sedimentation coefficients, the results from analysing the boundary can give a false impression concerning purity. A more sensitive test for purity is to estimate the slope of $\ln C$ from the plateau against t, again using statistical regression analysis (C was taken as being the denominator of eq. (1)).

2.3. Measurement of translational diffusion

Diffusion coefficients were calculated from records of intensity fluctuation spectroscopy [9]. The broadening of the Rayleigh line was measured using a digital autocorrelator having 24 channels (Precision Devices Ltd, Malvern, Worcs, U.K.). A focussed beam from a 50 mW Ar-ion laser (Spectral Physics, California) was used and the scattered radiation collected at an angle of 90° to the incident beam. Preliminary calcula-

Table 1
Sedimentation coefficients ($S_{20,w}$) for phosphoglycerate kinase. Three estimates are given for each sample, $S_{\bar{x}}$ is calculated from the second moment, S_{max} from the schlieren peak, and S_p from the decrease in concentration of the plateau. Concentrations were calculated from areas under the schlieren peak extrapolated back to zero time

conc. (g ℓ$^{-1}$)	S_{max} (ps)	$S_{\bar{x}}$ (ps)	S_p (ps)
0.85	0.319 ±0.006	0.307 ±0.009	0.817 ±0.203
2.68	0.327 ±0.006	0.332 ±0.007	0.713 ±0.057
3.13	0.323 ±0.017	0.333 ±0.025	0.258 ±0.213
3.88	0.319 ±0.005	0.336 ±0.006	0.490 ±0.061
4.39	0.307 ±0.008	0.328 ±0.012	0.705 ±0.075
5.00	0.316 ±0.004	0.327 ±0.006	0.450 ±0.074
5.68	0.299 ±0.006	0.334 ±0.009	0.449 ±0.060
6.16	0.308 ±0.007	0.324 ±0.006	0.694 ±0.127
6.69	0.312 ±0.005	0.326 ±0.007	0.746 ±0.117

tions showed that scattering from phosphoglycerate kinase would not show significant angular dependence so this was neglected. The normalised second-order correlation function $[g^2(t)]$ is related to the translational diffusion coefficient by the relationship

$$g^2(t) = 1 + A \exp(-2K^2 D_T \tau), \quad (2)$$

where A = constant determined by temporal and spatial coherence, K = scattering vector, D_T = diffusion coefficient, τ = delay time [Zt, where Z is the channel number (1 to 24) and t a sampling time calculated from coherence considerations]. Hence $\ln g^2(t)$ is linearly related to t and the slope of this line = $-2K^2 D_T$. No curvature was detected in any of our experiments when tested at the 95% probability level following subtraction of base-lines (see below).

In estimating D_T, two further factors are important. First the choice of the correct sampling time (t) is important and for the present work the criteria defined by Chu [10] were used giving the optimum sampling time for photoglycerate kinase to be between 2 μs and 3 μs. The second experimental variable which must be measured is the base-line of the exponential decay. This base-line is caused by dc signals and traces of heterogeneity in the sample; it was estimated from correlation records collected at sample times of 100 μs. Occasionally these estimates were higher than expected so these experiments were rejected. It is thought the spread in estimates for D_T (table 2) is caused partly by compounding the errors incurred in estimating baselines with those from the records collected at short sampling times (because the spread between experiments is outside the standard errors of the individual estimates of D_T) and partly by varying degrees of heterogeneity of the samples.

3. Results

3.1. Sedimentation velocity

The values for $S_{20,w}$ estimated from the second moment calculations ($S_{\bar{x}}$, table 1) were generally higher than the $S_{20,w}$ estimated from the schlieren peaks (S_{max}, table 1). Both sets of data fitted straight lines for the concentration dependence (defined in eq. (3)) to give the following parameters: $S^0_{\bar{x}}$ = 0.328 ± 0.006 ps, $g_{\bar{x}}$ = 0.01 ± 0.005 ℓ g^{-1}; S^0_{max} = 0.319 ± 0.007 ps, $g_{max} = -6 \pm 5 \times 10^{-3}$ ℓ g^{-1}.

$$S = S^0(1 - gc). \quad (3)$$

Although sedimentation constants calculated from the two measurements are not significantly different, the slopes describing the concentration dependence are dissimilar. The S^0_{max} agreed closely with the reported value of 0.312 ± 0.002 ps [5]. The observed zero concentration dependence calculated from the schlieren peaks also confirmed the observations of earlier work and it was this low dependence which suggested weak association for phosphoglycerate kinase [5]. The concentration dependence for $S_{20,w}(\bar{x})$ showed the expected negative slope (positive g, eq. (3)). Generally a higher value was recorded for $S_{20,w}$ estimated by dilution of the plateau and this reflected the level of contamination of foreign material in the samples. In general the $S_{20,w}$ ranged between 0.4 ps and 0.8 ps

Table 2
Diffusion coefficients ($D_{20,2}$) for phosphoglycerate kinase. Values are quoted for the two sampling times (2 μs and 3 μs) where these were measured

Conc. g ℓ$^{-1}$	$D_{20,w}$ (× 10^{11} m^2 s^{-1})	
	2 μs	3 μs
0.45	6.78 ±0.27	6.95 ±0.23
1.36	7.21 ±0.28	6.84 ±0.17
3.89	7.27 ±0.13	–
	6.68 ±0.17	6.78 ±0.19
5.60	–	6.77 ±0.10
		6.81 ±0.08
6.16	6.58 ±0.20	–
11.52	6.31 ±0.11	–
14.44	7.29 ±0.06	–

with a mean of about 0.6 ps (table 1). This high $S_{20,w}$ (p) was consistent with results obtained from two dimensional acrylamide gel electrophoresis (not shown) where traces of macromolecules having relative masses around 100,000 and 140,000 were detected. In some preparations additional spots were noted for molecules having relative masses around 30,000. These traces were found for both crystalline and non-crystalline preparations. The presence of these larger macromolecules presumably caused the difference in g (eq. (3)) for the two sources of coefficients ($S_{\bar{x}}$ and S_{max}). Repeated attempts to remove the contaminant (e.g. gel chromatography) were unsuccessful and the differences between the estimates shown in table 1 represent the results from different concentrations as well as from various attempts to reduce the contamination to lower levels. It was noted when attempting to study concentrations greater than 10 g ℓ$^{-1}$ that invariably there slowly formed a precipitate and this limited the maximum concentration that could be studied. This precipitation appeared to be an irreversible step since it could not be redissolved by simple dilution of the solution.

3.2. Diffusion measurements

A second source of hydrodynamic measurements was from the diffusion measurements (table 2) and under the conditions used for these experiments the diffusion of most macromolecules show negligible concentration dependence. In fact a linear regression analysis showed that the results from table 2 could be represented by a straight line to give $D^0_{20,w} = 6.89 \pm 0.13 \times 10^{-11}$ m^2 s^{-1} and slope of $-7 \pm 21 \times 10^{-3}$ ℓ g^{-1}, making the slope effectively zero. The large error in the slope reflects the variation in purity between the samples as well as systematic errors accrued by subtracting estimated baselines.

It seems they are not caused by instrumental inaccuracies as the results in table 2 indicate, (estimates for $D_{20,w}$ measured at two sample times were similar) nor by lack of precision (the standard errors are relatively constant at about 3% of the value for $D_{20,w}$). Within the limits set by those errors the results of $D_{20,w}$ are consistent and fulfil expected relationships for macromolecules like phosphoglycerate kinase.

4. Discussion

Three basic experimental parameters for phosphoglycerate kinase emerge, $S^0_{20,w} = 0.328 \pm 0.006$ ps, $D^0_{20,w} = 6.89 \pm 0.13 \times 10^{-11}$ m^2 s^{-1}, and concentration dependence for sedimentation (g, eq. (3)) = 0.01 ± 0.005 ℓ g (the low or zero concentration dependence for diffusion only confirms expected results). The presence of the contaminating larger species was a constant worry, especially since we were never able to completely remove these contaminants. The reappearance of large molecules after purification indicates an associating system, but the level of the concentration dependence shows that this was not a reversible system involving fast reaction rates. Workers have reported the precipitation of phosphoglycerate kinase caused by heat stable factors [11] and we feel our present observations are related to this non-reversible denaturation of the enzyme. It also seems the molecule can be readily split into smaller fragments and re-examination of our earlier ultracentri-

fugal equilibrium data [5] suggests that the presence of these components were the reason for the earlier high estimate of $D_{20,w}$ (7.45×10^{-11} m^2 s^{-1}) to give a lower molecular weight for phosphoglycerate kinase (40080). Combining the present estimates of $S^0_{20,w}$ and $D^0_{20,w}$ in the Svedberg equation gives a molecular weight of 45 800 ± 1700, using a specific volume of 0.748 cm^3 g^{-1} calculated from the amino acid composition: (insufficient pure material was available to make accurate estimates possible but it is unlikely that the calculated value differs appreciably from the real value under these experimental conditions); this new figure corresponds closely with estimates from X-ray crystallography (personal communication from Dr. H. Watson, University of Bristol, U.K.). Thus, our present analyses appear to have removed any perturbations that could have been produced by heterogeneity in the samples making it possible to use these estimates to calculate shapes and solvent-solute interactions.

Following the accepted procedure of classical hydrodynamics the ratio of experimental diffusion constant to the diffusion constant calculated for a sphere of the mass and density of phosphoglycerate kinase [13] was found to be 1.305 ± 0.039 (all the errors quoted in subsequent discussion are standard errors compounded from the various sources of experimental errors). From this ratio can be calculated an asymmetry ratio by first allowing for solvation [14], equivalent to 0.46 g H_2O/g protein [5] to give 1.114 ± 0.033; this is the experimental ratio which must be related to the X-ray coordinates. From this ratio was calculated the semi-major to semi-minor axis (a and b) using Perrin's equation [15] for a prolate ellipsoid giving $a \approx 5$ nm, $b \approx 1.7$ nm; the nearest equivalent dimensions extracted from X-ray crystallography studies [1] are $a \approx 4.2$ nm, $b \approx 2.7$ nm. This mismatch between calculated and experimental coordinates produced through using a regular uniform shape is a common problem; a more exact portrayal of the molecule is shown in fig. 1 (we are grateful to Dr. N. Walker, University of Bristol, for supplying us with the details for this diagram). From this model the molecule was represented by three spheres in order to calculate the frictional coefficient using Bloomfield's method [3]; the radii of the spheres were taken as 1.808 nm, 1 nm and 1.989 nm. Although the older model of Bloomfield's is less refined than his later model [17], it is difficult to apply this later model to calculations on a single unit, unless the amino acid sequences were known. At present this is not possible because the sequence of phosphoglycerate kinase is unknown; however, it does seem from the results given by De La Torre and Bloomfield [17] that even if the more refined model could be used there would be only about a 1% improvement for this molecule compared with the less exact procedure [3]. The calculated frictional ratio for the model in which the spheres were assembled in a straight line was 1.115 (which compares with the experimental of 1.114 ± 0.033) while that for a V-shaped assembly having an angle of 135° instead of 180° between the arms gave a frictional ratio of 1.020. These results show that in solution the overall shape of phosphoglycerate kinase does not differ much from the crystallographic model, making it essentially a linear molecule with limited amplitude of flexing.

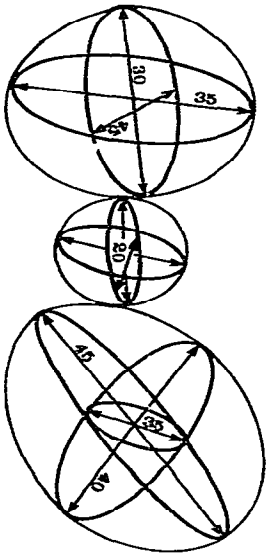

Fig. 1. Schematic model of phosphoglycerate kinase using overall dimensions from X-ray crystallography kindly supplied by Dr. N. Walker (Department of Biochemistry, University of Bristol, U.K.). The approximate dimensions given on the figure are in Å.

Comparisons of experimental and calculated frictional coefficients depend partly on what values of hydration are used, and here we used those obtained

from NMR. Other techniques may give lower estimates of hydration so it is reasonable to question whether the NMR method is valid for these calculations. If in place of 0.46 g H_2O/g protein a value for the hydration had been 0.2 g/g, then the measured asymmetry ratio increases to 1.207. This new ratio is considerably greater than the calculated value for the linear molecule (1.115) and since the calculations do not involve a knowledge of the hydration, this gives confidence that the NMR estimate of hydration is valid for diffusion studies.

Information on the total solvent-solute interactions which lead to concentration dependence of sedimentation and diffusion coefficients can be calculated from the present data. The accepted way to represent concentration dependence of diffusion coefficients is to express concentration as a volume fraction ϕ (in order to retain a solvent and not a laboratory frame of reference for diffusion) when [16]

$$D = (kT/f^0)(1 + B_2 - K_f)\phi, \tag{4}$$

ϕ was calculated from the concentration (g ℓ^{-1}) of phosphoglycerate kinase by multiplying by 1.208×10^{-3} ℓ g^{-1} (0.748 cm^3 g^{-1} plus a solvation layer, 0.46 g H_2O g^{-1}), f^0 is the frictional coefficient at infinite dilution, B_2 the second virial coefficient and K_f the concentration dependence of the frictional factor as determined from the dependence of the sedimentation coefficient (making it the volume fraction equivalence to g, eq. (3)). Since there was negligible concentration dependence for D, then B_2 was estimated to be 0.010 ± 0.005 ℓ g^{-1}, or $1.1 \pm 0.5 \times 10^{-7}$ ℓ g^{-2} M^{-2}. This experimental value for B_2 compared favourably with the value calculated using the model of Ishihara and Hayashida [18] (1.44×10^{-7} ℓ g^{-2} M^{-2}) from the experimental asymmetrical frictional ratio (1.114). If the hydration had been over-estimated by NMR and 0.2 g/g used in place of 0.46 g/g, then the calculated B_2 becomes 1.11×10^{-7} ℓ g^{-2} M^{-2}. Although this agrees more closely with the experimental value, the difference in the two calculated values of B_2 falls within the experimental error. This lack of precision in the experimental B_2 means that the comparisons between experimental and calculated coefficients are less convincing that those made earlier for frictional ratios in deciding whether the hydration estimated from NMR is the correct value for hydrodynamic measurements. The experimental value for K_f (eq. (4)) was found to be 8.00 ± 3.8 which, as expected, is larger than the value expected for a hard sphere (ranging from 6.55–7.2) [16]. Finally from the dependence of D on ϕ (eq. (5)) [18] can be calculated the interactive volume of phosphoglycerate kinase (eq. (6)) [19]

$$D = \frac{(\partial\pi/\partial c)_{T,P}}{f} \frac{(1 - \phi)}{(1 - \phi G)} \tag{5}$$

$$G = (\tfrac{8}{4}\pi a^3) \int d\mathbf{r}^3 \mathbf{j} \cdot \mathbf{D}^{\mathbf{T}}(\mathbf{r}) \cdot \mathbf{j} \{\exp[-w(\mathbf{r})/kT] - 1\}/D_0 \tag{6}$$

where $\mathbf{j}$ is the unit vector, $\mathbf{D}^{\mathbf{T}}(\mathbf{r})$ is the Oseen tensor, w is the energy required to spatially translate the molecule and includes all the work done against the weak forces involved in the solvent-solute interactions. $(\partial\pi/\partial c)_{T,P}$ is the osmotic gradient of the solution. For the present data $G = -5.93 \pm 2.8$ which when combined with the volume of phosphoglycerate kinase (5.8×10^{-20} cm^3) gives the value for the volume integral (eq. (6)) of $-3.4 \pm 1.6 \times 10^{-19}$ cm^3. The cube root of this volume is about 7.0 ± 1.1 nm, which is nearly three times the radius of the equivalent hard sphere of phosphoglycerate kinase (2.4 nm). The numerical value for this relationship between G and the volume of the sphere resembles closely that found for oxyhaemoglobin [20]. At its simplest the excess volume is that occupied by the hard sphere and represents an average space in which a molecule is free to move randomly (equivalent to about half its original radius) before interacting with a second molecule of its own type. In some respects this volume can be equated with the extent of the perturbation of solvent structure caused by the introduction of the large molecule into the solvent.

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