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Ultracentrifugal studies of the effect of molecular crowding by trimethylamine *N*-oxide on the self-association of muscle glycogen phosphorylase *b*

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The suitability of sedimentation equilibrium for characterizing the self-association of muscle glycogen phosphorylase *b* has been reappraised. Whereas sedimentation equilibrium distributions for phosphorylase *b* in 40 mM Hepes buffer (pH 6.8) supplemented with 1 mM AMP signify a lack of chemical equilibrium attainment, those in buffer supplemented additionally with potassium sulfate conform with the requirements of a dimerizing system in chemical as well as sedimentation equilibrium. Because the rate of attainment of chemical equilibrium under the former conditions is sufficiently slow to allow resolution of the dimeric and tetrameric enzyme species by sedimentation velocity, this procedure has been used to examine the effects of thermodynamic nonideality arising from molecular crowding by trimethylamine *N*-oxide on the self-association behaviour of phosphorylase *b*. In those terms the marginally enhanced extent of phosphorylase *b* self-association observed in the presence of high concentrations of the cosolute is taken to imply that the effects of thermodynamic nonideality on the dimer–tetramer equilibrium are being countered by those displacing the T $\rightleftarrows$ R isomerization equilibrium for dimer towards the smaller, nonassociating T state. Because the R state is the enzymically active form, an inhibitory effect is the predicted consequence of molecular crowding by high concentrations of unrelated solutes. Thermodynamic nonideality thus provides an alternative explanation for the inhibitory effects of high concentrations of glycerol, sucrose and ethylene glycol on phosphorylase *b* activity, phenomena that have been attributed to extremely weak interaction of these cryoprotectants with the T state of the enzyme.

Keywords: sedimentation equilibrium; sedimentation velocity; phosphorylase *b* self-association; molecular crowding.

At neutral pH, phosphorylase *b* is a stable dimer comprising two identical subunits, each of which contains an active site, separate allosteric activatory and inhibitory sites, and a glycogen storage site [1]. Allosteric activation of the enzyme by AMP induces the formation of tetramers, which are also favoured by a decrease in temperature or the presence of sulfate ions [2–5]. On the basis that adoption of the R state is a prerequisite for tetramer formation [3,6–8], the position of the dimer–tetramer equilibrium provides a means of monitoring effector-induced transitions between the inactive (T state) and active (R state) conformations of the enzyme subunits [4,9,10]. The present investigation was initiated with a view to determining the likely consequences of molecular crowding by high concentrations of inert solutes, which also have the potential to favour protein self-association [11–13] and hence to act as nonspecific effectors of the phosphorylase reaction. Trimethylamine *N*-oxide (TMAO) has been used as the space-filling cosolute [14–16].

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Abbreviation: TMAO, trimethylamine *N*-oxide

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Whereas initial characterization of the dimer–tetramer equilibrium entailed sedimentation velocity studies of glycogen phosphorylase *b* [4], the use of sedimentation equilibrium has been reported subsequently [17]. In this investigation, the results were analyzed on the basis that chemical equilibrium prevailed throughout the sedimentation equilibrium distributions, the usual assumption in studies of proteins for which the various oligomeric states are in rapid association equilibrium. However, caution is required in applying the sedimentation equilibrium technique to the phosphorylase *b* system, for which the dimer–tetramer interconversion under some conditions is sufficiently slow to allow separation of the two species by sedimentation velocity [4,18–20]. Before examining the effect of TMAO on the dimer–tetramer equilibrium, further sedimentation equilibrium studies on glycogen phosphorylase *b* have therefore been undertaken to ascertain the validity of the assumption that the results reflect the attainment of self-association equilibrium as well as sedimentation equilibrium.

Detailed analysis of sedimentation equilibrium distributions at 15 °C for phosphorylase *b* in 40 mM Hepes buffer (pH 6.8) supplemented with 1 mM AMP signifies a lack of chemical equilibrium attainment, but conformity is found in buffer supplemented additionally with potassium sulfate [17] of the requirements of a dimerizing system in chemical as well as sedimentation equilibrium. Because the rate of chemical equilibrium attainment under the former conditions is sufficiently slow to allow resolution of the dimeric

and tetrameric enzyme species by sedimentation velocity, this procedure has been used to examine the effects of thermodynamic nonideality arising from molecular crowding by TMAO on the self-association behaviour of phosphorylase *b*. The marginally enhanced extent of phosphorylase *b* self-association observed in the presence of high concentrations of this cosolute implies that the effects of thermodynamic nonideality on the dimer–tetramer equilibrium are being countered by those displacing the T $\rightleftarrows$ R isomerization equilibrium for dimer towards the smaller, nonassociating T state. Because the R state is the enzymically active form, molecular crowding by high concentrations of unrelated solutes is thus predicted to exert an inhibitory effect on phosphorylase *b* activity. Thermodynamic nonideality should thus contribute to the decreased activity exhibited by phosphorylase *b* in the presence of glycerol, ethylene glycol and sucrose [21,22], a phenomenon which has thus far been considered solely in terms of cryoprotectant interaction with the T state of the enzyme.

EXPERIMENTAL PROCEDURES

Preparation of enzyme solutions

Phosphorylase *b* was isolated from rabbit skeletal muscle by the method of Fischer and Krebs [23] except that dithiothreitol replaced cysteine [5]. After four crystallizations, the AMP-free phosphorylase *b* preparations were stored at −20 °C in 2-phosphoglycerate/NaOH, pH 6.8) supplemented with 50% glycerol (v/v). Protein concentrations were determined spectrophotometrically on the basis of an absorption coefficient ($A^{1\%}_{1cm}$) of 13.2 at 280 nm [24]; the corresponding coefficient at other wavelengths was deduced from the above value and the ratio of absorbances at the two wavelengths. These weight-concentrations were converted to a base-molar scale by dividing by 194 800, the relative molecular mass (M_r) of dimeric phosphorylase *b* [25], the smallest stable species at neutral pH.

Analytical ultracentrifugation

Solutions of phosphorylase *b* were first dialyzed against 40 mM Hepes buffer (pH 6.8) to obtain solutions in dialysis equilibrium with this buffer, and then supplemented with AMP (1 mM) just prior to loading into the sample sector of a 12-mm double-sector cell. The diffusate to be placed in the reference sector was also supplemented with 1 mm AMP. Sedimentation equilibrium experiments were conducted at 15 °C in a Beckman XL-I ultracentrifuge operated at 6000–12 000 r.p.m. in a standard four-hole rotor. The resulting solute distributions were recorded spectrophotometrically at either 290 or 295 nm by means of the absorption optical system. The optical system was calibrated in each experiment by recording the distribution upon attainment of speed to allow comparison of this measure of the absorbance with the value determined for the loaded solution in a conventional spectrophotometer. Baselines for the sedimentation equilibrium scans were obtained by overspeeding to create a solvent plateau, which was then recorded [26].

Analysis of the sedimentation equilibrium scans has been based on the integral form of the basic sedimentation equilibrium expression for each species [27,28], namely:

$$z_i(r) = z_i(r_F)\exp[M_i(1-\bar{v}_i\rho_s)\omega^2(r^2-r_F^2)/(2RT)] \quad (1)$$

where $z_i(r)$, the molar thermodynamic activity of species i at radial distance r, is described in terms of its activity at a reference radial distance (r_F) and an exponential term containing the molecular mass (M_i) and partial specific volume ($\bar{v}_i$) of species i, the buffer density (ρ_s), the angular velocity (ω), the the squares of the radial distances and the absolute temperature (T): R is the universal gas constant. The use of relatively low solute concentrations has been considered to justify the substitution of molar concentrations (C_i) for thermodynamic activities, whereupon results for a system comprising dimer ($i = 2$) and tetramer ($i = 4$) species may be analyzed in terms of the expression [29]

$$\bar{C}(r) = C_2(r)\psi_2(r) + 2C_4(r)\psi_4(r) \quad (2)$$

where $\bar{C}(r)$ is the base-molar concentration of phosphorylase *b*, the weight-concentration divided by M_r of the smallest species (dimer); and where

$$\psi_i(r) = \exp[M_i(1-\bar{v}_i\rho_s)\omega^2(r^2-r_F^2)/(2RT)] \quad (3)$$

In the analysis a partial specific volume of 0.737 mL·g^{-1}, obtained by applying a temperature correction of 0.002 mL·g^{-1} to the experimental value of 0.739 mL·g^{-1} measured at 20 °C [30], has been used for the dimeric and tetrameric species, a simplification which allows Eqn (2) to be written in terms of the single independent variable $\psi_2(r)$ on the grounds that $\psi_4(r) = [\psi_2(r)]^2$. With this simplification Eqn (2) becomes [29]:

$$\bar{C}(r)/\psi_2(r) = C_2(r_F) + 2C_4(r_F)\psi_2(r) \quad (4)$$

The dependence of $\bar{C}(r)/\psi_2(r)$ upon $\psi_2(r)$ thus has potential for evaluation of the concentration of dimeric species at the chosen reference radial position as the ordinate intercept, irrespective of the rate of association equilibrium attainment.

Sedimentation velocity runs were conducted at 15 °C and rotor speeds of 40 000–48 000 r.p.m., the absorption optical system again being used to record radial scans at 3-min intervals throughout the 2.5-h duration of the experiments. Sedimentation coefficients were evaluated by means of the time-derivative analysis, $g(s^*)$ [31]. The fraction of dimer (f_2) was estimated as $f_2 = A_2/(A_2 + A_4)$, where A_2 and A_4 are the respective areas of the dimer and tetramer peaks in the $g(s^*)$ vs s^* distribution, a procedure that allowed determination of the association constant, $K_{2,4}$, as

$$K_{2,4} = (1-f_2)/(2f_2{}^2\bar{C}) \quad (5)$$

where $\bar{C}$, the base-molar concentration of phosphorylase *b*, has been taken as that of the initial solution. In other words, the results have been interpreted on the basis of the 'rectangular' approximation without regard to the consequences of the Johnston–Ogston effect [32].

Calculation of second virial coefficients for TMAO and phosphorylase *b*

The likely consequences of thermodynamic nonideality of phosphorylase *b* arising from the presence of an inert cosolute M (TMAO) have been calculated on the statistical–mechanical basis of excluded volume [33]. On

this basis, the thermodynamic activity coefficient of enzyme species *i*, γ_i, is related to molar species concentrations by the expression [34,35]:

$$\gamma_i = \exp\left[2B_{i,i}C_i + \sum_{i \neq j} B_{i,j}C_j + \ldots\right] \quad (6)$$

where $B_{i,i}$ is the second virial coefficient describing thermodynamic nonideality of species *i* because of its own concentration, and the $B_{i,j}$ is the corresponding virial coefficient describing thermodynamic nonideality of species *i* resulting from the presence of another species (*j*) present at concentration C_j. In the present experiments, the high concentration of TMAO ensures dominance of the cosolute concentration (C_M) term, whereupon Eqn (6) becomes

$$\gamma_i \approx \exp(B_{i,M}C_M) \quad (7)$$

Prediction of the consequences of such thermodynamic nonideality requires the assignment of magnitudes to $B_{2,M}$ and $B_{4,M}$, the respective second virial coefficients reflecting excluded volume interactions of dimer and tetramer with cosolute. Because of the lack of charge on the cosolute (TMAO), these virial coefficients equate with the covolumes of M and the enzyme species, the volume from which the centres of the two molecules are mutually excluded. Calculation of $B_{i,M}$ is thus conditional upon assignment of regular three-dimensional shapes to M and each of the enzyme species.

On the basis that the colligative properties of small cosolutes such as glycerol, proline, sucrose and betaine (trimethylglycine) are commensurate with their thermodynamic description as effective spheres [35,36], TMAO is ascribed a radius, R_M, of 0.3 nm, in accordance with estimates obtained for similar cosolutes from isopiestic [35,37] and vapour pressure osmometry [38] measurements. On the other hand, dimeric phosphorylase *b* is more appropriately modelled as a prolate ellipsoid with major semiaxis, a_2, and semiminor axes, b_2, in the ratio 1.84 : 1 [39]. Combination of this information with the effective hydrodynamic radius (R_e) of 5.19 nm deduced from the sedimentation coefficient of 8.70 S for dimer and the relationship $a_2b_2^2 = R_e^3$ yields estimates of 7.80 nm and 4.24 nm for a_2 and b_2, respectively. By similar reasoning the tetramer, comprising two such units side-by-side [3], is taken to be an oblate ellipsoid with $a/b = 1.92$ and $R_e = 6.74$ nm, a situation which gives rise to major semiaxes, a_4, of 8.38 nm and a semiminor axis, b_4, of 4.36 nm. Values of 422 L·mol^{-1} for $B_{2,M}$ and 833 L·mol^{-1} for $B_{4,M}$ are obtained from the expressions [40,41]:

$$B_{2,M} = N[(4\pi {R_M}^3/3) + (4\pi {a_2}^2 b_2/3) + 2\pi a_2 b_2 R_M\{(1-\varepsilon^2)^{1/2} + (\sin^{-1}\varepsilon)/\varepsilon\} + 2\pi a_2 {R_M}^2\{1 + [(1-\varepsilon^2)/(2\varepsilon)] \times \ln[(1+\varepsilon)/(1-\varepsilon)]\}] \quad (8)$$

$$B_{4,M} = N[(4\pi {R_M}^3/3) + (4\pi {a_4}^2 b_4/3) + [\pi a_4 b_4 R_M/(1-\varepsilon^2)^{1/2}]\{1 + [(1-\varepsilon^2)]/(2\varepsilon)\} \times \{\ln[(1+\varepsilon)/(1-\varepsilon)]\} + [2\pi b_4 {R_M}^2/(1-\varepsilon^2)^{1/2}] \times \{(1-\varepsilon^2)^{1/2} + (\sin^{-1}\varepsilon)/\varepsilon\}] \quad (9)$$

where the ellipticity, ε, is defined by the relationship $\varepsilon^2 = 1-(b/a)^2$.

RESULTS AND DISCUSSION

Studies of phosphorylase *b* in dilute solution

As noted in the introduction, equilibrium attainment between dimeric and tetrameric forms of phosphorylase *b* is sufficiently slow under some conditions to allow the composition of the centrifuged mixture to be deduced from the proportions of the resolved boundaries corresponding to dimeric and tetrameric species. This aspect of the sedimentation velocity behaviour of phosphorylase *b* in an experiment conducted at 48 000 r.p.m. is illustrated in Fig. 1, which comprises two clearly resolved boundaries, the sedimentation coefficients of which (after correction to $s_{20,w}$) coincide with the respective values of 8.7 S and 13.4 S for dimer and tetramer. Interpretation of the areas of these two $g(s^*)$ peaks in terms of Eqn (5) yields a dimerization constant, $K_{2,4}$, of 3.0×10^4 M^{-1} for phosphorylase *b* in the present Hepes/AMP environment, a value which is in reasonable agreement with that of 2.4×10^4 M^{-1} deduced previously by sedimentation velocity under similar (but not identical) conditions [4].

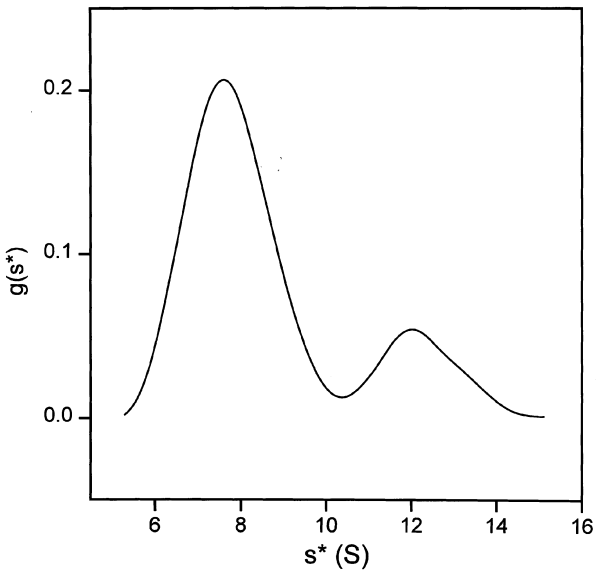

Fig. 1. Sedimentation velocity behaviour of phosphorylase *b* in 40 mM Hepes (pH 6.8) supplemented with 1 mM AMP. Apparent sedimentation coefficient distribution, $g(s^*)$ vs. s^*, calculated by the method of Stafford [31] from experiments conducted at 15 °C.

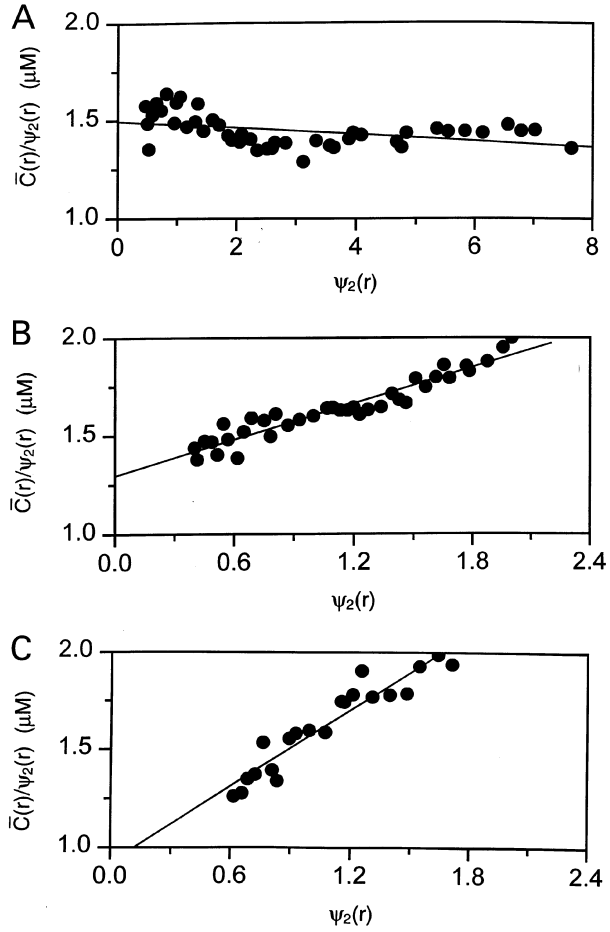

Fig. 2. Use of the ψ analysis to test whether sedimentation equilibrium distributions also reflect self-association equilibrium. Results for phosphorylase *b* in 40 mM Hepes buffer (pH 6.8) supplemented with 1 mM AMP are analyzed in terms of Eqn (4) with r_F selected on the basis of a common value, 1.60 μM, for $\bar{C}(r)$ in experiments conducted at 15 °C and (a) 10 000 r.p.m., (b) 7000 r.p.m. and (c) 6000 r.p.m.

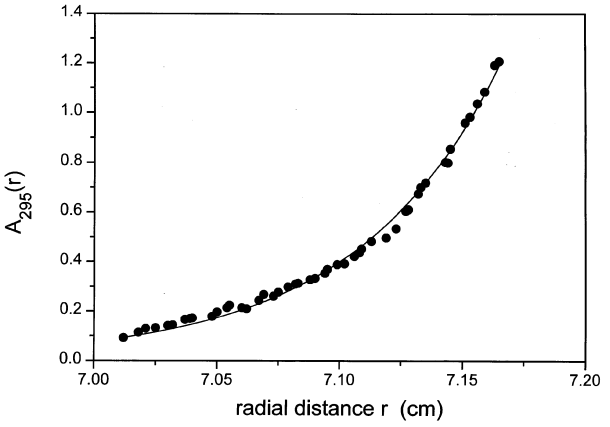

Fig. 3. Conformity of the sedimentation equilibrium distribution for phosphorylase *b* at 10 000 r.p.m. with the behaviour of a single solute. The solid line signifies the best-fit description of the experimental sedimentation equilibrium distributiion obtained by nonlinear regression analysis in terms of Eqn (1).

This evaluation of an equilibrium constant from the size of boundaries formed by resolving the mixture into its separate species is incompatible with the concept of any chemical interconversion to restore equilibrium during the course of the experiment. Although the time-scale of a sedimentation equilibrium experiment is far greater, the validity of assuming the attainment of chemical as well as sedimentation equilibrium under these conditions certainly needs to be checked. Sedimentation equilibrium distributions obtained for phosphorylase *b* at a range of rotor speeds have therefore been analyzed in terms of Eqn (4), an analysis which is not reliant upon attainment of chemical equilibrium.

In the event that chemical equilibrium is attained throughout the sedimentation distribution, the concentration of dimer, $C_2(r_F)$ deduced as the ordinate intercept of the dependence of $\bar{C}(r)/\psi_2(r)$ upon $\psi_2(r)$ from separate experiments should be invariant when r_F is chosen for each distribution on the basis of a common value of $\bar{C}(r_F)$. Application of this criterion for the establishment of chemical equilibrium to sedimentation equilibrium distributions for phosphorylase *b* obtained at 6000–10 000 r.p.m. is summarized in Fig. 2, which is based on a value of 1.60 μM for $\bar{C}(r_F)$. Clearly, these results defy description in terms of a common ordinate intercept, $C_2(r_F)$, and a common slope, $2C_4(r_F)$, the behaviour of a dimer–tetramer system in association equilibrium. Although the inverse dependence of the ordinate intercept upon rotor speed would be consistent with the concept of a pressure-dependent chemical equilibrium, this possibility has been precluded in a previous investigation [4]. It is therefore concluded that sedimentation equilibrium patterns for phosphorylase *b* under the present conditions should be considered in terms of a noninteracting mixture of dimer and tetramer, rather than that of a dimer–tetramer system in reversible association equilibrium.

The essentially horizontal (in fact slightly negative) slope of Fig. 2a implies the absence of tetrameric phosphorylase *b* in the distribution recorded at the highest speed (10 000 r.p.m.). Indeed, analysis of the distribution (Fig. 3) in terms of the sedimentation equilibrium expression for a single species, Eqn (1), yields a molecular mass of 194 000 (±5000), a value that is marginally smaller than the M_r of 194 800 deduced for dimer from the amino-acid sequence [25]. This insignificant disparity accounts for the slightly negative slope of the dependence of $\bar{C}(r)/\psi_2(r)$ upon $\psi_2(r)$ in Fig. 2a. The implication that the solution has been depleted of tetrameric phosphorylase *b* is borne out by calculations based on the sedimentation coefficient for tetramer ($s_{15,b}$ = 12.2 S), which show that a boundary characterized by such a sedimentation coefficient would have traversed the distance between the air–liquid meniscus and the cell base within 4 h of the 24-h experiment. The extent of tetramer depletion decreases with decreasing rotor speed, and hence leads to the progressive decrease in ordinate intercept and increase in positive slope observed in Fig. 2.

Inasmuch as the present results are clearly incompatible with interpretation in terms of chemical equilibrium attainment throughout the sedimentation equilibrium distributions, they call into question the interpretation of corresponding results for phosphorylase *b* reported previously [17]. This earlier study refers to enzyme in 50 mM

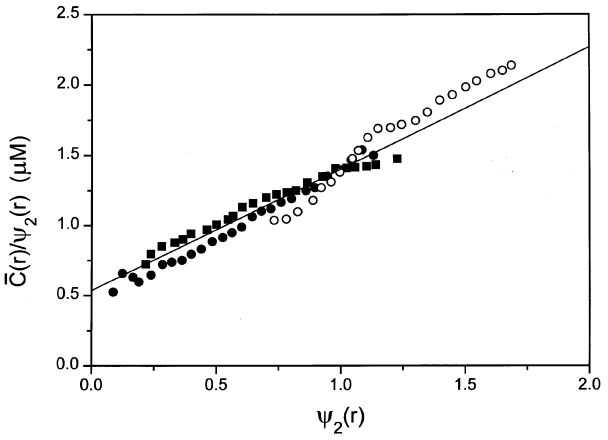

Fig. 4. Evidence for attainment of chemical as well as sedimentation equilibrium in distributions for phosphorylase *b* in 50 mM glycylglycine buffer (pH 6.8) supplemented with 0.125 M K_2SO_4, 0.2 mM EDTA, 1 mM AMP and 4 mM glucose 1-phosphate. Results, calculated from the distributions reported in [17], refer to experiments conducted at 17 °C and either 10 000 r.p.m. with loading concentrations of 0.37 (●) or 0.92 (■) mg·mL^{-1} enzyme, or 7200 r.p.m. with a loading concentration of 2.0 mg·mL^{-1} (○). The line is the relationship predicted by Eqn (4) for a rapidly equilibrating dimer–tetramer system with the selected $\bar{C}(r)$ of 1.40 μM and the published dimerization constant [17] of 1.5×10^6 M^{-1}.

glycylglycine buffer (pH 6.8, 15 °C) supplemented not only with AMP (1 mM) but also K_2SO_4 (0.125 M), conditions under which the self-association is much stronger. Furthermore, the attainment of self-association equilibrium is much faster, the rate constant for tetramer dissociation being 0.008 s^{-1} [5]. Analysis of these results in terms of Eqn (4) with $\bar{C}(r_F) = 1.40$ μM is summarized in Fig. 4, where the essentially single dependence of $\bar{C}(r)/\psi_2(r)$ upon $\psi_2(r)$ for data from distributions recorded at 7200 r.p.m. (open symbols) and 10 000 r.p.m. (closed symbols) conforms reasonably well to that predicted by Eqn (4) and

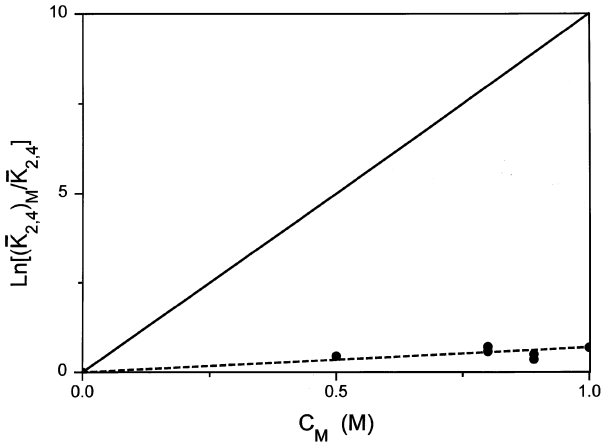

Fig. 5. Dependence of the self-association behaviour of phosphorylase *b* upon the concentration of osmoprotectant TMAO present in the solution. Effective dimerization constants obtained by sedimentation velocity are plotted in the form suggested by Eqns (11) and (14). See text.

the law of mass action for a dimer–tetramer system with the reported $K_{2,4}$. This experimental verification of the earlier presumption of chemical as well as sedimentation equilibrium attainment justifies the earlier analysis of these distributions and the conclusion drawn that $K_{2,4} = 1.5 \times 10^6$ M^{-1} [17].

Although the present use of the psi function [29] to determine whether a sedimentation distribution reflects a stable mixture of dimer and tetramer or a dimer–tetramer system in rapid association equilibrium is the first such application, the principle of the procedure has been applied for many years. The method described here replaces that in which the criterion for a sedimentation equilibrium distribution to be in chemical equilibrium entails establishment of the identity of the weight-average molecular mass $\bar{M}$ for the same $\bar{C}$ from distributions obtained at different rotor speeds [42–44]. Overlap of the dependence of $\bar{M}(r)$ upon $\bar{C}(r)$ deduced from the various sedimentation equilibrium distributions thus provided the means of justifying the consideration of results in terms of a system in chemical as well as sedimentation equilibrium, which requires their description in terms of a single $\bar{M}(r)$–$\bar{C}(r)$ relationship, namely

$$\bar{M}(r) = M_2[1 + 4K_{2,4}C_2(r)]/[1 + 2K_{2,4}C_2(r)] \qquad (10)$$

for a dimer–tetramer system. The condition that $\bar{M}(r)$ for a given $\bar{C}(r)$ be constant thus also reflects the requirement that $C_2(r)$ be invariant for the same $\bar{C}(r)$ in sedimentation equilibrium distributions obtained at different rotor speeds. Its replacement by an equivalent procedure based on direct analysis of the sedimentation equilibrium distributions merely avoids the error and uncertainty introduced [45] by their differentiation to obtain the transformed variable $\bar{M}(r)$ [46].

Effect of molecular crowding on phosphorylase *b* self-association

Perturbation of the dimer–tetramer equilibrium by specific effectors of phosphorylase *b* activity is a consequence of their displacement of the T ⇄ R isomerization towards the dimeric state of the enzyme [4,9,10]. The possibility therefore exists that thermodynamic nonideality arising from the presence of high concentrations of totally unrelated cosolutes can also act as a modulator of phosphorylase *b* activity by virtue of the molecular crowding effect on the dimer–tetramer equilibrium [11–13]. In the present studies with TMAO as the space-filling cosolute the apparent dimerization constants have been determined by the sedimentation velocity approach because of the slow interconversion between dimeric and tetrameric phosphorylase *b*.

For a dilute solution of a dimer–tetramer system in buffer supplemented with a high concentration of inert cosolute, M, the dependence of the measured dimerization constant $(K_{2,4})_M$, upon C_M is described by the relationship [12,35]

$$\ln[(K_{2,4})_M/K_{2,4}] \approx (2B_{2,M} - B_{4,M})C_M \qquad (11)$$

in which $K_{2,4}$, the thermodynamic dimerization constant, may be approximated by the value obtained in the absence of cosolute. Because of its small size, the cosolute remains essentially uniformly distributed throughout the solution

subjected to sedimentation velocity, whereupon C_M in Eqn (11) may be taken as the cosolute concentration in the buffer against which the enzyme is predialyzed. As noted above, $B_{2,M}$ and $B_{4,M}$ are second virial coefficients describing the thermodynamic nonideality arising from the respective physical (excluded volume) interactions of dimer and tetramer with cosolute. Results for the effect of TMAO concentration on ln $[(K_{2,4})_M/K_{2,4}]$ for phosphorylase *b* (●, Fig. 5) are certainly consistent with description in terms of a linear dependence. However, the extent of self-association enhancement is relatively minor in comparison with the theoretical consequences of molecular crowding (—) predicted by Eqn (11) and the estimated magnitude of $(2B_{2,M} - B_{4,M})$ that emanates from the estimated second virial coefficients (see Experimental procedures). Although such disparity could reflect concomitant preferential binding of TMAO to the dimeric state of phosphorylase *b*, the overestimate of the experimental slope could also indicate inadequacy of the simple dimer–tetramer model used for the excluded volume calculations.

As noted above, the self-association of phosphorylase *b* is conditional upon conversion of the dimeric T state to the R state [3,6–8]. A more appropriate model of the system under thermodynamically ideal conditions is therefore the reaction scheme:

$$2T_2 \overset{Y}{\rightleftarrows} 2R_2 \overset{K_{2,4}}{\rightleftarrows} R_4$$

whereupon the measured dimerization constant should be regarded as the constitutive parameter, $K_{2,4}$ [47 (49]:

$$\bar{K}_{2,4} = K_{2,4}[Y/(1 + Y)]^2 \approx K_{2,4}Y^2 \qquad (12)$$

where the approximate form reflects the present situation wherein *Y*, the isomerization constant governing the $T_2 \rightleftarrows R_2$ conversion in the presence of AMP, is taken to be much smaller than unity to comply with the Monod model [50] for the allosteric activatory effect of AMP on phosphorylase *b*. The consequences of including a concentration C_M of inert cosolute (TMAO) are thus twofold. In addition to the effect of thermodynamic nonideality on the dimer–tetramer equilibrium discussed above, there is the corresponding effect on the isomerization equilibrium, which is displaced towards the smaller, nonassociating T state in accordance with the expression [35,37,51–53]

$$(Y_M) \approx Y \exp[(B_{T2,M} - B_{R2,M})C_M] \qquad (13)$$

Combination of Eqns (11)–(13) yields the relationship

$$\ln[(\bar{K}_{2,4})_M/\bar{K}_{2,4}] \approx [(2B_{2R,M} - B_{4R,M}) + 2(B_{2T,M} - B_{2R,M})]C_M \qquad (14)$$

as the expression for the dependence of the measured dimerization constant upon TMAO concentration. A decrease in the predicted slope from the value of 11 L·mol^{-1} for the simple dimer–tetramer model to a value of 0.7 L·mol^{-1} (—, Fig. 5) would require $B_{2R,M}$ to exceed $B_{2T,M}$ by only 5 L·mol^{-1} (i.e. by 1.2% of $B_{2T,M}$). On the grounds that a relative covolume change of this magnitude is seemingly reasonable in that corresponding values of 10 and 2.8% have been observed for isomeric states of rabbit muscle pyruvate kinase [51] and yeast hexokinase [53], respectively, the minor effect of TMAO on phosphorylase *b* self-association is considered to be consistent with the consequences of molecular crowding by an inert spacefilling cosolute. However, the results could also be interpreted in terms of preferential TMAO interaction with the T state of the enzyme.

The above interpretation of the effect of TMAO on phosphorylase *b* self-association implies that enzyme inhibition should result from the presence of high concentrations of unrelated solutes because of the displacement of the $T \rightleftarrows R$ isomerization equilibrium towards the small, inactive T state. As noted above, such displacement of the isomerization equilibrium could also reflect preferential binding of cosolute to the T state, the interpretation used to account for the inhibition of enzyme activity observed in the presence of high concentrations of glycerol, ethylene glycol and sucrose [21,22]. In this regard the need to consider the possible involvement of cosolute in preferential interactions with one isomer is emphasized by the fact that enhanced phosphorylase *b* activity is observed in the presence of other cryoprotectants [21,22,54], an outcome that can only be ascribed to preferential interaction with the larger, active R state. Although the present interpretation of the TMAO effect on phosphorylase *b* self-association in terms of thermodynamic nonideality is thus deficient because of its failure to take into account any consequences of preferential interactions, it draws attention to a corresponding shortcoming of the interpretation placed on the activity measurements – failure to consider the consequences of molecular crowding by the cryoprotectant. Because thermodynamic nonideality invariably favours the smaller isomeric state, the extremely weak dissociation constants inferred from the inhibition measurements overestimate the strength of chemical interaction with the T state: those inferred from the extent of activation underestimate the strength of interaction with the R state.

The present study has also revealed a potential pitfall in the use of the extent of tetramer formation as an index of the position of the $T \rightleftarrows R$ isomerization equilibrium position [4,9,10]. On this basis, the virtually unchanged magnitude of $K_{2,4}$ in the presence of 1 M TMAO implies a corresponding invariance of the effective isomerization constant for the $T \rightleftarrows R$ transition. However, the seemingly ideal self-association behaviour may be reflecting effects of thermodynamic nonideality on the dimer–tetramer equilibrium that are being countered by those emanating from a relatively minor expansion of dimer during its conversion from the T state to the enzymically active, dimerizing R state. Furthermore, the effects of thermodynamic nonideality on the $T \rightleftarrows R$ isomerization equilibrium could be pronounced, despite insensitivity of the dimer–tetramer equilibrium position to such effects. For example, interpretation of the twofold enhancement of $K_{2,4}$ observed for phosphorylase *b* in the presence of 1 M TMAO on the basis of Eqn (14) leads to the conclusion that the effective isomerization constant, Y_M, is 170-fold smaller than the value (*Y*) applying in dilute solution. Thus, although the R state of phosphorylase *b* certainly provides an alternative to phosphorylase *a* as a potential catalyst for glycogen phosphoryation and degradation under the conditions employed in conventional kinetic experiment *in vitro*, this calculation suggests the possibility of a diminished catalytic role for phosphorylase *b* in the crowded physiological environment, whereupon initiation of glycogenolysis would then remain the prerogative of muscle glycogen phosphorylase *a*, the

phosphorylated form of the enzyme resulting from kinase action on phosphorylase *b*.

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