Biophysical Chemistry, 31 (1988) 287–294
Elsevier



BPC 01302

# Effect of sucrose on the dimerization of α-chymotrypsin

## Allowance for thermodynamic nonideality arising from the presence of a small inert solute

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Received 5 February 1988
Revised manuscript received 13 May 1988
Accepted 7 June 1988

Thermodynamic nonideality; Excluded volume; α-Chymotrypsin dimerization; Sucrose activity coefficient

The space-filling effects of sucrose on the dimerization of α-chymotrypsin have been investigated by sedimentation equilibrium studies on the enzyme in acetate-chloride buffer, pH 3.9, $I$ 0.2. From the extent of enhancement of the apparent dimerization constant in the presence of 0.05–0.16 M sucrose, it is concluded that this effect of thermodynamic nonideality finds quantitative explanation in terms of excluded volume. However, the suggested approximation that the radius of an inert small solute would be sufficiently small to be neglected in the calculation of covolumes (D.J. Winzor and P.R. Wills, Biophys. Chem. 25 (1986) 243) has not withstood the more stringent test afforded by the present study of α-chymotrypsin dimerization. A value of 0.34 nm for the effective thermodynamic radius of sucrose was inferred from the covolume for self-interaction obtained by frontal gel chromatography on Sephadex G-10 under the conditions of the ultracentrifugal studies. Finally, results of sedimentation equilibrium experiments on α-chymotrypsin in the presence of 0.1 M glycerol were also shown to be consistent with interpretation in terms of the model of space-filling effects entailing complete exclusion of small solute from the hydrated protein domain.

## 1. Introduction

Studies of the effects of inert macromolecular solutes on protein interactions have led to the prediction and demonstration that self-association of proteins and enzymes is enhanced by the presence of these solutes [1–5]. Furthermore, this enhancement of protein self-association is amenable to quantitative interpretation by statistical-mechanical application of the excluded volume concept [6–9]. Although effects of small solutes have traditionally been considered to reflect preferential solvation [10–14], they also find equivalent interpretation in terms of excluded volume [15]. Such analysis has the advantage of making possible their quantitative specification in terms of composition-dependent activity coefficients for the interacting species. In the two considerations of thermodynamic nonideality arising from the presence of sucrose [15,16], the approximation was made that the radius of the small solute contributed negligibly to the covolume radius, whereupon the hydrated volumes of the interacting species (isomers in both instances) were substituted for the covolumes in the quantitative interpretation of space-filling effects. However, analysis of published data [17] on the concentration dependence of the thermodynamic activity of sucrose in terms of a second virial coefficient did lead to a finite value for the apparent radius of sucrose (0.27 nm) that was only 56% of its hydrodynamic counterpart [15]. Since this radius of 0.27

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nm is clearly only an empirical value derived for the description of thermodynamic nonideality due to self-interaction of sucrose, the question at issue is whether it should also be applied for predicting excluded volume effects of sucrose on macromolecular interactions. The system chosen for study is the dimerization of α-chymotrypsin in acetate-chloride buffer, pH 3.9, $I$ 0.2, conditions under which the self-association is sufficiently strong [18–21] for experiments to be restricted to an enzyme concentration range of 0–1 mg/ml, and hence conditions under which thermodynamic nonideality arising from enzyme-enzyme covolume effects may justifiably be ignored.

The present investigation has entailed a series of sedimentation equilibrium experiments, of meniscus-depletion design [22], in the presence of 0–0.16 M sucrose. In addition, the second virial coefficient and hence apparent thermodynamic radius of sucrose under the present conditions (pH 3.9, $I$ 0.2) has been determined by frontal gel chromatography on Sephadex G-10, in accordance with a procedure described previously [23].

## 2. Experimental

### 2.1. Preparation of α-chymotrypsin solutions

α-Chymotrypsin (three-times crystallized, salt-free and freeze-dried) was obtained from Worthington Biochemical Corp. (Freehold, NJ). Solutions were prepared by direct dissolution of the enzyme in acetate-chloride buffer, pH 3.9, $I$ 0.2 (0.18 M sodium chloride-0.02 M sodium acetate, pH adjusted with acetic acid), supplemented with the required concentration (0–0.16 M) of reagent-grade sucrose: glycerol (0.1 M) was substituted for sucrose in one series of experiments. Any autolysis fragments were removed by zonal gel chromatography of the enzyme solution (1.5 ml, 20 mg/ml) on a column of Sephadex G-75 (2.4 × 16.5 cm) preequilibrated with the same buffer, the high concentration of α-chymotrypsin being used in these experiments to enhance dimerization of the enzyme and hence improve its separation from slower-migrating autolysis fragments [21]. Material from the leading half of the eluted peak was considered to comprise reversibly dimerizing α-chymotrypsin in dialysis equilibrium with the pre-equilibrating acetate-chloride buffer, pH 3.9, $I$ 0.2. An absorption coefficient ($A^{1\%}_{1\,cm}$) of 20.1 at 280 nm [24] was used for spectrophotometric determination of α-chymotrypsin concentration.

### 2.2. Equilibrium sedimentation

Solutions of α-chymotrypsin prepared as described above were diluted to approx. 1 mg/ml with the preequilibrating acetate-chloride-sucrose buffer, pH 3.9, $I$ 0.2, and then subjected to ultracentrifugation for 24 h at 20 °C and rotor speeds of 22 000–28 000 rpm in a Beckman model E ultracentrifuge fitted with electronic speed control. The solute distributions in these high-speed [22] sedimentation equilibrium experiments were recorded as Rayleigh interferograms, which were measured on a Nikon two-dimensional comparator. After conversion of the fringe displacements to protein concentrations on the basis of a specific refractive increment of 0.186 ml/g at 546 nm [25,26], the $[\bar{c}(r), r]$ data were analyzed with the $\Omega$ function [27] using values of 25 000 for the monomeric molecular weight [28] and 0.736 ml/g for the partial specific volume [29]. An Anton Paar precision density meter was used for the determination of buffer densities at 20 °C.

### 2.3. Calculation of activity coefficients for macromolecular species

The activity coefficient, $y_i$, of species $i$ may be described in terms of a second virial coefficient, $\alpha_{ij}$, via the expression [7]

$$y_i = \exp\left\{\sum(\alpha_{ij} m_j)\right\} \tag{1}$$

where $j$ encompasses all species (including $i$), and

$$\alpha_{ij} = U_{ij} - M_j \bar{v}_j + \frac{Z_i Z_j (1 + \kappa r_i + \kappa r_j)}{2I(1 + \kappa r_i)(1 + \kappa r_j)} \tag{2}$$

$U_{ij}$ is the covolume of species $i$ and $j$ (both of which are assumed to be spherical), the second term denotes the anhydrous molar volume of $j$, and the third describes charge-charge interactions

in conventional nomenclature [7]. In the present experimental design the concentration of sucrose, $m_M$, is much greater than the concentrations of monomeric ($m_A$) and dimeric ($m_C$) α-chymotrypsin, and hence terms containing concentrations of A and C assume negligible proportions in the expression for $Y_i$ (eq. 1). Furthermore, the fact that sucrose is uncharged ($Z_M = 0$) eliminates any charge-charge contribution to $\alpha_{iM}$, whereupon

$$\alpha_{iM} = U_{iM} - M_M\bar{v}_M \tag{3a}$$

For sucrose the anhydrous partial molar volume ($M_M\bar{v}_M$) is 0.21 l/mol [11], and it therefore remains to evaluate the two covolumes from the expression

$$U_{iM} = 4\pi N(r_i + r_M)^3/3 \tag{3b}$$

where $r_i$ and $r_M$ denote the hydrated radii of $i$ (A or C) and sucrose, respectively. Although $r_A$ and $r_C$ are thermodynamic rather than hydrodynamic quantities [30], the absence of values for the former has led to the use of Stokes radii, an approximation for which there is some experimental support in the case of two proteins, ovalbumin [31,32] and hemoglobin [32].

Combination of the sedimentation coefficient ($s^\circ_{20,w}$) of 2.4 S for monomeric α-chymotrypsin [33] with its molecular weight of 25000 [28] and partial specific volume of 0.736 ml/g [29] leads to a value of 2.44 nm for $r_A$ on the basis of spherical geometry. Consideration of dimeric α-chymotrypsin to be a sphere with twice the volume ($r_C = 2^{1/3}r_A$) then gives a value of 3.07 nm for the hydrated radius of C. For small solutes the effective thermodynamic radius differs considerably from its Stokes counterpart [15], and accordingly must be evaluated experimentally.

### 2.4. Gel chromatography of sucrose on Sephadex G-10

In order to evaluate the second virial coefficient for self-interaction of sucrose under the conditions used in the sedimentation equilibrium studies (pH 3.9, $I$ 0.2), the concentration dependence of the weight-average elution volume of sucrose was determined by frontal gel chromatography [34] on a column (2.5 × 21.6 cm) of Sephadex G-10. To quantify the extent of any change in column volume due to osmotic shrinkage of the gel beads [35], the height of the column was routinely monitored by means of a cathetometer, and the void volume (taken as the elution volume of bovine serum albumin) determined for each run. In retrospect, this decrease in volume amounted to less than 0.5% and was accordingly neglected.

Solutions (55 ml) comprising sucrose (0.02–0.35 M) and bovine serum albumin (1 mg/ml) in acetate-chloride buffer (pH 3.9, $I$ 0.2) were applied to the column at a flow rate of 0.48 ml/min, the column effluent being collected as 1.4-ml fractions in previously tared tubes. Each tube was reweighed to determine the precise weight of the eluate fraction, whose volume and sucrose concentration were then determined by density measurement in the Anton Paar density meter: the protein concentration of each fraction was assayed spectrophotometrically at 280 nm. To determine the total accessible volume of the Sephadex G-10 column, a boundary in $Cl^-$ was generated by applying the acetate-chloride buffer (pH 3.9, $I$ 0.2) to the column preequilibrated with acetate buffer devoid of sodium chloride (pH 3.9, $I$ 0.02). The elution profile for $Cl^-$ was determined by titrating each fraction with 0.05 M silver nitrate.

The elution volume, $V_M$, obtained from the median bisector of the advancing elution profile, was first converted to a partition coefficient, $\sigma_M$, by means of the relationship [36,37]

$$\sigma_M = (V_M - V_0)/(V_t - V_0) \tag{4}$$

for which the void volume ($V_0$) and total accessible volume ($V_t$) of the column were 43.4 and 70.9 ml, respectively. These partition coefficients, obtained for a series of sucrose concentrations, $m_M$, were then used in conjunction with the expression [23]

$$\sigma_M = \sigma_M^0 \exp\{\alpha_{MM} m_M(1 - \sigma_M)\} \tag{5}$$

to obtain $\sigma_{MM}$ as the slope of a plot of $\ln \sigma_M$ vs. $m_M(1 - \sigma_M)$.

## 3. Results and discussion

### 3.1. Activity coefficient of sucrose (pH 3.9, I 0.2)

A direct plot of results obtained in the frontal gel chromatography study of sucrose on Sephadex G-10 equilibrated with acetate-chloride buffer, pH 3.9, $I$ 0.2, is presented in fig. 1a. In that regard the marginally curvilinear line is based on the ordinate and slope of fig. 1b, a replot of the results in accordance with the logarithmic form of eq. 5: least-squares calculations signify a slope and hence second virial coefficient, $\alpha_{MM}$, of $0.58 \pm 0.16$ l/mol. Combination of this value with the anhydrous molar volume ($M_M\bar{v}_M$) of 0.21 l/mol

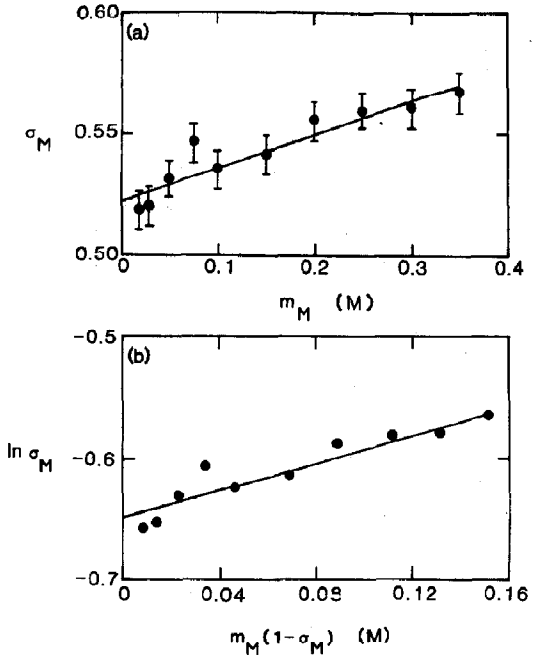


Fig. 1. Evaluation of the second virial coefficient, $\alpha_{MM}$, for self-interaction of sucrose by frontal gel chromatography on a column (2.5×21.6 cm) of Sephadex G-10 equilibrated with acetate-chloride buffer, pH 3.9, $I$ 0.2. (a) Dependence of the partition coefficient upon sucrose concentration. (b) Plot of results in accordance with the logarithmic form of eq. 5, from which values of 0.58 l/mol for the second virial coefficient ($\alpha_{MM}$) and 0.52 for the limiting partition coefficient ($\sigma_M^0$) are obtained from the slope and ordinate intercept, respectively. The curve drawn in panel a is the theoretical relationship predicted by eq. 5 and these parameters.

[11] in eq. 3 yields a covolume for self-interaction of sucrose ($U_{MM}$) of 0.79 l/mol, and a consequent effective thermodynamic radius of 0.34 nm. From isopiestic measurements on sucrose in water [17], the corresponding estimate of this radius is 0.27 nm [15]. There is thus substantial agreement between these two measurements of sucrose activity, under nonidentical conditions and by different means: (i) that the self-interaction of sucrose is satisfactorily described in terms of a second virial coefficient; and (ii) that the value of $\alpha_{MM}$ signifies an effective thermodynamic radius for this small solute that is considerably smaller than its hydrodynamic (Stokes) counterpart of 0.47 nm [38].

### 3.2. Effect of sucrose on the dimerization of α-chymotrypsin

Results of sedimentation equilibrium experiments on α-chymotrypsin in acetate-chloride buffer, pH 3.9, $I$ 0.2 (○), and in the same medium supplemented with 0.15 M sucrose (●) are subjected to $\Omega$ analysis [27] in fig. 2, which clearly indicates a difference between the ordinate intercepts of these plots based on a reference concentration, $\bar{c}(r_F)$, of 0.46 mg/ml: the inset shows that the enhanced dimerization in the presence of sucrose is also readily apparent from plots of the equilibrium distributions in more conventional but less sensitive format. An interesting point arises in this application of the $\Omega$ analysis, which has in the past been used to define the activity of monomer, $a_A(r_F)$, in a mixture of monomeric and polymeric species with total concentration $\bar{c}(r_F)$. As noted in the original report of the analysis [27], the ordinate intercept is defined as

$$\lim_{\bar{c}(r)\to 0} \Omega = \frac{y_A(r_F)[c_A(r_F)/\bar{c}(r_F)]}{y_A(r)[c_A(r)/\bar{c}(r)]} \qquad (6)$$

which, in a system comprising self-associating solute alone, is $a_A(r_F)/\bar{c}(r_F)$ on the grounds that $y_A(r)$ and $c_A(r)/\bar{c}(r)$ both tend to unity as $\bar{c}(r) \to 0$. However, for the present system with a high concentration ($m_M$) of small inert solute (sucrose), $c_A(r)/\bar{c}(r)$ still tends to unity, whereas $y_A(r)$ is governed by the expression (eq. 3)

$$y_A(r) = \exp\{[U_{AM} - M_M\bar{v}_M]m_M(r)\} \qquad (7)$$

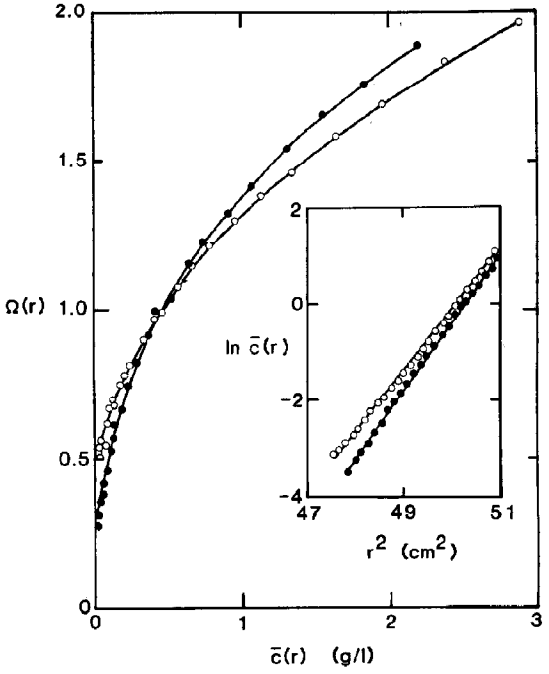


Fig. 2. Quantitative analysis, by means of the $\Omega$ function [27], of sedimentation equilibrium distributions for $\alpha$-chymotrypsin in acetate-chloride buffer, pH 3.9, $I$ 0.2 (○), and in the same buffer supplemented with 0.15 M sucrose (●). The ordinate intercept corresponds to the proportion of monomer present in the reference total enzyme concentrations, $\bar{c}(r_F)$, of 0.46 mg/ml. (Inset) Same results plotted in more conventional format to show the enhanced dimerization of enzyme in the presence of sucrose.

Since the concentration of sucrose varies by only 4% across the liquid column at sedimentation equilibrium, $m_M(r)$ is considered to be essentially constant, whereupon $y_A(r) = y_A(r_F)$, and eq. 6 becomes

$$\lim_{\bar{c}(r)\to 0} \Omega = \frac{c_A(r_F)}{\bar{c}(r_F)} \qquad (8)$$

The different ordinate intercepts in fig. 2 thus reflect an effect of sucrose on the proportion of monomeric enzyme present in the reference concentration (0.46 mg/ml) of $\alpha$-chymotrypsin; and hence an effect of sucrose on the concentration of monomer throughout the entire equilibrium distribution.

A problem inherent in the application of the $\Omega$ analysis is the uncertainty of the extrapolation to the ordinate intercept in order to obtain $c_A(r_F)/\bar{c}(r_F)$ [39]. However, for any value of the intercept and hence of $c_A(r_F)$ it is possible to generate the entire $[c_A(r), \bar{c}(r)]$ distribution, whereupon the apparent dimerization constant may be determined for each radial distance, $r$, from the expression

$$X_{app} = [\bar{c}(r) - c_A(r)]/[c_A(r)]^2 \qquad (9)$$

In view of the uncertainty in the extrapolation, the most appropriate intercept in fig. 2 has therefore been selected as the value which leads to the smallest standard error in $X_{app}$. This procedure, which has in fact been adopted throughout the history of the $\Omega$ analysis, achieves the same end as the nonlinear regression analysis of the $\Omega(r) - \bar{c}(r)$ plot recommended by Morris and Ralston [39] to improve the definition of the ordinate intercept.

The dependence of $X_{app}$ for $\alpha$-chymotrypsin upon sucrose concentration ($m_M$) is presented (●), in semilogarithmic format, in fig. 3, where the solid line denotes the best-fit linear relationship

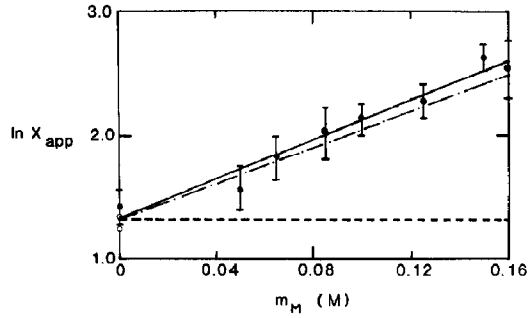


Fig. 3. Effect of sucrose concentration upon the apparent dimerization constant for $\alpha$-chymotrypsin in acetate-chloride buffer, pH 3.9, $I$ 0.2: the solid symbols denote the present results, whereas the open symbols refer to published equilibrium constants [18,21] under these conditions in the absence of sucrose. (———) Best-fit description of the results obtained by least-squares calculations, and also the relationship predicted on the basis of spherical geometry and a radius of 2.44 nm for monomeric enzyme: (·—·—·) relationship calculated [40,41] by consideration of the dimer to be prolate ellipsoid with axial ratio of 2: (- - - - -) plot predicted for both models by eq. 11b with neglect of the sucrose radius in covolume calculations.

obtained by least-squares calculations. Its ordinate intercept of $1.3 \pm 0.4$ l/g implies a dimerization constant, $X$, of $3.7 \pm 0.4$ l/g in the absence of sucrose, which compares favorably with the present estimate of $4.1 \pm 0.3$ l/g and previously reported values of 3.8 l/g [18] and 3.5 l/g [21] (open symbols in fig. 3) under the same conditions (pH 3.9, $I$ 0.2). We now need to reconcile the slope of 8.0 ($\pm 1.3$) for the best-fit linear relationship in fig. 3 with predictions based on thermodynamic nonideality.

Comparison of eq. 9 with the definition of the thermodynamic dimerization constant, $X$, in terms of species activities shows that

$$X_{app} = X(y_A^2/y_C) \tag{10}$$

By substituting eq. 3a for each $y_i$ this expression then becomes

$$X_{app} = X \exp\{(2U_{AM} - U_{CM} - M_M\bar{v}_M)m_M\} \tag{11a}$$

or

$$\ln X_{app} = \ln X + (2U_{AM} - U_{CM} - M_M\bar{v}_M)m_M \tag{11b}$$

which is clearly consistent with the experimental results of fig. 3 in its prediction of linear dependence of $\ln X_{app}$ upon sucrose concentration, $m_M$. Furthermore, combination of the value of 0.34 nm for the effective thermodynamic radius of sucrose (fig. 1) with the calculated hydrated radii of 2.44 and 3.07 nm for monomer ($r_A$) and dimer ($r_C$), respectively (see section 2.3), leads to a predicted slope of 8.2, which is in excellent agreement with the experimental value ($8.0 \pm 1.3$). Reasonable agreement between theory and experiment is still maintained if the macromolecular parameters are assigned other magnitudes. In that regard the second theoretical relationship with positive slope in fig. 3 (·–·–·) summarizes the dependence calculated [40,41] for the situation in which a prolate ellipsoid with axial ratio of 2 is substituted for spherical dimer. This line, with a slope of 6.8, also describes the predicted dependence for the model based solely on spherical geometry but with the slightly smaller radius of 2.3 nm [42] for monomeric enzyme. On the other hand, if the contribution of sucrose to the covolumes is disregarded ($r_M = 0$), it follows that $U_{CM} = 2U_{AM}$ for all three models and that the predicted slope is $-M_M\bar{v}_M$, namely, $-0.21$ (- - - - - in fig. 3). Thus, empirical though the effective thermodynamic radius of sucrose may be, the data of fig. 3 clearly attest to the importance of taking into account its magnitude (0.3 nm) in quantitative prediction of the space-filling effects of this small solute on macromolecular interactions.

### *3.3. Effect of glycerol on the dimerization of α-chymotrypsin*

Although the major thrust of this study has been to investigate the effects of thermodynamic nonideality arising from the use of sucrose as inert, space-filling solute, a few experiments have also been performed to examine the effect of glycerol on α-chymotrypsin dimerization. Our reason for so doing is that measurements of partial specific volume have been interpreted as showing that glycerol, unlike sucrose, partially penetrates hydrated protein species [11,12]. Consequently, calculations based solely on the excluded volume concept should overestimate the extent of dimerization enhancement effected by this frequently used inert small solute.

Fig. 4 presents, in the manner suggested by the logarithmic form of the law of mass action for a monomer-dimer system, the results of duplicate high-speed [22] sedimentation equilibrium experiments on α-chymotrypsin in acetate-chloride buffer (pH 3.9, $I$ 0.2) supplemented with 0.1 M glycerol. The straight line, drawn with the mandatory slope of 2 through the mean of the experimental points, corresponds to an apparent dimerization constant, $X_{app}$, of $5.8 \pm 0.5$ l/g, which is clearly greater than the value of $3.7 \pm 0.4$ l/g inferred for the thermodynamic dimerization constant ($X$) from fig. 3. Moreover, the experimentally determined enhancement factor of $1.4 \pm 0.2$ compares extremely favorably with the value of 1.5 for $X_{app}/X$ predicted by eqs. 3b and 11a with $r_A = 2.44$ nm and $r_C = 3.07$ nm, as before, and $r_M$ taken as 0.17 nm for glycerol [15]. Thus, despite the counterindications reported earlier [11,12],

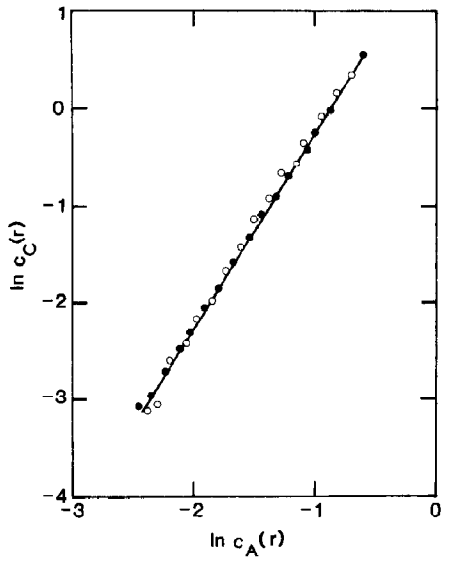


Fig. 4. Evaluation of the apparent equilibrium constant for the dimerization of α-chymotrypsin in acetate-chloride buffer (pH 3.9, $I$ 0.2) supplemented with 0.1 M glycerol, the values of monomer concentration, $c_A(r)$, having been obtained by $\Omega$ analysis [27] of sedimentation equilibrium distributions from duplicate experiments.

these results signify that little, if any, error is introduced by interpreting the space-filling effects of sucrose and glycerol at the present concentrations (< 0.2 M) in terms of a common model in which the small solute is considered to be excluded completely from the hydrated protein domain.

## 4. Concluding remarks

This investigation has served four main functions. Firstly, by demonstrating that the enhanced dimerization of α-chymotrypsin by sucrose may be interpreted quantitatively in terms of space-filling effects (figs. 2 and 3), this study reinforces an earlier assertion that the concept of excluded volume may be applied to small as well as macromolecular inert solutes [15]. Secondly, the suggested assumption [15] that the radius of the small solute is sufficiently small to be neglected in the calculation of covolumes has not withstood the more stringent test afforded by the present study of α-chymotrypsin dimerization, the inadequacy of this approximation being illustrated in fig. 3. Since a value of the effective thermodynamic radius of the small inert solute is thus required for quantitative appraisal of thermodynamic nonideality effects, the present investigation has served a third function by its demonstration of a frontal gel chromatographic procedure for the determination of this quantity for sucrose (fig. 1). Finally, whereas studies of preferential solvation by measurement of partial specific volume have led to the conclusion that effects of glycerol differ from those of sucrose by virtue of its ability to bind slightly to proteins, the present comparison of their effects on α-chymotrypsin dimerization indicates that the thermodynamic nonideality effected by both of these small solutes at moderate concentrations (< 0.2 M) finds quantitative rationalization in terms of excluded volume.

## Acknowledgements

The technical assistance of C.J. Leeder is gratefully acknowledged, as is the support of this investigation by the Australian Research Grants Scheme.

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