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Some Observations of the Sedimentation of Chicken Heart Glyceraldehyde 3-Phosphate Dehydrogenase*

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ABSTRACT: In dilute salt solutions glyceraldehyde 3-phosphate dehydrogenase from chicken heart has a molecular weight of 137,600 as determined by the method of sedimentation equilibrium. In a solution of 1.3 M potassium phosphate, the protein exhibits an apparent molecular weight of 86,200 by the same method. The determination of the sedimentation coefficient of the protein as a function of phosphate concentration and the analysis of the dependence led to the conclusion that positive preferential hydration is responsible for the decrease in measured apparent molecular weights in these solutions rather than a dissociation of the tetramer to subunits.

Glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle is known to be composed of four identical subunits (Harris and Perham, 1965; Harrington and Karr, 1965). Enzymological kinetic studies and direct studies of the binding of diphosphopyridine nucleotide have suggested a certain degree of interaction between the identical subunits (Velick, 1953; Listowsky *et al.*, 1965; DeVijlder and Slater, 1968; Conway and Koshland, 1968). Therefore, a probe of the interactions between subunits should be very helpful in interpreting the mechanism by which this enzyme can alter its activity.

Some studies of this nature have, in fact, been undertaken. Jaenicke *et al.* (1968) have investigated the molecular weight of the enzyme under various conditions in an attempt to find conditions of subunit dissociation. Measuring sedimentation coefficients and diffusion coefficients to calculate the molecular weight, they obtained the monomer molecular weight when the protein was succinylated or by oxidized performic acid and also under denaturing conditions, such as in 6 M guanidine hydrochloride, in 10 M urea, and at pH 12.5. The tetrameric molecular weight was found in phosphate, pyrophosphate, and borate buffers, 0.15–0.20 in ionic strength in the pH range of 6.2–10. At ionic strengths of 1.5 and 3 in phosphate, however, apparent molecular weights of 123,000 and 88,000 were found, leading to the conclusion that the protein was dissociating in concentrated phosphate solutions.

Recently, Hoagland and Teller (1969), utilizing the method of sedimentation equilibrium, reported a study of the molecular weight of the enzyme. Elegant statistical analysis of data allowed them to demonstrate that the protein was dissociating to a dimer in 0.1 M Tris (pH 7) at 5° with an equilibrium constant of about 5×10^{-7} M; which indicates that the tetramer is well-favored thermodynamically at concentrations normally used for sedimentation velocity and diffusion experiments.

In view of the results of Jaenicke *et al.* (1968), a probe of the subunit interactions of chicken heart GDPH[1] was undertaken. The enzyme has a very similar amino acid composition to that of rabbit muscle as shown by Allison and Kaplan (1964) and it is expected that the overall physical parameters should be very similar.

Materials and Methods

The crystalline chicken heart GDPH used in this study was a generous gift from Dr. William Allison. Solutions were prepared by removing an aliquot of the protein–ammonium

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[1] Abbreviation used is: GPDH, glyceraldehyde 3-phosphate dehydrogenase.

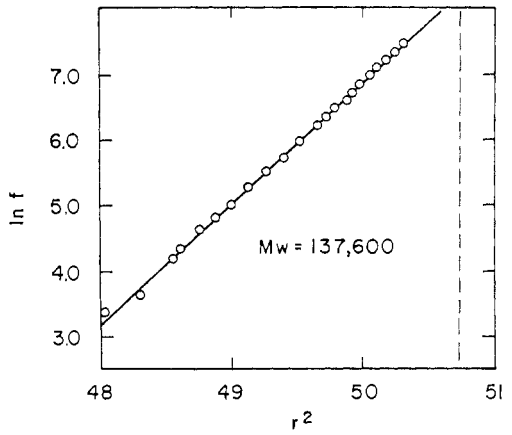

FIGURE 1: Natural logarithm of the fringe displacement *vs.* the square of the radial distance. Conditions were 15,190 rpm, 25°, 0.1 M KCl (pH 5.44) and protein concentration of 0.57 g/l.

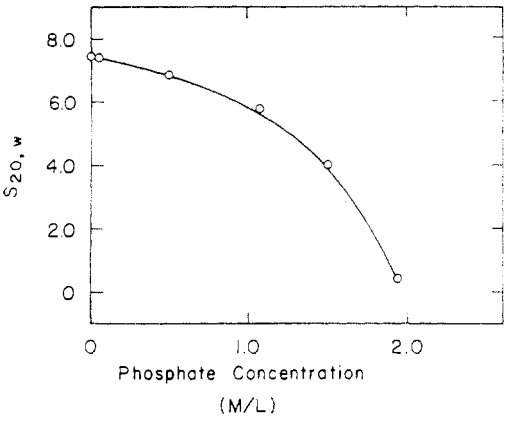

FIGURE 2: $s_{20,w}$ plotted *vs.* the concentration of phosphate (potassium buffer at pH 6.97) in moles per liter.

sulfate slurry and dialyzing at 4° *vs.* the appropriate solvent overnight. On the basis of dry weight determinations at 106° in air, the absorptivity of the protein at 280 mμ was found to be 10.2 dl/g with a A_{280}:A_{260} ratio of 1.17. Treatment with charcoal according to Velick (1953) yielded what is termed apoprotein, which exhibited an absorptivity at 280 mμ of 8.2 dl/g with a A_{280}:A_{260} ratio of 1.80.

Sedimentation velocity experiments were carried out using a Spinco Model E ultracentrifuge equipped with schlieren optics. For concentrated salt solutions, sedimentation coefficients were measured using Epon-filled aluminum synthetic boundary cells; otherwise, Kel-F single-sector cells were used. The sedimentation coefficients were generally corrected to $s_{20,w}$ values according to Svedberg and Pederson (1940). The sedimentation equilibrium experiments were carried out using Epon-filled aluminum double-sector centerpieces with interference optics. All runs were controlled at 25° with the RTIC unit.

Densities and viscosities were extracted from the "International Critical Tables" or "Timmermans Physico-chemical Constants of Binary Systems" for the various solutions. In the case of the potassium phosphate buffers at pH 6.97, densities and viscosities were calculated from fraction of composition in the manner of Kawahara and Tanford (1966). Pyconometric measurements of two concentrated phosphate solutions demonstrated that the method was valid to within ±0.003 g/cc for density values.

Results and Discussion

The molecular weight of the chicken heart enzyme was measured in 0.1 M potassium chloride–0.01 M mercaptoethanol (pH 5.75) at 25° using the method of sedimentation equilibrium. Figure 1 shows the conventional plot of the natural logarithm of the fringe displacement, ln *f*, *vs.* the square of the distance from the center of rotation, r^2. Assuming a value of 0.736 cc/g at 25° for the partial specific volume calculated from composition (Cohn and Edsall, 1943), a molecular weight of 137,600 ± 6,000 is obtained with no evidence of dissociation at the lowest concentrations measured by interference optics. This value is in agreement with the value of 145,000 ± 6,000 obtained by Harrington and Karr (1965) for the rabbit muscle protein.

It was clear that a perturbant of the interactions between subunits was necessary to observe the dissociation equilibria. Therefore, the sedimentation coefficient, $s_{20,w}$, was measured under several probing conditions. Protein concentrations were in the region of 2–4.5 g/l., but no correction for concentration dependence of $s_{20,w}$ was applied.

The parameter, $s_{20,w}$, was found to be 7.43 and 7.45 S in a solution of 0.1 M potassium chloride–0.1 M mercaptoethanol–0.05 M phosphate buffer (pH 6.8) and in a solution of 0.1 M potassium chloride–0.01 M mercaptoethanol (pH 5.44), demonstrable of the tetrameric structure at either pH. In 0.507 M guanidine hydrochloride, GDPH precipitated. The protein which remained in solution sedimented with a $s_{20,w}$ value of 7.6 S, typical of the tetrameric structure, whereas under similar conditions hemoglobin is successfully dissociated (Kawahara *et al.*, 1965). High concentrations of potassium chloride also brought about precipitation at neutral pH values, whereas at pH values above 10.5, the protein was quite soluble and the measured $s_{20,w}$ value was 2.74 S; a gross unfolding, however, was suggested from an appreciable increase in levorotation. Since the study of Jaenicke *et al.* (1968) suggested that the rabbit muscle protein dissociates in concentrated phosphate solutions, a similar probe of the dissociation of the chicken enzyme was undertaken. The enzyme was salted out in concentrated phosphate solutions, enough protein remaining in solution to measure the $s_{20,w}$ value.

Figure 2 shows the dependence of $s_{20,w}$ on the concentration of potassium phosphate buffer (pH 6.97). A decrease in $s_{20,w}$ with increase of the potassium phosphate concentration is observed; the value of $s_{20,w}$ does not level off, however, at some value appropriate for the dimer or monomer. This is more clearly demonstrated in Figure 3 where $s\eta$ is plotted *vs.* ρ, where s is the sedimentation coefficient, η is the viscosity of the solvent, and ρ is the density of the solvent. This plot demonstrates almost a linear extrapolation to zero sedimentation at a density of 1.231 g/cc corresponding to a concentration of about 1.97 M phosphate. This indicates that the protein is preferentially solvated at the higher phosphate concentrations, without necessarily dissociating.

Since at a density of 1.231 g/cc there is no sedimentation, the system must be in chemical equilibrium. It is possible

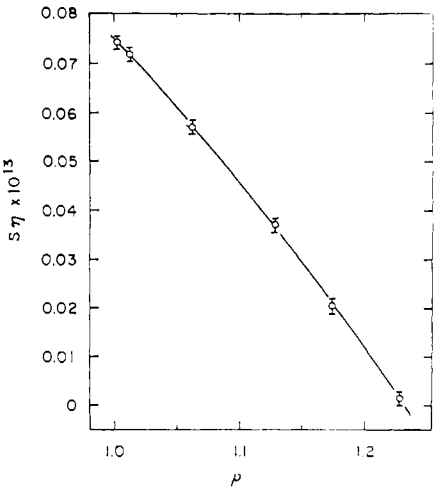

FIGURE 3: The product of the sedimentation coefficient of the protein and viscosity of the solvent *vs.* the density of the solvent.

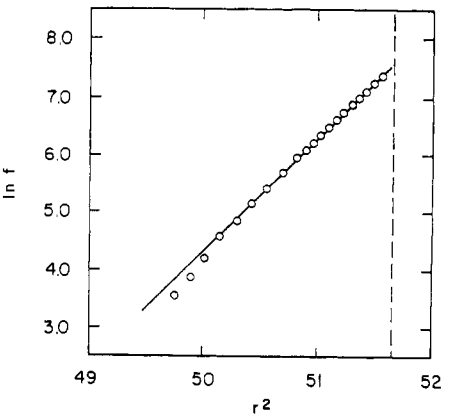

FIGURE 4: Natural logarithm of the fringe displacement *vs.* the square of the radial distance. Conditions were 26,000 rpm, 25°, 1.30 M potassium phosphate buffer (pH 6.97) and protein concentration of 0.4 g/l.

therefore, to solve for the amount of preferential interaction by the use of eq 1 (Casassa and Eisenberg, 1964), where ϕ_2 is

$$\phi_2 - \phi_2' = \left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}\left[\frac{1}{\rho_s} - \phi_3\right] \qquad (1)$$

the partial specific volume of the protein as measured pycnometrically in a dilute salt, ϕ_2' is the partial specific volume of the protein as measured by comparing the density of a protein solution to that of its dialysate, $[\partial g_3/\partial g_2]_{T,\mu_1,\mu_3}$ is the amount of solute preferentially bound to anhydrous protein in grams per gram of protein, g_i is the concentration of component i per gram of component 1 (water), ρ_s is the density of the solution, and ϕ_3 is the partial specific volume of the salt.

Since the solvent is potassium phosphate buffer of pH 6.97, solution component 3 is, in fact, a mixture of two species, K_2HPO_4 and KH_2PO_4. These two salt species are in dynamic equilibrium in solution; their mixture may, therefore, be considered as a single thermodynamic component. The molecular weight, M_3, of component 3 is then 159.33, since at pH 6.97 the mixture of phosphate salts is 61% K_2HPO_4 and 39% KH_2PO_4. Therefore, with $\phi_2 = 0.736$ cc/g, $\phi_2' = 1/\rho_s = 0.812$ cc/g, and $\phi_3 = 0.203$ cc/g calculated from density data of the phosphate buffers, $[\partial g_3/\partial g_2]_{T,\mu_1,\mu_3}$ is found to be -0.125 g/g. This is related to preferential hydration by

$$\left[\frac{\partial g_1}{\partial g_2}\right]_{\mu_3,\mu_1} = -\frac{1000}{m_3 M_3}\left[\frac{\partial g_3}{\partial g_2}\right]_{\mu_3,\mu_1} = +0.366 \text{ g/g} \qquad (2)$$

Therefore, in 1.97 M phosphate, GPDH is preferentially hydrated to the extent of +0.366 g of H_2O/g of protein.

The observation of positive preferential hydration rather than dissociation was further tested by measuring the molecular weight of GPDH by sedimentation equilibrium in a solution of 1.30 M phosphate, at which concentration sufficient "dissociation" would be predicted. Figure 4 shows a plot of ln f *vs.* r^2 for such an experiment on the apoenzyme. The parameter $M(1 - \phi_2'\rho)$ measured was found to be 13,200 ± 600. If ϕ_2' is taken to be 0.736 cc/g, *i.e.*, assuming no preferential interactions, the apparent molecular weight is calculated to be 86,400 ± 3,900, close to the number obtained by Jaenicke *et al.* (1968). The sedimentation velocity results have shown, however, that ϕ_2' must increase to 0.812 cc/g in 1.97 M phosphate, so that ϕ_2' should be greater than 0.736 cc/g in 1.30 M phosphate. Because ϕ_2' values are not easy to measure accurately in high salt concentrations and since the sedimentation velocity data were available, the data were analyzed with the following assumptions (Hill and Cox, 1965): (a) ϕ_2' increases from 0.736 cc/g in a monotonic fashion to 0.812 cc/g in high phosphate concentrations; (b) no molecular weight change is involved in the measurement of the sedimentation coefficient as a function of phosphate concentration.

Then, the variation of s with phosphate concentration is a function only of η, ρ, and ϕ_2'. According to the Svedberg equation (Svedberg and Pederson, 1940)

$$s = \frac{M(1 - \phi_2'\rho)}{Nf} \qquad (3)$$

where N is Avogadro's number and f is the frictional coefficient of the protein.

If the protein is treated as a sphere, the frictional coefficient can be expressed as

$$f = 6\pi\eta R_{eff}\frac{f}{f_0} \qquad (4)$$

where $f_0 = 6\pi\eta R_{eff}$ and R_{eff} can be considered as the radius of a molecule of the given mass packed as a sphere. Thus we have

$$R_{eff} = \left[\frac{3M}{4\pi N}\phi_2'\right]^{1/3} \qquad (5)$$

Combining eq 3, 4, and 5 gives

$$s\eta = k\frac{[1 - \phi_2'\rho]}{(\phi_2')^{1/3}} \qquad (6)$$

TABLE I

Phosphate Concn (moles/l.)	ϕ_2'	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$
0.05	0.736		
0.50	0.750	−0.020	+0.246
1.07	0.768	−0.048	+0.269
1.50	0.789	−0.083	+0.296
1.94	0.810	−0.121	+0.360
(1.97)	(0.812)[a]	(−0.125)[a]	(+0.366)[a]

[a] True thermodynamic parameters.

where k is the accumulation of constants and the quantity f/f_0. Further, the quantity f/f_0 is assumed to be independent of phosphate concentration and, therefore, k may be evaluated and used as a constant independent of phosphate concentration. Using the dilute salt value of $\phi_2' = 0.736$ cc/g, and the measured s value of 8.3 S in 0.1 M KCl at 25°, k is calculated to be 2.54×10^{-14} sec P $g^{-1/3}$ cm. The parameter ϕ_2' may then be evaluated as a function of the measured s value and the solution variables η and ρ. Table I contains the calculated values of ϕ_2' along with those of $[\partial g_3/\partial g_2]_{T,\mu_1,\mu_3}$ and $[\partial g_1/\partial g_2]_{T,\mu_1,\mu_3}$ at several phosphate concentrations.

Interpolation results in a value of ϕ_2' of 0.778 cc/g in 1.30 M phosphate. With this value, the molecular weight from the sedimentation equilibrium experiment is calculated to be 126,000 ± 11,000. Considering the assumptions involved in the model and the inherent error of the data, it is felt that this result is within experimental error of the tetramer molecular weight. Thus, an apparent molecular weight obtained by the method of sedimentation equilibrium, which is low, neglecting preferential interactions, can be easily corrected to the tetrameric molecular weight by taking into account positive preferential hydration. Furthermore, since the weight-average molecular weight obtained from the slope of the curve in Figure 4 does not obey any mass action law type of association over a 200-fold concentration range in the equilibrium experiment, it is clear that the experimental results are self-consistent with the occurrence of positive preferential hydration in concentrated phosphate solutions.

The variation in preferential solvation as a function of phosphate concentration shown in Table I does not mean necessarily that the absolute amounts of solvent components immobilized in the immediate domain of the protein molecules vary in similar manner. In fact, if these quantities remained constant, the preferential interactions observed would, of necessity, have to vary with change in bulk solvent composition, since they represent essentially a comparison of the compositions of bulk solvent and solvent immobilized by the protein (Timasheff and Inoue, 1968). The equation for the preferential binding of water is

$$\left[\frac{\partial m_1}{\partial m_2}\right]_{T,\mu_1,\mu_3} = \nu_1 - \frac{m_1}{m_3}\nu_3 \qquad (7)$$

where m_i is the molality of component i and ν_i is the number of molecules of component i bound to one molecule of protein at the given solvent composition. For example, the data of Table I may be satisfied with a model based on the assumption that glyceraldehyde 3-phosphate dehydrogenase carries with it a constant amount of water inaccessible to salt and also binds a small amount of phosphate at sites which become saturated at low salt concentration. Calculations using eq 7 show that these data are consistent with constant values of ν_1 of 740 molecules of water and of ν_3 of 3 molecules of salt per subunit of the enzyme. This would amount to a protein hydration of 0.4 g of bound water/g of protein. The present study demonstrates quite clearly the important contribution which a shell of bound solvent may make to the thermodynamics of macromolecules in mixed solvents.

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