ELSEVIER

Biophysical Chemistry 130 (2007) 89–92

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

# Reconsideration of sedimentation equilibrium distributions reflecting the effects of small inert cosolutes on the dimerization of α-chymotrypsin

Donald J. Winzor [a,*], Chetan N. Patel [b,1], Gary J. Pielak [b,c]

[a] *Department of Biochemistry, University of Queensland, Brisbane, Queensland 4072, Australia*
[b] *Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599, USA*
[c] *Departments of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599, USA*

Received 14 July 2007; accepted 24 July 2007
Available online 7 August 2007

**Abstract**

A reported discrepancy between quantitative estimates of the extent of enhanced α-chymotrypsin dimerization in the presence of sucrose is traced to different consequences of using an incorrect value of the buoyant molecular weight in the analysis of sedimentation equilibrium distributions. Support is thereby provided for the earlier contention that the effect of sucrose, as well as of glucose and raffinose, on dimerization may be rationalized quantitatively in terms of molecular crowding by an inert cosolute.


*Keywords:* Chymotrypsin; Molecular crowding; Sedimentation equilibrium; Thermodynamic nonideality

## 1. Introduction

In the initial examination by sedimentation equilibrium of the effect of sucrose on the dimerization of α-chymotrypsin [1] the increase in apparent dimerization constant was shown to agree with that predicted by excluded-volume interpretation of the resulting thermodynamic nonideality [2]. However, a subsequent sedimentation equilibrium study of the same system [3] gave poorer agreement between predicted and experimentally observed enhancement because of a much lower value for the measured estimate. The purpose of the present communication is to identify the source of this disparity.

## 2. Theoretical considerations

### 2.1. A self-associating single-solute system

Consider a protein undergoing reversible dimerization ($2A \leftrightarrows C$) that is dialyzed against solvent (buffer) before being subjected to ultracentrifugation at angular velocity $\omega$ and absolute temperature $T$. At sedimentation equilibrium the distribution of monomer is given by [4]

$$z_A(r) = z_A(r_F)\psi_A(r) \tag{1a}$$

$$\psi_A(r) = \exp\left[M_A(1-\overline{\nu}_A\rho)\omega^2\left(r^2-r_F^2\right)/(2RT)\right] \tag{1b}$$

which relates the molar thermodynamic activity of monomer at radial distance $r$, $z_A(r)$, to that at a selected reference radial position, $z_A(r_F)$. $M_A$ is the monomer molecular weight and $\overline{\nu}_A$ its partial specific volume (taken as that for the protein), $\rho$ is the buffer density and $R$ the universal gas constant. $\psi_A(r)$ is a renormalization of the independent variable $r$ for a specified buoyant molecular weight $[M_A(1-\overline{\nu}_A\rho)]$, rotor speed and temperature. As shown previously [4], the radial dependence of base-molar protein concentration (weight-concentration divided by $M_A$), $\overline{C}(r)$, is described by the relationship

$$\overline{C}(r) = z_A(r_F)\psi_A(r) + 2(K_2 - B_{AA})[z_A(r_F)\psi_A(r)]^2 + \ldots \tag{2}$$

where $K_2 = z_C(r)/[z_A(r)]^2$ is the thermodynamic dimerization constant, and $B_{AA}$ is the osmotic second virial coefficient for monomer self-interaction. Nonlinear regression analysis of the

* Corresponding author. Fax: +61 7 3365 4699.
*E-mail address:* d.winzor@uq.edu.au (D.J. Winzor).
[1] Present address: Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020, USA.

0301-4622/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2007.07.006

$[\psi_A(r), \overline{C}(r)]$ data set in terms of Eq. (2) thus yields $z_A(r_F)$ and a constant, $(K_2 - B_{AA})$, as the two curve-fitting parameters.

For many proteins the self-association can be quantified in sufficiently dilute solution for the characterization to be conducted under conditions that are usually described as approaching thermodynamic ideality. In fact, such neglect of thermodynamic nonideality is merely an implicit assumption that $K_2 \gg B_{AA}$ for the system under consideration [5], whereupon Eq. (2) becomes

$$\overline{C}(r) \approx z_A(r_F)\psi_A(r) + 2K_2[z_A(r_F)\psi_A(r)]^2. \quad (3)$$

This simplified expression may justifiably be applied to the present α-chymotrypsin system (pH 3.9, $I$ 0.2 M), for which $K_2 = 50{,}000\ M^{-1}$ [1,3] compared with a value of only $200\ M^{-1}$ for $B_{AA}$ [4].

### 2.2. Protein dimerization in the presence of a small cosolute

We now consider the situation in which the protein undergoing reversible dimerization is dialyzed exhaustively against buffer supplemented with a molar concentration $C_M$ of small inert cosolute ($M$) before being subjected to ultracentrifugation. In this situation the distribution of monomer activity at sedimentation equilibrium continues to be given by Eqs. (1a) and (1b); and a corresponding expression (with $M$ instead of $A$ subscripts) applies to the distribution of cosolute. Provided that the buoyant molecular weight of cosolute, $M_M(1-\overline{v}_M\rho)$, is sufficiently small for only minor redistribution of $M$ to occur at sedimentation equilibrium, the cosolute may be regarded as part of the solvent. However, the equilibrium distribution of molar monomer concentration ($C_A$) is then described by the expression [6–8]

$$C_A(r) \approx C_A(r_F)\psi_A'(r) \quad (4a)$$

$$\psi_A'(r) = \exp\left[M_A(1 - \phi_A'\rho_d)\omega^2\left(r^2 - r_F^2\right)/(2RT)\right] \quad (4b)$$

$$\rho_d = \rho + (1 - \overline{v}_M\rho)M_M C_M \quad (4c)$$

in which the buoyancy factor is described in terms of the density of cosolute-supplemented diffusate ($\rho_d$) and the apparent partial specific volume of the protein ($\phi_A'$).

From an experimental viewpoint the preferable course of action is to determine $M_A(1-\phi_A'\rho_d)$ as the buoyant molecular weight in the limit of zero protein concentration [6,8]; but for the present retrospective circumstance we shall take advantage of the theoretical relationship [6]

$$M_A(1 - \phi_A'\rho_d) = M_A(1 - \overline{v}_A\rho) - (1 - \overline{v}_M\rho)B_{AM}M_M C_M(r_F) + \ldots \quad (5)$$

in which $C_M(r_F)$, the molar cosolute concentration at the chosen reference radial position, may reasonably be approximated by $C_M$, its concentration in the diffusate. For an uncharged cosolute such as sucrose the second virial coefficient for the monomer–cosolute interaction ($B_{AM}$) may be calculated as

$$B_{AM} = 4\pi N(R_A + R_M)^3/3 \quad (6)$$

where $R_A$ and $R_M$ denote the respective radii of monomer and cosolute. Avogadro's number ($N$) is required in Eq. (6) to convert the second virial coefficient (excluded volume) from a molecular to a molar basis.

As before, the extent of dimerization may be quantified on the basis of the counterpart of Eq. (3),

$$\overline{C}(r) = C_A(r_F)\psi_A'(r) + 2K_2^{app}[C_A(r_F)\psi_A'(r)]^2 + \ldots \quad (7)$$

where [1,3]

$$K_2^{app} = K_2\exp[(2B_{AM} - B_{CM})C_M + \ldots]. \quad (8)$$

Whereas Eq. (7) is the expression for nonlinear curve-fitting to evaluate $K_2^{app}$ and $C_A(r_F)$, Eq. (8) renders possible the comparison of experimental findings with predictions based on the excluded volume concept of thermodynamic nonideality [2].

### 2.3. The omega approach

Prior to development of the characterization of self-association based on $\psi_A(r)$ [4], the direct evaluation of $K_2$ for a single-solute system was effected by means of the omega function [9]

$$\Omega_A(r) = \left[\overline{C}(r)/\overline{C}(r_F)\right]\exp\left[M_A(1 - \overline{v}_A\rho)\omega^2\left(r_F^2 - r^2\right)/(2RT)\right] \quad (9a)$$

which we can now write [cf Eq. (1b)] as

$$\Omega_A(r) = \left[\overline{C}(r)/\overline{C}(r_F)\right]/\psi_A(r). \quad (9b)$$

The omega function provided the first means of evaluating directly the thermodynamic activity of monomer from sedimentation equilibrium distributions for a self-associating solute. On the grounds that

$$\lim_{C(r)\to 0} \Omega_A(r) = \Omega_o = z_A(r_F)/\overline{C}(r_F) \quad (10)$$

the combination of $\Omega_o$, the ordinate intercept of the dependence of $\Omega_A(r)$ upon $\overline{C}(r)$, with $\overline{C}(r_F)$ yielded the monomer activity at the reference radial position, $z_A(r_F)$, and hence $z_A(r)$ for all $\overline{C}(r)$ as $z_A(r_F)\psi_A(r)$ [9].

By analogy with the above reasoning for the psi analysis, the application of the same approach to sedimentation equilibrium distributions reflecting protein dimerization in the presence of a small cosolute requires the rewriting of Eq. (9b) as

$$\Omega_A'(r) = \left[\overline{C}(r)/\overline{C}(r_F)\right]/\psi_A'(r) \quad (11)$$

with $\psi_A'(r)$ defined by Eq. (4a) and (4c), as well as the replacement of Eq. (10) by [1]

$$\lim_{C(r)\to 0} \Omega_A'(r) = \Omega_o' = C_A(r_F)/\overline{C}(r_F). \quad (12)$$

The consequent evaluation of $C_A(r_F)$ from $\Omega_o'$, and hence of $C_A(r)$ throughout the sedimentation equilibrium distribution, then allows the determination of $K_2^{app}$ as the best-fit value of $[\overline{C}(r) - C_A(r)]/[C_A(r)]^2$.

## 3. Consideration of experimental results

In both sedimentation equilibrium studies of the effect of sucrose on the dimerization of α-chymotrypsin in acetate—chloride buffer, pH 3.9, $I$ 0.2 M, the buoyant molecular weight was calculated incorrectly as $M_A(1-\overline{\nu}_A\rho_d)$ [1,3]. The normalized radius parameter in both investigations was therefore

$$\psi''_A(r) = \exp\left[M_A(1-\overline{\nu}_A\rho_d)\omega^2\left(r^2-r_F^2\right)/(2RT)\right] \quad (13)$$

instead of $\psi'_A(r)$ as defined by Eq. (4b). Because the later study [3] merely incorporated the incorrect substitution of $\psi''_A(r)$for $\psi'_A(r)$ into the treatment of results according to Eq. (7), the actual expression used in that analysis was

$$\overline{C}(r) = C_A(r_F)[\psi'_A(r)]^y + 2K_2^{app}\{C_A(r)[\psi'_A(r)]^y\}^2 + \ldots \quad (14)$$

where $y=(1-\overline{\nu}_A\rho_d)/(1-\phi'_A\rho_d)$. Discussion of the quantitative consequences of that mistake on the determination of $K_2^{app}$ is delayed until consideration has also been given to the corresponding problem in the earlier investigation [1], which preceded development of the psi analysis [4].

On the grounds that the omega function in that investigation [1] was defined incorrectly as $\Omega''_A(r)=[\overline{C}(r)/\overline{C}(r_F)]/[\psi'_A(r)]^y$ it follows that the ordinate intercept ($\Omega''_o$) of the resulting dependence of $\Omega''_A(r)$ upon $\overline{C}(r)$ was given by

$$\Omega''_o(r) = \left[C_A(r_F)/\overline{C}(r_F)\right]/[\psi'_A(r)]^{y-1}. \quad (15)$$

Although the ordinate intercept was mistakenly taken as $[C_A(r_F)/\overline{C}(r_F)]$, Shearwin and Winzor [1] then obtained $C_A(r)$ throughout the distribution as

$$C_A(r) = \Omega''_o(r)\overline{C}(r_F)\psi''_A(r) = \Omega'_o\overline{C}(r_F)[\psi'_A(r)]^{1-y}[\psi'_A(r)]^y = \Omega'_o\overline{C}(r_F)\psi'_A(r) \quad (16)$$

which is correct. Self-cancellation of the error has thus led to a situation wherein correct values of $K_2^{app}$ were obtained despite invalidity of the interpretation accorded the omega plot upon which they were based.

At this stage we have established the probable correctness of $K_2^{app}$ values upon which the assertion was made [1] that the observed enhancement of α-chymotrypsin dimerization in the presence of sucrose is entirely consistent with interpretation in terms of thermodynamic nonideality arising from the monomer–sucrose excluded-volume interaction. It now remains to ascertain the quantitative manifestations of employing Eq. (14) instead of Eq. (7) for the evaluation of apparent dimerization constants by psi analysis in the subsequent investigation [3]. For that purpose we compare results from the two analyses of a sedimentation equilibrium distribution, [$r$, $\overline{C}(r)$], at 22,000 rev/min and 20°C for α-chymotrypsin in 0.15 M sucrose [3]. As on that occasion, monomeric enzyme has been assigned a molecular weight of 25,200, a partial specific volume of 0.736 ml/g, and a molecular radius of 2.44 nm, the corresponding values for sucrose being taken as 342, 0.616 ml/g and 0.34 nm. On the basis of an unsupplemented buffer density of 1.0055 g/ml [4], that of the sucrose-containing diffusate is calculated [Eq. (4c)] to be

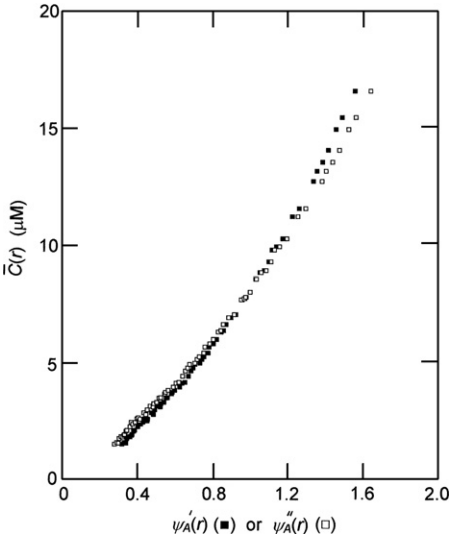


Fig. 1. Psi plots of an experimental sedimentation equilibrium distribution for α-chymotrypsin in acetate–chloride buffer (pH 3.9, $I$ 0.2 M) supplemented with 0.15 M sucrose. Solid and open symbols refer to the respective analyses of the experimental [$r$, $\overline{C}(r)$] distribution according to Eqs. (7) and (14).

1.0250 g/ml. From Eq. (5) the correct buoyant molecular weight of monomer is 5493, whereas that derived from the product of partial specific volume ($\overline{\nu}_A$) and supplemented diffusate density ($\rho_d$) is 6189: the value of $y$ in Eq. (14) is thus 1.127.

Analysis of the experimental [$r$, $\overline{C}(r)$] distribution according to the correct expression [Eq. (7)] is summarized in Fig. 1 by the solid symbols, which signify an apparent equilibrium constant of 170,000 (±10,000) $M^{-1}$. However, analysis of the same distribution according to Eq. (14) leads to the open symbols in Fig. 1 and an apparent dimerization constant of 64,000 (±3000) $M^{-1}$. Use of an incorrect value for the buoyant molecular weight of monomer [3] thus led to substantial underestimation of $K_2^{app}$. The consequence of this error on the dependence of apparent dimerization constant upon sucrose concentration is presented in Fig. 2, which plots the results from all of the earlier sedimentation equilibrium distributions [3] in accordance with the logarithmic form of Eq. (8). Values of $K_2^{app}$ obtained using the correct buoyancy term (●) signify a covolume difference, $(2B_{AM}-B_{CM})$, of 9.0 (±1.6) L/mol, which is significantly higher than the earlier estimate [3] of 1.9 (±0.3) L/mol obtained from the open symbols in Fig. 2. Furthermore, the revised estimate, which confirms the earlier report [1] of 8.0 (±1.2) L/mol for the covolume difference, encompasses the theoretical prediction of 8.3 L/mol for $(2B_{AM}-B_{CM})$ that is calculated from Eq. (8) on the statistical-mechanical basis of excluded volume [2]. The disparity between results from the two sedimentation equilibrium investigations of α-chymotrypsin dimerization in the presence of sucrose [1,3] is thus resolved.

Reanalysis of sedimentation equilibrium distributions for α-chymotrypsin in the presence of 0–0.5 M glucose [3] increases the estimate of $d\ln K_2^{app}/dC_M$ from the earlier value of 1.0 (±0.3) to 4.9 (±1.4) L/mol, the revised value being in much better agreement with the statistical-mechanical prediction of 5.9 L/mol for $(2B_{AM}-B_{CM})$ based on spherical

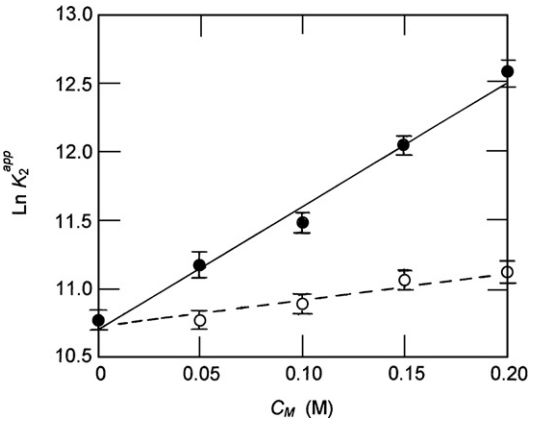


Fig. 2. Effect of sucrose concentration on apparent dimerization constants obtained from experimental sedimentation equilibrium distributions for α-chymotrypsin (pH 3.9, I 0.2 M). Solid symbols denote values of $K_2^{app}$ obtained by applying Eq. (7), whereas open symbols signify corresponding estimates from analysis according to Eq. (14), which incorporates an erroneous buoyancy term that was used in the experimental investigations [1,3]. The solid line is the theoretical dependence predicted by Eq. (8).

geometry for all species. A detailed comparison of findings from reanalysis of enzyme distributions in the presence of 0–0.2 M raffinose [3] is precluded by upward curvilinearity of the dependence of ln $K_2^{app}$ upon saccharide concentration (an exacerbation of a trend already evident in the original plot [3]). What is therefore required is the slope of the limiting tangent to the dependence as $C_M \rightarrow 0$. To that end the revised estimate of 12 L/mol for dln $K_2^{app}/dC_M$ inferred from the apparent dimerization constant at lowest raffinose concentration (0.05 M) is again reasonably close to the predicted slope of 11.1 L/mol that stems from excluded-volume considerations.

## 4. Concluding remarks

In summary, the present study has established the theoretical validity of apparent dimerization constants obtained by omega analysis [9] in the original sedimentation equilibrium study [1] of α-chymotrypsin in the presence of sucrose (*M*), despite the use of an incorrect buoyancy term in their determination. However, the use of that erroneous buoyancy term in a subsequent investigation of the same system [3] has led to underestimation of $K_2^{app}$ in the presence of various saccharidic cosolutes (including sucrose) because of the different method (psi analysis [4]) employed to analyze the sedimentation equilibrium distributions. We therefore conclude that the disparities between experimental values of $d\ln K_2^{app}/dC_M$ in the later study [3] and predictions based on excluded-volume calculations reflect this error in analysis rather than nonconformity with the behaviour predicted by the statistical-mechanical treatment of thermodynamic nonideality [2] arising from molecular crowding by the cosolute.

## Acknowledgements

Research in the Pielak laboratory is supported by a U.S. National Institutes of Health Pioneer Award (5DPIOD783) as well as grants from the U.S. National Science Foundation (MCB 0516547) and the Petroleum Research Fund of the American Chemical Society (42748-AC4).

## References

[1] K.E. Shearwin, D.J. Winzor, Effect of sucrose on the dimerization of α-chymotrypsin: allowance for thermodynamic nonideality arising from the presence of a small inert cosolute, Biophys. Chemist. 31 (1988) 287–294.
[2] W.G. McMillan, J.E. Mayer, The statistical thermodynamics of multicomponent systems, J. Chem. Phys. 13 (1945) 276–305.
[3] C.N. Patel, S.M. Noble, G.T. Weatherly, A. Tripathy, D.J. Winzor, G.J. Pielak, Effects of molecular crowding by saccharides on α-chymotrypsin dimerization, Protein Sci. 11 (2002) 997–1003.
[4] P.R. Wills, M.P. Jacobsen, D.J. Winzor, Direct analysis of solute self-association by sedimentation equilibrium, Biopolymers 38 (1996) 119–130.
[5] D.J. Winzor, M. Deszczynski, S.E. Harding, P.R. Wills, Nonequivalence of second virial coefficients from sedimentation equilibrium and static light scattering studies of protein solutions, Biophys. Chemist. 128 (2007) 46–55.
[6] M.P. Jacobsen, P.R. Wills, D.J. Winzor, Thermodynamic analysis of the effects of small inert cosolutes in the ultracentrifugation of noninteracting proteins, Biochemistry 35 (1996) 13173–13179.
[7] E.F. Casassa, H. Eisenberg, Thermodynamics of multicomponent solutions, Adv. Protein Chem. 19 (1964) 287–395.
[8] M. Deszczynski, S.E. Harding, D.J. Winzor, Negative second virial coefficients as predictors of protein crystal growth: evidence from sedimentation equilibrium studies that refutes the designation of those light scattering parameters as osmotic virial coefficients, Biophys. Chemist. 120 (2006) 106–113.
[9] B.K. Milthorpe, P.D. Jeffrey, L.W. Nichol, Direct analysis of sedimentation equilibrium results with polymerizing systems, Biophys. Chemist. 3 (1975) 169–176.