Interpretation of the Reversible Inhibition of Adenosine Deaminase by Small Cosolutes in Terms of Molecular Crowding†

Thierry G. A. Lonhienne and Donald J. Winzor*

Department of Biochemistry, School of Molecular and Microbial Sciences, University of Queensland, Brisbane, Queensland 4072, Australia

Received April 26, 2001; Revised Manuscript Received June 14, 2001

ABSTRACT: Published results on the inhibitory effects of small cosolutes on adenosine deamination by adenosine deaminase [Kurz, L. C., Weitkamp, E., and Frieden, C. (1987) *Biochemistry 26*, 3027−3032; Dzingeleski, G., and Wolfenden, R. (1993) *Biochemistry 32*, 9143−9147] have been reexamined. Results for sucrose, dioxane, methanol, and ethanol are shown to be qualitatively consistent with thermodynamic interpretation in terms of molecular crowding effects arising from the occurrence of a minor increase in enzyme volume and/or asymmetry during the kinetic reaction—a conformational transition that could be either preexisting or ligand induced. Direct evidence for the existence of the putative isomeric transition is provided by active enzyme gel chromatography on Sephadex G-100, which demonstrates a negative dependence of enzyme elution volume upon substrate concentration and is therefore consistent with substrate-mediated conformational changes that favor a larger (or more asymmetric) isomeric state of the enzyme. There are thus experimental grounds for adopting the present description of the inhibitory effects of unrelated cosolutes on the kinetics of adenosine deamination by adenosine deaminase in terms of thermodynamic nonideality.

The hydrolytic activity of calf-intestinal adenosine deaminase is decreased by high concentrations of small cosolutes such as sucrose, dioxane, methanol, ethanol, and acetonitrile (*1*, *2*). Although the initial observation of the phenomenon with sucrose was ascribed to a viscosity effect (*1*), that interpretation was precluded subsequently by use of other cosolutes as viscogenic agents (*2*). Instead, the reversible inhibitory effect was attributed to hypersensitivity of the enzyme to solvent water—a conclusion based on a correlation between the logarithm of k_c/K_m and water concentration. However, a difficulty with that interpretation is the experimental observation that molar concentration rather than molar thermodynamic activity of water seems to govern the kinetics of enzyme catalysis (*2*).

In the search for a more plausible explanation of the experimental findings, we have explored the possibility that the cosolute-dependent impairment of catalysis by adenosine deaminase is merely the consequence of thermodynamic nonideality arising from changes in the distribution of enzyme between different isomeric states. As demonstrated elsewhere (*3*−*10*), thermodynamic nonideality as the result of molecular crowding by small cosolutes affords a powerful means for detecting the coexistence of different conformational states of a protein.

EXPERIMENTAL SECTION

Materials. Calf-intestinal adenosine deaminase and adenosine were used as supplied by Sigma Chemical Co. The enzyme was dialyzed against phosphate-chloride buffer (0.025 M NaH_2PO_4−0.026 M Na_2HPO_4−0.047 M NaCl), pH 6.9, I 0.15. Concentrations of enzyme in the dialyzed solutions were determined spectrophotometrically at 278 nm on the basis of an absorption coefficient ($A^{1\%}_{1cm}$) of 8.15 (*11*) and a molecular mass of 40.6 kDa (*12*).

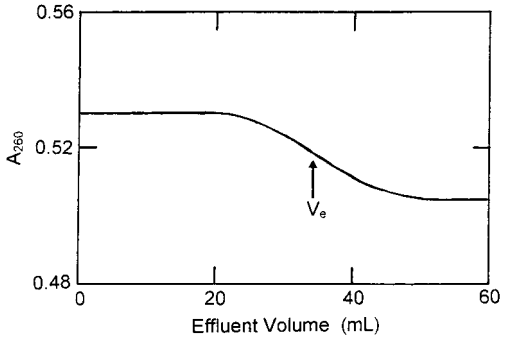

FIGURE 1: Elution profile obtained in active enzyme gel chromatography (*13*) of adenosine deaminase (100 μL, 12 nM) in phosphate-chloride buffer (pH 6.9, *I* 0.15) on a column of Sephadex G-100 (2.0 × 19.7 cm) preequilibrated with 40 μM adenosine in the same buffer. V_e denotes the elution of the enzyme zone.

Active Enzyme Gel Chromatography. In active enzyme gel chromatography experiments (*13*), an aliquot (100 μL, 12 nM) of dialyzed adenosine deaminase was applied to a column of Sephadex G-100 (2.0 × 19.7 cm) preequilibrated with solutions of adenosine (15−300 μM) in the diffusate from the above dialysis step. Elution was effected with the same adenosine-supplemented diffusate at a flow-rate of 0.63 mL/min and the column effluent monitored at 260 nm. The elution volume of enzyme was taken as the midpoint of the gradient in absorbance that resulted from adenosine deamination during passage of the enzyme zone through the column (Figure 1).

† This investigation was supported by a grant from the Australian Research Council.

* To whom correspondence should be addressed. Phone: +61-7-3365 4863. Fax: +61-7-3365 4699. E-mail: winzor@biosci.uq.edu.au.

10.1021/bi010857o CCC: $20.00
Published on Web 07/10/2001

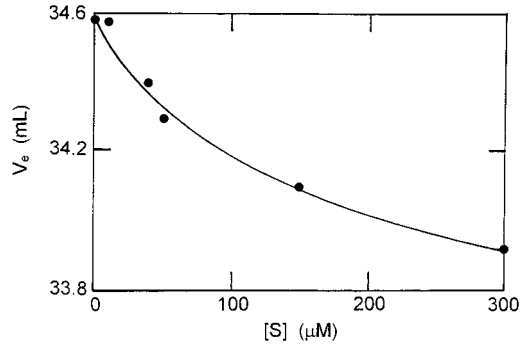

FIGURE 2: Effect of substrate concentration on the elution volume of adenosine deaminase obtained by active enzyme gel chromatography (*13*) on a column (2.0 × 19.7 cm) of Sephadex G-100 preequilibrated with the indicated concentration of adenosine in phosphate-chloride buffer (pH 6.9, *I* 0.15).

RESULTS AND DISCUSSION

Experimental results have been published on the effects of several small cosolutes on the kinetics of adenosine deamination by adenosine deaminase (*1*, *2*). Those results are to be reinterpreted in the light that the observed inhibition reflects molecular crowding of an enzyme conformational equilibrium somewhere within the catalytic pathway. Because such effects of thermodynamic nonideality can only displace an isomerization toward the smaller form of the enzyme, catalytic activity would necessarily be associated with an expanded conformational state of adenosine deaminase. We begin by seeking independent experimental evidence of the putative enzyme isomerization.

The effect of substrate concentration upon enzyme elution volume in active-enzyme gel chromatography (*13*) is summarized in Figure 2, which clearly establishes the existence of a systematic decrease in elution volume of the enzyme with increasing substrate concentration. This evidence of a substrate-mediated increase in Stokes radius would be in keeping with the displacement of either a preexisting or a substrate-induced enzyme transition in favor of the larger conformational state as the result of substrate binding. There are therefore some experimental grounds for considering the inhibitory effects of unrelated cosolutes on the kinetics of adenosine deamination to be a consequence of thermodynamic nonideality on an equilibrium involving different conformational states of adenosine deaminase. Unfortunately, further testing of this postulate by additional active enzyme gel chromatography experiments to demonstrate a cosolute effect on the elution volume of adenosine deaminase is precluded by cosolute-mediated changes is the swelling characteristics of Sephadex G-100.

To cover the possibility that the isomerization may be either preexisting or substrate-induced, the enzyme catalyzed conversion of small substrate, S, to product, P, is considered to occur via the mechanism (*7*)

$$E^* \overset{Y}{\rightleftarrows} E + S \overset{K}{\rightleftarrows} ES \overset{X}{\rightleftarrows} ES^{\dagger} \overset{k}{\rightarrow} E + P \qquad (1)$$

It is assumed that the rate constant governing product formation (k) is rate determining, in which case the various enzyme and enzyme−substrate species are in a state of equilibrium governed by the indicated thermodynamic constants: $Y = [E^*]/[E]$, $K = [ES]/([E][S])$, $X = [ES^{\dagger}]/[ES]$. Under the thermodynamically ideal conditions that apply to the kinetics of catalysis in the absence of cosolute, the conventional rate parameter (k_c) and Michaelis constant (K_m) are defined in terms of the above mechanism by the relationships

$$k_c = kX/(1 + X) \qquad (2a)$$

$$K_m = (1 + Y)/[K(1 + X)] \qquad (2b)$$

The inclusion of an inert cosolute introduces thermodynamic nonideality into the three reversible steps, the equilibrium positions of which tend to be displaced toward the smaller isomeric state of the enzyme or enzyme−substrate species.

Because enzyme kinetic experiments are performed under conditions of constant temperature and pressure, the equilibria should be described in terms of the molal concentration (m_i) and molal activity coefficient (y_i) of each participating species, i (*14*). In the experiments under consideration the extremely low enzyme concentration ensures that the activity coefficient is dominated by the term in cosolute concentration (*7*), which then assumes the form $\exp(B_{i,M} - M_M\bar{v}_M)m_M\rho_s$ where ρ_s is the solvent density, $B_{i,M}$ the second osmotic virial coefficient describing physical interaction of species i with cosolute, and $M_M\bar{v}_M$ the cosolute partial molar volume (*15*). The effect of a concentration m_M of inert cosolute on the three reversible interactions in the reaction scheme may thus be described in terms of the following apparent equilibrium constants (*15*)

$$X_{app} = X \exp[(B_{ES,M} - B_{ES^{\dagger},M})m_M\rho_s + ...] \qquad (3a)$$

$$Y_{app} = Y \exp[(B_{E,M} - B_{E^*,M})m_M\rho_s + ...] \qquad (3b)$$

$$K_{app} = Ky_S \exp[(B_{E,M} - B_{ES,M})m_M\rho_s + ...] \qquad (3c)$$

For the present system with an uncharged cosolute the second virial coefficient is simply the covolume of species i and cosolute: that is, $B_{i,M} = 4\pi N(R_i + R_M)^3/3$ for spherical macromolecular and cosolute species with radii R_i and R_M, respectively. A change in enzyme size (volume) as the result of either a substrate-induced conformational change ($B_{ES,M} \neq B_{ES^{\dagger},M}$) or a preexisting isomerization ($B_{E,M} \neq B_{E^*,M}$) thus has the potential to alter the magnitude of the relevant apparent equilibrium constant in the presence of cosolute from its thermodynamic value. Inasmuch as the binding of a small substrate to the enzyme should lead to no detectable change in size ($R_E = R_{ES}$; $B_{E,M} = B_{ES,M}$), the only effect of cosolute on the apparent association equilibrium constant for ES formation (K_{app}) stems from any change in the activity coefficient of substrate, y_S—a parameter that is taken as unity to simplify the present illustrative analysis.

In the presence of cosolute the expressions for k_c and K_m need to be written in terms of X_{app} and Y_{app}, whereupon

$$(k_c)_M/k_c = \frac{(1 + X)\exp[(B_{ES,M} - B_{ES^{\dagger},M})m_M\rho_s + ...]}{1 + X\exp[(B_{ES,M} - B_{ES^{\dagger},M})m_M\rho_s + ...]} \qquad (4a)$$

$$(K_m)_M/K_m = \frac{(1 + X)\{1 + Y\exp[(B_{E,M} - B_{E^*,M})m_M\rho_s + ...]\}}{(1 + Y)\{1 + X\exp[(B_{ES,M} - B_{ES^{\dagger},M})m_M\rho_s + ...]\}} \qquad (4b)$$

The first point to note in relation to the experimental kinetic data for adenosine deaminase is the independence of

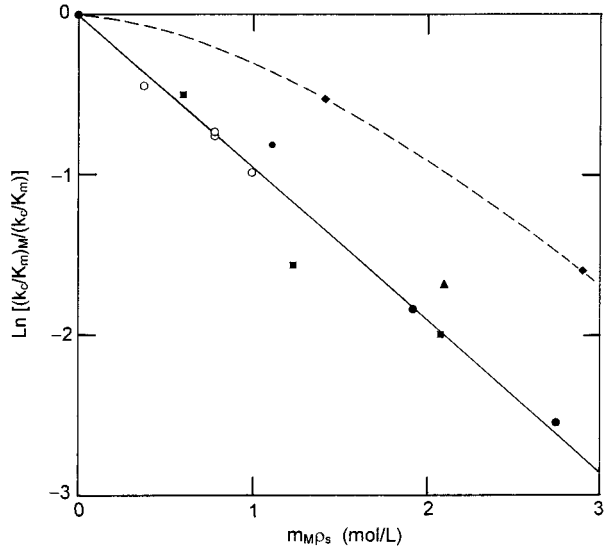

FIGURE 3: Effect of inert cosolutes on the magnitude of k_c/K_m for the deamination of adenosine by adenosine deaminase, the results being plotted in accordance with the logarithmic form of eq 5: (○, ●) sucrose; (■) dioxane; (▲) ethanol; (◆) methanol. Open symbols refer to results inferred from Figure 1 of Kurz et al. (*1*), whereas closed symbols refer to data inferred from Figure 2 of Dzingeleski and Wolfenden (*2*).

k_c upon cosolute concentration (*2*). Although concentration dependence of k_c was reported in the other study (*1*), that conclusion was predominantly the consequence of the measured maximal velocity at the highest solute concentration. As noted previously (*6*, *7*), the absence of any term in Y in eq 4a signifies that independence of $(k_c)_M$ upon cosolute concentration is a feature of situations where the enzyme isomerization is preexisting. On the other hand, substitution of the condition $X \gg 1$ into eq 4a shows that invariance of $(k_c)_M$ also occurs in situations where a substrate-induced conformational change is governed by a very large isomerization constant (*15*).

The experimental dependencies of ln $[(k_c/K_m)_M/(k_c/K_m)]$ upon $m_M\rho_s$ (*1*, *2*) are summarized in Figure 3 for sucrose, dioxane, methanol, and ethanol: solid symbols denote results obtained by Dzingeleski and Wolfenden (*2*), whereas the open symbols refer to the original observations with sucrose as cosolute (*1*). Only data for cosolute concentrations below 3 molal have been included because the statistical-mechanical treatment is being restricted to consideration of nearest-neighbor interactions (i.e., to consideration of the consequences of second virial coefficient terms). Any attempt to interpret these results on the statistical-mechanical basis of excluded volume must rationalize the magnitude of the slope of the common dependence for sucrose and dioxane, and also the obvious disparity of the slope for methanol.

Substitution of the condition for a substrate-induced isomerization ($Y = 0$) in eq 4b leads to the expression

$$[(k_c/K_m)_M/(k_c/K_m)] = \exp[(B_{ES,M} - B_{ES^{\dagger},M})m_M\rho_s + ...] \quad (5a)$$

On the other hand, for a system with $X = 0$ [whereupon $(k_c)_M = k_c$] the corresponding expression is

$$[(k_c/K_m)_M/(k_c/K_m)] = [1 + Y\exp[(B_{E^*,M} - B_{E,M})m_M\rho_s + ...]/(1 + Y) \cong \exp[(B_{E^*,M} - B_{E,M})m_M\rho_s + ...] \quad (5b)$$

in which the approximate form reflects the assumption that Y is large enough ($Y \gg 1$) for substrate addition to cause observable displacement of the preexisting equilibrium in favor of the active isomer. For either situation (eq 5a or 5b), an initially linear dependence of the logarithm of $[(k_c/K_m)_M/(k_c/K_m)]$ upon $m_M\rho_s$ is predicted, the slope of the common dependence for sucrose and dioxane describing the covolume difference for cosolute and the isomeric pair involved in the relevant conformational equilibrium. From the results for sucrose and dioxane shown in Figure 2, a magnitude of −0.95 L/mol may thus be assigned either to $(B_{ES,M} - B_{ES^{\dagger},M})$ or to $(B_{E^*,M} - B_{E,M})$.

To interpret the magnitude of this difference in terms of the extent of the conformational expansion, we need an estimate of $B_{E,M} = B_{ES,M}$, the covolume for cosolute and enzyme prior to substrate-induced isomerization, or, for a preexisting isomerization, of $B_{E^*,M}$, the corresponding parameter for the predominant enzyme species in the absence of substrate. An effective thermodynamic radius, R_M, of 0.32 nm has been obtained from isopiestic and freezing-point depression measurements on sucrose (*16*), and we shall assume that the Stokes radius of enzyme in the absence of substrate provides an adequate value of either $R_E = R_{ES}$ (for substrate-induced isomerization) or R_{E^*} (for preexisting enzyme isomerization). On the basis of the Stokes radius of 2.3 nm reported for human adenosine deaminase (*17*), the enzyme-sucrose covolume prior to expansion of the enzyme is 45.4 L/mol: the corresponding covolume for sucrose and the expanded form of enzyme is therefore 46.4 L/mol. On the ground that $(R_{ES^{\dagger}} + R_M)$ or $(R_E + R_M)$ is therefore 2.64 nm, the radius of the enzyme has increased from 2.30 to 2.32 nm (a 1% increase in radius or 3% increase in volume). Such a change in the effective spherical volume of the hydrated enzyme may simply reflect a shape change (an increased asymmetry) and, therefore, may not be identified unequivocally as an increase in the actual volume of the solvated enzyme molecule.

This analysis of the kinetic results (Figure 3) in terms of thermodynamic nonideality has indicated that the addition of substrate gives rise to a relatively small increase in enzyme asymmetry and/or volume, irrespective of whether the conformational change is substrate-induced ($B_{ES^{\dagger},M} > B_{ES,M}$; $R_{ES^{\dagger}} > R_{ES}$) or preexisting ($B_{E,M} > B_{E^*,M}$, $R_E > R_{E^*}$). A thermodynamic interpretation of the results shown in Figure 3 is therefore that molecular crowding by the cosolute is displacing an enzyme isomerization equilibrium in favor of a smaller (or more symmetrical) state—in a direction counter to catalysis.

Although the kinetic results obtained with methanol are amenable to similar qualitative interpretation, the disparity from those for sucrose and dioxane requires comment because the likely differences between R_M for sucrose, dioxane, and methanol do not lead to any effective change in the slope of Figure 3 predicted by eq 5a or 5b. In that regard, the above analysis is based on the premise that the cosolute is chemically inert. Evidence for violation of that requirement by methanol and ethanol comes from examination of their self-interaction behavior by analysis of freezing point depression data (*16*). On the basis that the latent heat of fusion is sufficiently independent of temperature over the relatively small range covered by the freezing point measurements to allow its expression in terms of the freezing point

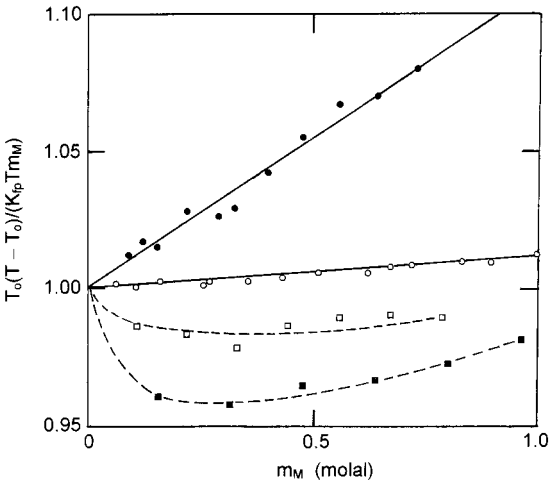

FIGURE 4: Analysis of freezing point depression data for sucrose (●), dioxane (○), ethanol (□), and methanol (■) in terms of eq 6 to establish the validity of their thermodynamic consideration as chemically inert, impenetrable spheres. A positive linear dependence is symptomatic of chemical inertness, whereas a negative dependence signifies either solute self-association or its interaction with solvent.

depression constant (K_{fp}), the covolume for self-interaction of cosolute, $B_{M,M}$, is given by the relationship

$$T_o(T - T_o)/(K_{fp}Tm_M) = 1 + (B_{M,M} - M_M\bar{v}_M)m_M\rho_s + ... \quad (6)$$

where T_o and T are the respective freezing points of solvent and cosolute solution with molal concentration m_M: the presence of the partial molar volume term, $M_M\bar{v}_M$, reflects the conversion of a molal second virial coefficient to its osmotic counterpart (*14*, *16*). Results for sucrose and dioxane as well as for methanol and ethanol are presented in Figure 4, where the freezing point data for dioxane have been inferred from a study by Scatchard and Benedict (*18*). Those for the other two cosolutes have been taken from the Handbook of Physics and Chemistry, which was also the source of the value of 1.860 that has been used for K_{fp}. It is evident that the results for dioxane, like those for sucrose (*16*), exhibit the positive concentration dependence that is commensurate with the positive deviations from Raoult's Law that characterize the excluded-volume interactions of a chemically inert solute. On the other hand, the negative deviations from Raoult's Law that may be inferred from the data for methanol and ethanol presumably signify their interaction with solvent (water)—a factor which precludes their consideration as chemically inert solutes from the viewpoint of their molecular crowding effects in terms of eq 5a or 5b. Although the only point available for the effect of ethanol on adenosine deamination in the range covered by Figure 3 (▲) could be taken to conform with the behavior ascribed to sucrose and dioxane, its intermediate position between the two dependencies drawn in Figure 2 would also be consistent with the fact that the negative deviation from Raoult's Law is less extensive for ethanol than for methanol (Figure 4).

In summary, the results for all three cosolutes examined in Figure 3 are qualitatively consistent with interpretation as effects of thermodynamic nonideality arising from the occurrence of a minor increase in enzyme volume and/or asymmetry during either of the isomerization steps in the catalytic reaction scheme (eq 1). Furthermore, the equilibrium coexistence of the enzyme in two conformational states is indicated by the results presented in Figure 2, which also identify the larger isomer as the enzymically active conformational state.

An unresolved question is whether expansion of the enzyme occurs during an isomerization that is preexisting ($E^* \rightleftarrows E$) or one that is substrate-induced ($ES \rightleftarrows ES^\dagger$). In that regard, the finding that the effect of methanol on the K_I for nebularine parallels the corresponding dependence for K_m for substrate (*2*) would be more readily reconciled with preexistence of the conformational transition. However, the reported independence of K_I upon sucrose concentration for several competitive inhibitors of adenosine deaminase (*1*) is contrary to such a proposition. Direct experimental distinction between preexistence and substrate-induction of the conformational transition is difficult to obtain in situations where any preexisting equilibrium would heavily favor the smaller conformational state.

CONCLUDING REMARKS

By identifying the probable source of the inhibitory effects of small cosolutes on the kinetics of adenosine deamination by adenosine deaminase, this investigation has again emphasized the utility of thermodynamic nonideality as a probe of conformational changes in enzyme reactions. Even though almost two decades have elapsed since the use of cosolutes (macromolecular as well as small) was suggested as a potential means for exploring the existence of such isomerization (*19*, *20*), very little advantage has been taken of the approach. It is hoped that the successful outcome to this study of the adenosine deaminase system may convince others that there are relatively simple ways of obtaining experimental evidence for the existence of the putative substrate-mediated conformational changes that are often invoked to account for the kinetics of enzyme catalysis.

ACKNOWLEDGMENT

We gratefully acknowledge the informative discussions with Drs. Linda Kurz, Carl Frieden, and Richard Wolfenden that drew the attention of D.J.W. to the controversy surrounding the adenosine deaminase system.

REFERENCES

1. Kurz, L. C., Weitkamp, E., and Frieden, C. (1987) *Biochemistry 26*, 3027−3032.
2. Dzingeleski, G., and Wolfenden, R. (1993) *Biochemistry 32*, 9143−9147.
3. Winzor, D. J., and Wills, P. R. (1986) *Biophys. Chem. 25*, 243−251.
4. Shearwin, K. E., and Winzor, D. J. (1988) *Arch. Biochem. Biophys. 260*, 532−539.
5. Harris, S. J., and Winzor, D. J. (1988) *Arch. Biochem. Biophys. 265*, 458−465.
6. Bergman, D. A., and Winzor, D. J. (1989) *J. Theor. Biol. 137*, 171−189.
7. Bergman, D. A., Shearwin, K. E., and Winzor, D. J. (1989) *Arch. Biochem. Biophys. 274*, 55−63.
8. Shearwin, K. E., and Winzor, D. J. (1990) *Arch. Biochem. Biophys. 282*, 297−301.

9. Davis-Searles, P. R., Morar, A. S., Saunders, A. J., Erie, D. A., and Pielak, G. J. (1998) *Biochemistry 37*, 17048−17053.
10. Saunders, A. J., Davis-Searles, P. R., Allen, D. L., Pielak, G. J., and Erie, D. A. (2000) *Biopolymers 53*, 293−307.
11. Pfrogner, N. (1967) *Arch. Biochem. Biophys. 159*, 147−154.
12. Kelly, M. A., Vestling, M. M., Murphy, C. M., Hua, S., Sumpter, T., and Fenselau, C. (1996) *J. Pharm. Biomed. Anal. 14*, 1513−1519.
13. Ward, L. D., and Winzor, D. J. (1982) *Arch. Biochem. Biophys. 216*, 329−336.
14. Wills, P. R., Comper, W. D., and Winzor, D. J. (1993) *Arch. Biochem. Biophys. 300*, 206−212.
15. Winzor, D. J., and Wills, P. R. (1995) *Biophys. Chem. 57*, 103−110.
16. Winzor, D. J., and Wills, P. R. (1995) in *Protein−Solvent Interactions* (Gregory, R. B., Ed.) pp 483−520, Marcel Dekker, New York.
17. Van der Weyden, M. B., and Kelley, W. N. (1976) *J. Biol. Chem. 251*, 5448−5456.
18. Scatchard, G., and Benedict, M. A. (1936) *J. Am. Chem. Soc. 58*, 837−842.
19. Nichol, L. W., Sculley, M. J., Ward, L. D., and Winzor, D. J. (1983) *Arch. Biochem. Biophys. 222*, 574−581.
20. Winzor, D. J., Ford, C. L., and Nichol, L. W. (1984) *Arch. Biochem. Biophys. 234*, 15−23.

BI010857O