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Thermodynamic non-ideality as an alternative source of the effect of sucrose on the thrombin-catalyzed hydrolysis of peptide *p*-nitroanilide substrates

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Abstract

The inhibitory effect of sucrose on the kinetics of thrombin-catalyzed hydrolysis of the chromogenic substrate S-2238 (D-phenylalanyl–pipecolyl–arginoyl–*p*-nitroanilide) is re-examined as a possible consequence of thermodynamic non-ideality—an inhibition originally attributed to the increased viscosity of reaction mixtures. However, those published results may also be rationalized in terms of the suppression of a substrate-induced isomerization of thrombin to a slightly more expanded (or more asymmetric) transition state prior to the irreversible kinetic steps that lead to substrate hydrolysis. This reinterpretation of the kinetic results solely in terms of molecular crowding does not signify the lack of an effect of viscosity on any reaction step(s) subject to diffusion control. Instead, it highlights the need for development of analytical procedures that can accommodate the concomitant operation of thermodynamic non-ideality and viscosity effects.

Keywords: Enzyme kinetics; Molecular crowding; Thermodynamic non-ideality; Thrombin

1. Introduction

In mechanistic studies of catalysis by thrombin, the inhibitory effect of sucrose on kinetic parameters for the hydrolysis of chromogenic peptide substrates has been attributed to the increased relative viscosity of sucrose-supplemented reaction mixtures [1,2]. No consideration has been given to the possible consequences of thermodynamic non-ideality arising from molecular crowding by the high concentration of added cosolute [3–8]. Of interest in that respect is the report [9] of inhibitory effects of another inert cosolute, polyethylene glycol, on kinetic parameters for the thrombin-catalyzed hydrolysis of benzyl–arginoyl–*p*-nitroanilide—an extremely poor substrate for which the Michaelis constant may be regarded as the thermodynamic dissociation constant for the enzyme–substrate interaction [10]. To consider a cosolute effect on a thermodynamic parameter in terms of viscosity seems untenable, and hence an

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alternative source of the cosolute effect has been sought.

As noted elsewhere [5–8,11–15], molecular crowding by high concentrations of an inert cosolute provides a powerful probe of isomeric transitions within a protein. Furthermore, the existence of a Na^+-dependent transition in thrombin has been demonstrated by fluorescence intensity measurements [2]—a phenomenon also associated with enhancement of enzyme activity. It is therefore conceivable that the enhanced fluorescence observed in the presence of Na^+ could be reflecting the shift of a preexisting isomerization equilibrium towards a slightly larger (or more asymmetric) active conformational state, and that displacement of this preexisting equilibrium transition towards the smaller isomeric state as the result of molecular crowding by sucrose would afford an alternative explanation of the inhibitory effect currently being attributed [1,2] to viscosity.

The present investigation summarizes a search for experimental evidence that would allow distinction between effects of viscosity and thermodynamic non-ideality as the likely source of the inhibitory effect of sucrose on the thrombin-catalyzed hydrolysis of chromogenic substrate S-2238 (D-phenylalanyl–pipecolyl–arginoyl–*p*-nitroanilide). Although the results of that search favor interpretation of at least part of the inhibition in terms of thermodynamic non-ideality, the isomerization affected by molecular crowding is not the Na^+-dependent transition detected by fluorescence. Instead, the inhibitory effect of sucrose seems to reflect suppression of a substrate-induced isomerization of thrombin to a slightly expanded (or more asymmetric) transition state prior to the irreversible kinetic steps that lead to substrate hydrolysis.

2. Experimental

Human α-thrombin, prepared by the method of Fenton et al. [16], was a frozen sample of the preparation used previously [17]. Reagent-grade chemicals were used in the preparation of the buffer solutions used in this study, namely 0.05 M Tris/HCl–0.2 M NaCl, pH 8.0, and 0.05 M Tris/HCl–0.2 M choline chloride, pH 8.0. Sucrose (0.2 or 1.0 M) was an additional component in some experiments. Concentrations of thrombin solutions were spectrophotometrically determined on the basis of an absorption coefficient ($A_{1\ cm}^{1\%}$) of 18.3 at 280 nm [2,16].

2.1. Sedimentation equilibrium studies

Prior to molecular mass determination by sedimentation equilibrium, solutions of enzyme (200 μl, 0.15 mg/ml) were dialyzed against the two buffer solutions mentioned above and also against the choline chloride-containing buffer supplemented with 0.2 M sucrose. These dialyzed thrombin solutions were first spun to sedimentation equilibrium in a Beckman XL-I analytical ultracentrifuge operating at 20 °C and 12 000 rev. min^{-1}. The rotor speed was then increased to 28 000 rev min^{-1} to obtain sedimentation equilibrium distributions of meniscus-depletion design [18]. Equilibrium distributions at both rotor speeds were spectrophotometrically recorded at 280 and 500 nm, those at the latter wavelength being regarded as baselines for correction of the A_{280} recorded at each radial distance. The corrected equilibrium distributions were fitted to the relationship [19]:

$$A_{280}(r) = A_{280}(r_F)\exp\left[M(1-\bar{v}\rho)\omega^2(r^2-r_F^2)/(2RT)\right] \quad (1)$$

which expresses the absorbance at radial distance r, $A_{280}(r)$, in terms of that at an arbitrarily chosen radial position r_F for a solute with molecular mass M and partial specific volume $\bar{v}$ centrifuged at angular velocity ω and temperature T; r_F was taken as 7.050 cm for each distribution. An Anton-Paar density meter was used to determine the density, ρ, of each solvent (diffusate from the dialysis step). The partial specific volume of human thrombin was taken as 0.734 ml g^{-1}, the value calculated from the amino acid composition [20].

2.2. Gel chromatography on Sephadex G-100

In a series of zonal gel-chromatography studies, thrombin solution (50 μl, 0.7 mg ml^{-1}) was applied to a column of Sephadex G-100 (2.0 cm × 19.7 cm) preequilibrated at 0.63 ml min^{-1}

with one of the above buffer solutions. Blue dextran 2000 and potassium chromate were also included in the applied sample to obtain estimates of the void and total liquid-phase volumes (V_o and V_t, respectively) for the column. Elution volumes were estimated from the positions of the absorbance peaks (280 nm) in the effluent profile and were converted to partition coefficients K_D by the standard procedure [21,22].

2.3. Fluorescence titrations

The interaction of Na^+ with human thrombin at 25 °C has been quantified by measuring the increased fluorescence intensity at 340 nm [2] in response to a 280-nm incident beam in an ISS photon-counting spectrofluorimeter. As in that earlier study, a constant ionic strength was maintained by suitable admixture of the NaCl- and choline chloride-containing buffers to provide solutions with the requisite Na^+ concentration. A binding curve for the Na^+–thrombin interaction was also obtained in the presence of 1 M sucrose. Analysis of estimates for the fractional fluorescence enhancement, θ_F, in terms of the rectangular hyperbolic dependence upon metal ion concentration, $[L]$:

$$\theta_F = K_{EL}[L]/(1 + K_{EL}[L]) \quad (2)$$

provided a magnitude of the binding constant K_{EL}.

3. Results and discussion

In both mechanistic investigations of thrombin catalysis [1,2] it has been assumed that the decreased rate of hydrolysis in the presence of sucrose is a consequence of the increased viscosity of reaction mixtures [23,24]. The results reported in Fig. 2 of Wells and Di Cera [2] have therefore been interpreted in terms of a mandatory linear dependence of $1/k_{cat}$ upon relative viscosity, η_{rel}. However, closer inspection reveals curvilinearity of those plots (Fig. 1). Because viscosity is not therefore the sole factor responsible for the decreased rate of catalysis in the presence of sucrose, we assess the potential of thermodynamic non-ideality to account for the observed effects of

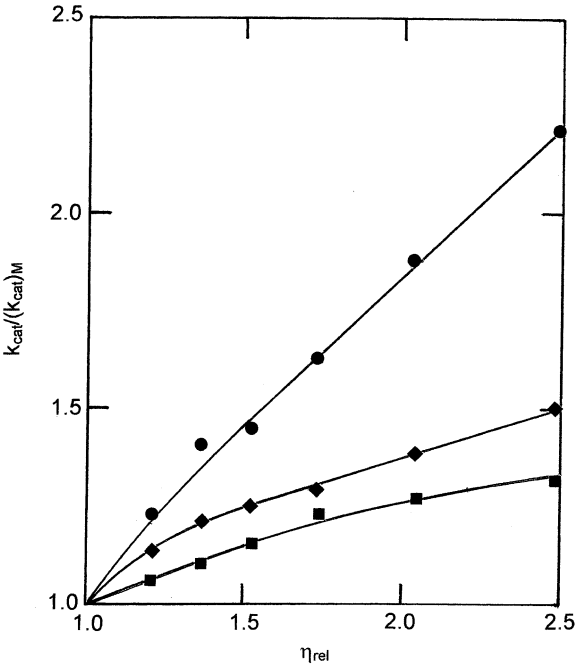

Fig. 1. Non-linearity of the dependence of $1/k_{cat}$ upon relative viscosity for the thrombin-catalyzed hydrolysis of S-2238 in Tris/HCl buffer containing 0.2 M choline chloride (●), KCl (◆) or NaCl (■). Results, taken from Fig. 2 of [2], have been normalized by expressing $(1/k_{cat})_M$, the value obtained in the presence of sucrose, to its value $(1/k_{cat})$ in the absence of the viscogenic agent.

sucrose on the kinetics thrombin-catalyzed hydrolysis of chromogenic substrate S-2238.

As noted in Section 1, any consideration of the inhibitory effect of sucrose on catalysis by thrombin in terms of thermodynamic non-ideality is conditional upon the existence of an equilibrium enzyme transition involving an increase in volume (or asymmetry) within the kinetic mechanism of enzyme action. In view of the enhanced fluorescence, as well as the enzyme activity of thrombin in the presence of Na^+ [2], it is certainly tempting to consider this transition as the isomerization reaction being sought.

3.1. Possible effects of molecular crowding on a preexisting enzyme isomerization

Although effects of thermodynamic non-ideality emanating from molecular crowding by small

cosolutes on such a preexisting enzyme isomerization equilibrium have been predicted [6] and indeed observed [7] in kinetic studies, the parameter subject to change was the Michaelis constant. On the grounds that cosolute inclusion in reaction mixtures gives rise to a decrease in k_{cat} but essentially no change in K_m for chromogenic substrate hydrolysis by thrombin [2], that simple model [6,7] with binding of the substrate restricted to the active isomer is thus precluded in the present instance. However, a dependence of k_{cat} upon cosolute inclusion is readily incorporated by modifying the model to the form:

$$\begin{array}{c} \overset{K_d}{} \quad \overset{k}{} \\ E + S \rightleftarrows ES \rightarrow E + P \\ Y \uparrow \downarrow \\ \overset{K_d}{} \\ E^* + S \rightleftarrows E^*S \end{array} \qquad (3)$$

in which K_d is the dissociation constant describing enzyme–substrate complex formation with either enzyme isomer. This restrictive requirement is introduced to accommodate the findings [1,2] that kinetic estimates of competitive inhibition constants (K_I) are unaffected by the presence of sucrose. Provided that k, the rate constant governing the product-forming step, is sufficiently small for concentrations of the various enzyme and enzyme–substrate species to be described in thermodynamic (equilibrium) terms, standard kinetic derivation on the basis of a steady state ($d[ES]/dt = 0$) yields the following expression for initial velocity, v:

$$v = \{k[E_t]/(1+Y)\}[S]/(K_d + [S]) \qquad (4)$$

where $[E_t]$ is the total enzyme concentration and $Y = [E^*]/[E]$ is the equilibrium constant governing the preexisting enzyme isomerization.. The parameters determined by conventional Michaelis–Menten analysis are thus amenable to mechanistic interpretation as:

$$k_{cat} = V_{max}/[E_t] = k/(1+Y) \qquad (5a)$$

$$k_{cat}/K_m = k/\{K_d(1+Y)\} \qquad (5b)$$

which signifies their sensitivity to effects of thermodynamic non-ideality because of a dependence upon the isomerization constant Y.

3.2. Effect of sucrose on the Na^+-dependent enzyme isomerization

Having established the potential for effects of thermodynamic non-ideality on a model with preexisting Na^+-dependent enzyme isomerization to give rise to the observed kinetic behavior of thrombin in the presence of sucrose, we need to ensure that the transition responsible for the enhanced fluorescence is, indeed, an isomerization. Qualitatively similar effects of molecular crowding would result from preferential catalysis by monomeric enzyme in association equilibrium with an oligomeric state [25,26]. Elimination of that possibility is of particular relevance, inasmuch as thrombin of bovine origin is known to undergo reversible dimerization [27,28].

Sedimentation equilibrium has been used to determine the molecular mass of human thrombin in the NaCl- and choline chloride-containing Tris/HCl buffers (pH 8.0), and also in the latter medium made 0.2 M with respect to sucrose. The rationale for this selection of conditions is that the NaCl-containing medium should enhance the proportion of the active enzyme species relative to its abundance in the NaCl-free environment. On the other hand, supplementation of the choline chloride medium with sucrose should enhance the proportion of enzyme in the inactive (or less active) state. Analyses of sedimentation equilibrium distributions obtained at 12 000 and 28 000 rev. min^{-1} under the three conditions are summarized in the first two lines of Table 1. The close conformity of all estimates of molecular mass with the monomer value of 36.5 kDa clearly eliminates self-association as the Na^+-dependent transition undergone by human thrombin. We therefore focus attention on the possibility that the enhanced fluorescence of human thrombin in the presence of NaCl [2] reflects the preferential binding of metal ion to a larger (or more asymmetric) isomeric state of the enzyme.

Partition coefficients (K_D) evaluated by zonal gel chromatography of human thrombin on

Sephadex G-100 are presented in the final line of Table 1. Failure to observe any difference between K_D for enzyme in the choline chloride and NaCl environments signifies that the increased fluorescence associated with the Na^+-dependent transition undergone by thrombin must reflect a local conformational change that is of insufficient magnitude to have a detectable effect on the overall size of the enzyme. Taken in isolation, the smaller partition coefficient obtained in the presence of sucrose seemingly implies displacement of a self-association equilibrium towards the polymeric state. However, that possible consequence of thermodynamic non-ideality [26] is precluded by the sedimentation equilibrium results. In view of the existence of a heparin-binding site on thrombin, it seems likely that the polysaccharide chain of the Sephadex serves as a weak affinity matrix for the enzyme, and that by acting as a competitive inhibitor of the affinity interaction, sucrose may restore the chromatographic process to pure gel filtration. A similar situation has been encountered with lysozyme, the elution of which from Sephadex is also facilitated by small saccharides [29].

Evidence that unequivocally eliminates the Na^+-mediated enzyme transition as a potential source of thermodynamic non-ideality has been afforded by a fluorimetric study of the effect of sucrose on the interaction between Na^+ ions and human thrombin (Fig. 2). As well as being experimentally indistinguishable, measurements of the fractional fluorescence enhancement in the absence (○) and presence (●) of sucrose (1 M) are well described by Eq. (4) and the binding constant of 36 M^{-1} deduced from a similar study [2] of the Na^+–thrombin interaction in the absence of saccharide (solid line in Fig. 2). These results signify that no gross conformational change accompanies the Na^+-mediated transition—a finding entirely consistent with the recently demonstrated similarity between the crystal structures of the slow (Na^+-free) and fast forms of thrombin [30].

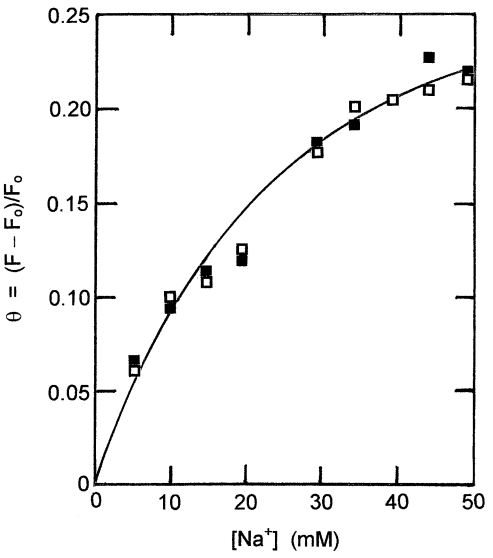

Fig. 2. Spectrofluorimetric investigation of the interactions of Na^+ with human thrombin in the absence (□) and presence (■) of sucrose (1 M). The solid line is the best-fit description obtained on the basis of Eq. (2) and a binding constant, K_{EL}, of 36 M^{-1} [2].

Table 1
Attempts to identify the Na^+-dependent thrombin transition by sedimentation equilibrium and gel chromatography on Sephadex G-100

Parameter	Buffer supplement		
	Choline chloride (0.2 M)	NaCl (0.2 M) (0.2 M)	Choline chloride (0.2 M) Sucrose (0.2 M)
M (kDa)[a]			
(12 000 rev. min^{-1})	36.3 (±0.3)	36.1 (±0.3)	35.6 (±0.4)
(28 000 rev. min^{-1})	36.5 (±0.6)	36.1 (±0.4)	35.5 (±0.4)
K_D (G-100)[b]	0.33 (±0.01)	0.33 (±0.01)	0.30 (±0.01)

Conditions: 0.05 M Tris/HCl buffer (pH 8.0) supplemented as indicated.
[a] Numbers in parentheses denote the uncertainty (±2 S.D.) of the estimate.
[b] Mean (±2 S.D.) of three estimates.

On the grounds that the presence of sucrose does not impart detectable thermodynamic non-ideality to this preexisting enzyme transition, we therefore consider the possibility that substrate-induced isomerization of thrombin could allow thermodynamic rationalization of the enzyme kinetic behavior originally attributed to viscosity [2].

3.3. Rationalization of the kinetic data in terms of a substrate-induced isomerization

We now consider the effects of thermodynamic non-ideality on thrombin catalysis in terms of an enzyme reaction conforming to the mechanism [3,6]:

$$\mathrm{E}+\mathrm{S}\overset{K_\mathrm{d}}{\rightleftarrows}\mathrm{ES}\overset{X}{\rightleftarrows}\mathrm{ES}^{\dagger}\overset{k}{\rightarrow}\mathrm{E}+\mathrm{P} \tag{6}$$

in which the substrate-induced transition is governed by isomerization constant X. Because enzyme kinetic experiments are conducted under conditions of constant temperature and pressure, equilibria are most readily described in terms of the molal concentration (m_i) and molal activity coefficient (y_i) of each participating species [31]. However, in the situation under consideration the extremely low enzyme concentration ensures that the activity coefficient of each enzyme species is dominated by the term in cosolute concentration [8]. The expression for y_i then assumes the form $\exp(B_{i,\mathrm{M}}-M_\mathrm{M}\nu_\mathrm{M})m_\mathrm{M}\rho_\mathrm{s}$, where ρ_s is the solvent density, $B_{i,\mathrm{M}}$ the second osmotic virial coefficient describing physical interaction of species i with cosolute M, and $M_\mathrm{M}\bar{\upsilon}_\mathrm{M}$ the molar volume of cosolute. Allowance for the effect of a molal concentration m_M of sucrose may thus be incorporated by means of the following apparent equilibrium constants [8]:

$$X_\mathrm{app}=X\exp\left[(B_{\mathrm{ES,M}}-B_{\mathrm{ES}^{\dagger},\mathrm{M}})m_\mathrm{M}\rho_\mathrm{s}+\ldots\right] \tag{7a}$$

$$(K_\mathrm{d})_\mathrm{app}=\left(K_\mathrm{d}/y_\mathrm{S}\right)\exp\left[(B_{\mathrm{ES,M}}-B_{\mathrm{ES}^{\dagger},\mathrm{M}})m_\mathrm{M}\rho_\mathrm{s}+\ldots\right] \tag{7b}$$

On the grounds that the second virial coefficient is simply the covolume of enzyme species i and sucrose for this system with an uncharged cosolute, $B_{i,\mathrm{M}}=4\pi N(R_i+R_\mathrm{M})^3/3$ for spherical enzyme and cosolute species with radii R_i and R_M, respectively [32]. An increase in volume (or asymmetry) associated with the $\mathrm{ES}\rightleftarrows\mathrm{ES}^{\dagger}$ transition thus has the potential to decrease the magnitude of X_app, and hence decrease the rate of product formation. On the other hand, the binding of a small substrate is considered to have little effect on the enzyme radius ($R_\mathrm{E}\approx R_\mathrm{ES}$). Consequently, the only effect of sucrose on $(K_\mathrm{d})_\mathrm{app}$ stems from a change in y_S, the activity coefficient of the substrate. This parameter is taken as unity to simplify the current illustrative analysis.

In the presence of cosolute the expression for k_cat needs to be written in terms of X_app, whereupon it follows that the effect of thermodynamic non-ideality on the catalytic rate constant is given by:

$$\left(1/k_\mathrm{cat}\right)_\mathrm{M}=\frac{1+X\exp\left[(B_{\mathrm{ES,M}}-B_{\mathrm{ES}^{\dagger},\mathrm{M}})m_\mathrm{M}\rho_\mathrm{s}+\ldots\right]}{k\exp\left[(B_{\mathrm{ES,M}}-B_{\mathrm{ES}^{\dagger},\mathrm{M}})m_\mathrm{M}\rho_\mathrm{s}+\ldots\right]} \tag{8a}$$

$$\approx\left[(1+X)/(kX)\right]+\left[1/(kX)\right](B_{\mathrm{ES,M}}-B_{\mathrm{ES}^{\dagger},\mathrm{M}})m_\mathrm{M}\rho_\mathrm{s} \tag{8b}$$

Similar considerations of $K_\mathrm{m}/k_\mathrm{cat}$, the other experimental parameter used by Wells and Di Cera [2], show that:

$$\left(K_\mathrm{m}/k_\mathrm{cat}\right)_\mathrm{M}=\left[K_\mathrm{d}/(kX)\right]X\exp\left[(B_{\mathrm{ES,M}}-B_{\mathrm{ES}^{\dagger},\mathrm{M}})m_\mathrm{M}\rho_\mathrm{s}\right] \tag{9a}$$

$$\approx\left[K_\mathrm{d}/(kX)\right]+\left[K_\mathrm{d}/(kX)\right](B_{\mathrm{ES,M}}-B_{\mathrm{ES}^{\dagger},\mathrm{M}})m_\mathrm{M}\rho_\mathrm{s} \tag{9b}$$

Positive linear dependence of $(1/k_\mathrm{cat})_\mathrm{M}$ and $(K_\mathrm{m}/k_\mathrm{cat})_\mathrm{M}$ upon $m_\mathrm{M}\rho_\mathrm{s}$ is thus the predicted outcome of the thermodynamic non-ideality effects of sucrose on thrombin catalysis.

The extent to which these predictions are borne out by experimental data on the effect of sucrose on the thrombin-catalyzed hydrolysis of S-2238 is summarized in Fig. 3a,b, which have been constructed from the results presented in Figs. 1 and 2 of Wells and Di Cera [2]. Viscosity tables in the *CRC Handbook of Biochemistry and Molecular*

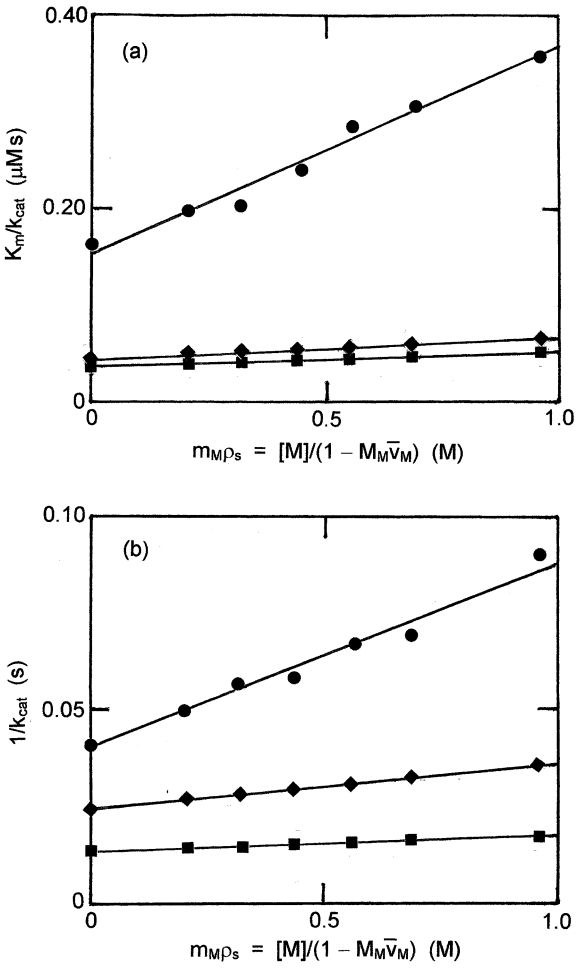

Fig. 3. Consideration of the effect of sucrose on S-2238 hydrolysis by thrombin as a consequence of thermodynamic nonideality, the results for (a) K_m/k_{cat} and (b) $1/k_{cat}$ being tested for conformity with Eqs. (8b) and (9b). Data for hydrolysis in Tris/HCl buffer supplemented with 0.2 M choline chloride (●), 0.2 M KCl (♦) or 0.2 M NaCl (■) have been inferred from Figs. 1 and 2 of [2].

Biology were used to convert each abscissa value from relative viscosity to molar sucrose concentration, which was then transformed to the required molar parameter, $m_M\rho_s$, on the basis of solution incompressibility [31].

An important observation to note from Fig. 3a,b is the adequacy of linear relationships to describe the dependence of $(K_m/k_{cat})_M$ and $(1/k_{cat})_M$ upon sucrose concentration—findings consistent with the theoretical predictions of Eqs. (8b) and (9b). From the viewpoint of estimating the extent of the putative expansion of the enzyme, it is informative to normalize the respective expressions for $(1/k_{cat})_M$ and $(K_m/k_{cat})_M$ by expressing them relative to their counterparts, $1/k_{cat}$ and K_m/k_{cat}, in the absence of sucrose. Specifically:

$$k_{cat}/(k_{cat})_M = \frac{1+X\exp\left[(B_{ES,M}-B_{ES^\dagger,M})m_M\rho_s\right]}{(1+X)\exp\left[(B_{ES,M}-B_{ES^\dagger,M})m_M\rho_s\right]} \quad (10a)$$

$$\left(K_m/k_{cat}\right)_M/\left(K_m/k_{cat}\right) = \exp\left[(B_{ES,M}-B_{ES^\dagger,M})m_M\rho_s\right] \quad (10b)$$

Despite the simpler form of the expression for the ratio of K_m/k_{cat} values, the two sets of experimental results are described by a common linear dependence of $\ln[k_{cat}/(k_{cat})_M]$ and $\ln[(K_m/k_{cat})_M/(k_m/k_{cat})]$ upon $m_M\rho_s$ (Fig. 4). This coincidence of dependence signifies a small magnitude for the isomerization constant ($X \ll 1$), whereupon the expression for the ratio of $1/k_{cat}$ values becomes identical with the right-hand side of Eq. (10b). In that regard the small value of X would be consis-

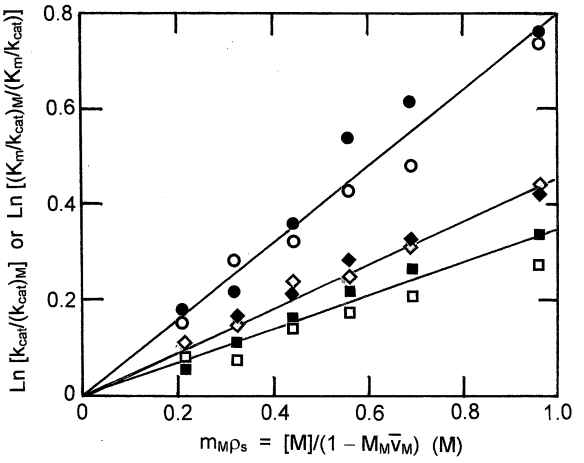

Fig. 4. Evaluation of the apparent change in covolume, $(B_{ES}{}^\dagger - B_{ES,M})$, from the data presented in Fig. 3 for the effect of sucrose on the thrombin-catalyzed hydrolysis of S-2238. Results for $[(K_m/k_{cat})_M/(K_m/k_{cat})]$ (*closed symbols*) and $k_{cat}/(k_{cat})_M$ (*open symbols*) are plotted in accordance with the logarithmic form of Eq. (10b).

tent with consideration of $ES^{\dagger}$ as the activated complex encountered in absolute reaction-rate theory [3,33].

Linear regression analysis of the combined data sets in terms of the logarithmic form of Eq. (10b) yields an estimate of 0.79 ($\pm$0.05) l mol^{-1} for $(B_{ES,M}-B_{ES^{\dagger},M})$ from the kinetic parameters obtained for thrombin in the choline chloride medium, but lower values of 0.45 ($\pm$0.03) and 0.35 ($\pm$0.04) l mol^{-1} emanate from kinetic measurements in the presence of KCl and NaCl, respectively (Fig. 4). We therefore proceed on the basis that the binding of a metal ion to thrombin may decrease the extent of the substrate-induced conformational change. In that regard, we note that the experimental parameters obtained in the presence of Na^+ and K^+ are all constitutive parameters, in the sense that the 0.2 M concentration of metal ion does not suffice to saturate the ligand (L) site, and that it is therefore necessary to consider the kinetic data not only in terms of the reaction scheme shown in Eq. (6), but also in terms of the corresponding reaction scheme for enzyme–ligand complex EL. The measured maximal velocity then defines an average catalytic rate constant, $\bar{k}_{cat}$, given by:

$$\bar{k}_{cat}=\left[(k_{cat})_E+K_{EL}[L](k_{cat})_{EL}\right]/\left(1+K_{EL}[L]\right) \quad (11a)$$

whereas its counterpart in the presence of sucrose, $(\bar{k}_{cat})_M$, becomes:

$$(\bar{k}_{cat})_M=\frac{(k_{cat})_E\exp\left[(B_{ES,M}-B_{ES^{\dagger},M})m_M\rho_s\right]+K_{EL}[L](k_{cat})_{EL}\exp\left[(B_{ELS,M}-B_{ELS^{\dagger},M})m_M\rho_s\right]}{(1+K_{EL}[L])} \quad (11b)$$

Combination of Eqs. (11a) and (11b) gives rise to the expression:

$$\bar{k}_{cat}/(\bar{k}_{cat})_M=\frac{(k_{cat})_E+K_{EL}[L](k_{cat})_{EL}}{(k_{cat})_E\exp\left[(B_{ES,M}-B_{ES^{\dagger},M})m_M\rho_s\right]+K_{EL}[L](k_{cat})_{EL}\exp\left[(B_{ELS,M}-B_{ELS^{\dagger},M})m_M\rho_s\right]} \quad (12)$$

which affords a means of estimating $(B_{ELS,M}-B_{ELS^{\dagger},M})$ provided that magnitudes can be assigned to the remaining parameters.

The only parameters for which magnitudes are directly available are $(B_{ES,M}-B_{ES^{\dagger},M})$ and $(k_{cat})_E$, which are taken as -0.79 l mol^{-1} (Fig. 4) and 23 s^{-1} (Table I in [2]), respectively. Values of $(k_{cat})_{EL}$ need to be determined from the k_{cat} values of 77 s^{-1} (Na^+) and 42 s^{-1} (K^+), also reported in Table I of Wells and Di Cera [2] in conjunction with the binding constants (K_{EL}) of 46 M^{-1} (Na^+) and 8 M^{-1} (K^+) that appear in Table III of the same investigation. Application of Eq. (11a) then leads to $(k_{cat})_{EL}$ values of 83 s^{-1} (L = Na^+) and 54 s^{-1} (L = K^+). The relevant parameter estimates are now incorporated into Eq. (12) with the value of $d\ln[k_{cat}/(k_{cat})_M]/d(m_M\rho_s)$ deduced from the relevant slope in Fig. 4 to obtain $(B_{ELS,M}-B_{ELS^{\dagger},M})$ as the remaining parameter of unknown magnitude. Such consideration of the experimental estimates of covolume differences to be weighted averages of parameters for the $ES \rightleftarrows ES^{\dagger}$ and $ELS \rightleftarrows ELS^{\dagger}$ transitions leads to respective estimates of 0.34 ($\pm$0.04) and 0.37 ($\pm$0.04) l mol^{-1} for $(B_{ELS,M}-B_{ELS^{\dagger},M})$ with Na^+ and K^+ as the metal-ion ligand, L.

Interpretation of the inhibitory effect of sucrose on the thrombin-catalyzed hydrolysis of chromogenic substrate S-2238 by thrombin in terms of thermodynamic non-ideality has thus led to the conclusion that the inhibition reflects displacement of a substrate-induced transition ($ES \rightleftarrows ES^{\dagger}$) toward the slightly smaller, inactive isomeric state (ES) as the result of molecular crowding by high concentrations of an inert cosolute (sucrose). In an environment devoid of alkali metal ions, the difference in covolume $(B_{ES^{\dagger},M}-B_{ES,M})$ is calculated to be 0.79 l mol^{-1}, whereas the corresponding difference for EL species $(B_{ELS^{\dagger},M}-B_{ELS,M})$ is 0.35 l mol^{-1}. To interpret the relative extent of the putative conformational expansion we need an estimate of $B_{E,M} \approx B_{ES,M}$, the covolume for cosolute and enzyme prior to substrate-induced isomerization. We use the effective thermodynamic radius, R_M, of 0.32 nm that has been obtained for sucrose from isopiestic and freezing-point-depression measurements [34], and adopt the viewpoint [35] that the effective thermodynamic radius for solvated enzyme exceeds the unsolvated radius, $R_u = [3MV/(4\pi N)]^{1/3}$, by 0.56 nm. On that basis, $R_E = R_{ES} = 2.76$ nm, whereupon the enzyme–sucrose covolume prior to expansion of the enzyme is 73.7 l mol^{-1}. The corresponding covolume for sucrose and the expanded form of thrombin in the choline

chloride environment is therefore 74.5 l mol^{-1}, which corresponds to a value of 3.10 nm for $(R^{\dagger}_{ES}+R_M)$, and hence a radius of 2.78 nm for $ES^{\dagger}$. These calculations signify that the substrate-induced transition gives rise to a 0.7% increase in effective radius (a 2% increase in effective volume) of thrombin. For the $ELS^{\dagger}$ species, the corresponding radius is 2.77 nm, which signifies a 0.36% increase in effective radius (a 1% increase in effective volume). However, as noted elsewhere [8,15], this change in effective spherical volume of the solvated enzyme may reflect an increase in asymmetry rather than a change in volume.

4. Concluding remarks

The present investigation has served several useful roles. First, because the use of sucrose as a viscogenic agent is a well-established protocol in mechanistic studies of enzyme action [1,2,23,24], this study of thrombin stresses the need to consider whether the inhibitory effect observed is, indeed, the consequence of increased buffer viscosity. In that regard we draw attention to the approach adopted by Dzingeleski and Wolfenden [36] to invalidate the classical enzyme-kinetic interpretation of sucrose effects on the adenosine deaminase reaction [37]—a system for which the inhibition by sucrose may also be rationalized as a consequence of thermodynamic non-ideality [15].

Secondly, although we have shown that the enzyme kinetic results published for the effect of sucrose on the hydrolysis of S-2238 by thrombin are seemingly amenable to quantitative interpretation in terms of molecular crowding, we do not wish to imply that there is no effect of viscosity. Inasmuch as some of the rate constants deduced [2] were of a magnitude commensurate with reactions under the influence of diffusion control, the inhibitory effect of sucrose may well reflect the combined effects of viscosity and thermodynamic non-ideality. Indeed, association rate constants of 10^7–10^8 M^{-1} s^{-1} for the initial enzyme–substrate interaction have now also been inferred from the temperature dependence of the kinetic parameters for the thrombin catalyzed hydrolysis of another chromogenic substrate, D-phenylalanyl–prolyl–*p*-nitroanilide [38]. The present attempt to interpret the results solely in terms of molecular crowding by sucrose may thus also represent an oversimplification of the situation. The next challenge is clearly the development of analytical procedures that allow for concomitant operation of thermodynamic non-ideality and viscosity effects.

Meanwhile, this investigation should at least serve to remind enzyme kineticists of the need to consider the consequences of thermodynamic non-ideality on the putative substrate-mediated conformational transitions that are often invoked to account for the kinetics of enzyme catalysis. Although it is appropriate to consider that enzyme solutions are sufficiently dilute to justify neglect of thermodynamic non-ideality under the usual in vitro conditions, that approximation may well cease to retain validity when the same reaction is examined in a more crowded molecular environment. Whereas this study has concentrated on the thermodynamic consequences of an artificially generated crowded environment, similar considerations may well apply to enzyme-catalyzed reactions occurring within the crowded physiological environment of the cell cytoplasm [39–41].

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