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Possible coupling of chemical to structural dynamics in subtilisin BPN′ catalyzed hydrolysis *

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The viscosity dependence of enzymatic catalysis was examined in subtilisin BPN′ catalyzed hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and *N*-succinyl-Ala-Ala-Pro-Phe-thiobenzyl ester. The viscosity of the reaction medium was varied by added glycerol, ethylene glycol, sucrose, glucose, fructose, poly(ethylene glycol) and Ficoll-400. Responses of the Michaelis-Menten parameters associated with hydrolysis were calculated from data obtained by spectrophotometric techniques. The reactions with these two substrates have catalytic rates well below the diffusion-controlled limit and thus enable us to study the viscosity effects on catalytic steps of non-transport nature. It was found that the K_m values for both amide and ester reactions remained relatively independent of cosolvents. On the other hand, while the k_{cat} values for amide were insensitive to cosolvents, those for ester were substantially attenuated except in the case of poly(ethylene glycol). The observed rate attenuations cannot be explained by changes in proton activity, water activity, dielectric constant of the reaction medium or shifts of any kinetically important pK_a. Instead, the results can be adequately described by microviscosity effects on the unimolecular deacylation step with a coupling constant of 0.65 ± 0.11. In addition, the different viscosity dependence in the acylation vs deacylation step can be rationalized in terms of fluctuation-dependent chemical dynamics of proton transfers in the context of the Bogris-Hynes model.

1. Introduction

Our understanding of enzyme catalysis has advanced steadily since the three-dimensional structure of proteins could be determined by X-ray diffraction. There is, however, still no consensus on the sources of the catalytic power of enzymes [1,2]. In particular, the role of dynamic properties of the protein matrix has not been correlated to bio-catalytic processes. It is generally accepted that transition state stabilization plays a critical role in catalysis. Intrinsic binding energy is believed to facilitate or be responsible for the catalytic free energy advantage, probably by a number of factors including the critical orientation and positioning of the reactive groups. Thus, the catalytic properties as related to macromolecular (protein) conformation are in general only confined to the active site. The energetic throttle is provided via transition state stabilization.

Although structural fluctuations have been suggested to be important in enzyme catalysis [3,4],

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Abbreviations: *N*-suc-AAPF-, *N*-succinyl-Ala-Ala-Pro-Phe-; -pNA, -*p*-nitroanilide; -SBzl, -thiobenzyl ester; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PEG, poly(ethylene glycol).

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there is no clear demonstration that such an effect is significant. If transition state stabilization is all that is required for catalysis, the one-way flux description provided by transition state theory would be adequate. The inadequacy of such a description has been gradually recognized as being due to the dynamic modulation by fluctuation properties of the reaction system [5]. These include fluctuating forces within the protein matrix itself and also through interaction with a fluctuating medium like a viscous solvent. The dynamic role of the solvent was first described by Kramers [6] as a random force induced barrier recrossing. The random force in this case is related to the friction coefficient by the fluctuation dissipation theorem [7]. Kramers' theory was later modified by Grote and Hynes [8] to include a decision time at the barrier region. If the decision time on the barrier top is short, then the solvents become static or irrelevant within the reaction time scale. The decision time is in general related to the height and shape of the barrier. If the barrier is sharp and high, the decision time is short and vice versa. As a result, the sharp barrier reactions will 'track' the short time friction and are not sensitive to the longer time, or collective effects that can increase the overall long time friction constant. One consequence of this is that the observed reaction rate will not necessarily track the increasing solvent viscosity as Kramers' theory would have predicted. The concept of 'viscosity' or 'friction coefficient' in all these theories mentioned above is quite different from the usual macroscopic viscosity of the solvent. On the other hand, viscosity has clearly the microscopic meaning of a local quantity for specific motions along a given reaction coordinate.

The applicability of Kramers' theory in protein systems has been demonstrated in the hydrogen exchange kinetics of lysozyme [9], ligand rebinding in myoglobin following laser photolysis [10], and possibly in the enzymes, carboxypeptidase A [4] and lactate dehydrogenase [11]. For the above-mentioned enzyme systems, the turnover rates are limited by certain conformational rearrangements upon substrate binding. As a result, the cosolvent influence can only be linked to the shifts in conformational equilibria of the reaction sequence, but not manifest in viscosity effects on barrier climbing during the chemical step. The aim of our investigation is to assess the relationship of structural fluctuations in a protein matrix to the chemical dynamics of the enzyme. Microscopic friction driven by molecular collisions with the solvent and internal fluctuations, which are both key factors in determining the response of the system when subject to external disturbance as manifested in the fluctuation dissipation theorem, underlie our experimental strategy. We propose to establish experimentally the degree of coupling of internal fluctuations to enzyme reaction dynamics from the measured viscosity dependence of the kinetic response. We have studied the kinetics of subtilisin BPN′ catalyzed hydrolysis of amide and ester. Subtilisins are a family of extracellular serine proteases with well-characterized kinetic properties [12]. The well-established kinetics is essential for our type of studies which then allows us to obtain the rate constants from Michaelis-Menten parameters. More importantly, the rather small k_{cat} values for the present reactions are not diffusion controlled in the usual sense of transport in the physical space. This will enable us to study the interplay of local friction and protein dynamics during barrier climbing of the chemical step.

2. Experimental

2.1. Materials

All materials were of reagent grade obtained from commercial sources. Cosolvent solutions were prepared by weighing the cosolvent into a fixed weight of buffer until the desired weight percentage was reached. Relative viscosity values for all cosolvent systems were obtained from the CRC Handbook of Chemistry and Physics (67th edn, 1987). For glycerol and ethylene glycol solutions, the ionic strength was kept constant by adding an appropriate amount of NaCl. The pH titration runs were conducted in 0.05 M phosphate buffer, 25°C. The pH were adjusted by mixing 0.5 M KH_2PO_4 and Na_2HPO_4 according to the Biochemists' Handbook (1961). The glass electrode readings for hydrogen ion activity in the presence of cosolvents were corrected according to Gelsema et al. [13].

Crystalline subtilisin BPN′ (protease type XXVII, 'Nagarase') was purchased from Sigma. Protein samples were purified prior to all kinetic measurements by an FPLC method modified from that described by Willis and Szabo [14]. The contaminants are mainly breakdown products resulting from autolysis. This procedure is thus important for accurate determinations of kinetic parameters. Typically, about 1 mg/ml of the crystalline protein was dissolved in 10 mM phosphate buffer (pH 6.2). The protein solution was then immediately injected onto an FPLC (Pharmacia) setup equipped with a Mono S HR 5/5 cation-exchange column (Pharmacia). The sample was loaded onto the column with buffer A (10 mM phosphate buffer, pH 6.2) and then eluted by increasing the percentage of buffer B (10 mM phosphate, pH 6.2, containing 0.5 M NaCl) to 15% in a linear gradient over 30 min at a flow rate of 1 ml/min. The eluant was monitored at 280 nm. Peaks were collected and tested for enzyme activity. Final protein concentration from the collected fraction was determined by using an extinction coefficient of $\epsilon_{280} = 3.22 \times 10^4$ M^{-1} cm^{-1} [15].

2.2. Kinetic assays

The two peptide substrates, *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl-X, where X is either *p*-nitroanilide for amide hydrolysis or thiobenzyl ester for ester hydrolysis, were purchased from Sigma.

The amide substrate used in the present study, *N*-suc-AAPF-pNA, first synthesized by Delmar et al. [16], is a convenient and sensitive substrate. These authors also showed that the spontaneous hydrolysis rate in 0.2 M Tris-HCl buffer (pH 8.0) was approx. 0.1% per day at 4° C. In addition, no organic solvent was needed to facilitate solubility beyond K_m in the assays.

The amide hydrolysis assays were conducted in 0.1 M Tris-HCl buffer (pH 8.6) at 25° C. Reactions were started by adding 25 μl purified enzyme to 1.6 ml of buffer containing the substrate and the desired amount of cosolvents. The progress of hydrolysis was monitored by the continuous increase in absorbance at 410 nm using a thermostated Cary model 219 spectrophotometer. An extinction coefficient of 8480 M^{-1} cm^{-1} [16] was used for *p*-nitroaniline, released from the hydrolysis, to obtain the initial velocity. The measurements were made at six substrate levels and repeated two or three times. The data were then analyzed graphically using a double-reciprocal or Lineweaver-Burk plot [17]. All the plots were linear and thus enabled us to calculate the Michaelis-Menten kinetic parameters [18]. V_{max} was given by the reciprocal of the intercept and K_m was equal to the ratio of the slope to intercept. k_{cat} was then calculated from the relation $k_{cat} = V_{max}/[\text{enzyme}]$. The kinetic parameters obtained from such graphical analysis have typical standard errors of 5–10% and repeated experiments are also statistically reproducible.

Thiobenzyl ester, as used in the present study, has been shown by Farmer and Hageman [19] to be useful in screening for protease in deficient mutants of *Bacillus subtilis*. The thiobenzyl ester has the advantage of being very much more stable than the *p*-nitrophenyl esters and presumably the *p*-nitrothiophenyl ester toward spontaneous hydrolysis at alkaline pH values. In addition, the use of Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) [20] (DTNB), to assay the thiol released during hydrolysis has provided a convenient continuous assay at visible wavelengths which is comparable in sensitivity to the most sensitive assay yet reported for chymotrypsin [21].

The hydrolysis of thiobenzyl ester was measured in 0.05 M KH_2PO_4-Na_2HPO_4 buffer (pH 7.0) at 25 ° C. The ester solutions were prepared in acetonitrile. Aliquots of the ester stock were then added to DTNB dissolved in buffer or buffer with desired cosolvent contents. The resulting levels of acetonitrile in all the assays ranged from about 2 to 6% (v/v). Backgrounds were measured, the enzyme added, and a rate obtained by following the increase in absorbance at 412 nm. An extinction coefficient of 13600 M^{-1} cm^{-1} [20] for the 3-carboxy-4-nitrophenoxide ion was used to calculate the initial velocity. The measurements were made at six substrate levels and k_{cat} and K_m were obtained from double-reciprocal plots as in the case of amide hydrolysis. The concentrations of DTNB were always maintained at about 5–10-times the ester concentrations used in the assay. Since only approx. 5% of the hydrolysis was measured, the concentration of DTNB was always in large excess.

The use of a reporting reaction to monitor the enzyme turnover rate needs further precautions when studying viscosity effects. We have performed the following control experiments in order to ensure that it is the enzyme turnover rate that we are actually measuring. We prepared two assays as in a typical kinetic run with identical substrate and enzyme concentrations. We then started the reaction in one assay with Ellman's reagent present and the other without. The benzyl mercaptan, released during hydrolysis, was produced at the same rate in both assays. There was a continuous increase in absorbance at 412 nm of the assay with Ellman's reagent because of the reporting reaction. The thiol released in the second assay without the Ellman's reagent was left unreacted and thus no absorbance change was observed. The enzyme reaction in both assays was left to proceed for about 30 min until hydrolysis had almost reached completion. The Ellman's reagent was then added to the second assay and the absorbance was measured. It was found that the second assay gave the same absorbance as the first almost instantaneously. We were not able to record such an absorbance increase within our mixing time, which was typically 10 s. The same procedure was then repeated in 40% (w/w) glycerol and the same phenomenon was observed. We thus did not observe the reporting reaction rate but the enzyme turnover rate.

3. Results

Table 1 lists the kinetic constants obtained in 0.1 M Tris-HCl buffer (pH 8.6) at 25 °C. The results are in good agreement with previously reported values.

Early evidence suggested that thiol esters were good substrates in chymotrypsin-catalyzed reactions [25]. The kinetics of the α-chymotrypsin-catalyzed hydrolysis of thiol esters have been compared to those of their oxygen counterparts [26,27]. These authors found that the absolute values of the rate constant for the acylation step are identical within experimental error for both esters at several pH values. Since in an S_N2 reaction, -SR group should be displaced at least 250-times faster than an -OR group [28,29], these results strongly suggest that oxygen and thiol esters are hydrolyzed via the same pathways, and that acylation consists of the formation and breakdown of a tetrahedral intermediate, the former being rate-limiting.

Table 1

Kinetic constants of subtilisin BPN′-catalyzed hydrolysis of *N*-suc-AAPF-pNA in 0.1 M Tris-HCl buffer (pH 8.6) at 25 °C

k_{cat} (s^{-1})	K_m (mM)	Reference
50.8 ± 1.5	0.14 ± 0.01	this work
50	0.14	22
57.0	0.17	23
44	0.207	24

Table 2 shows the kinetic constants of subtilisin BPN′ catalyzed hydrolysis of *N*-suc-AAPF-SBzl in 0.05 M phosphate buffer (pH 7.0) at 25 °C. We are not, however, aware of any reported kinetic parameters for thiobenzyl substrates with the same peptide sequence.

The hydrolysis of the synthetic substrate *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl-X by subtilisin BPN′ can be described by a two-step mechanism;

$$E + S \overset{K_s}{\rightleftharpoons} E.S \overset{k_2}{\rightarrow} \underset{\underset{P_i}{+}}{EA} \overset{k_3}{\rightarrow} E + A \qquad (1)$$

in which the kinetic parameters are defined as

$$k_{cat} = k_2 k_3/(k_2 + k_3); \; K_m = K_s k_3/(k_2 + k_3) \qquad (2)$$

where $K_s = (k_{-1} + k_2)/k_1$ is the dissociation con-

Table 2

Kinetic constants of subtilisin BPN′-catalyzed hydrolysis of *N*-suc-AAPF-SBzl in 0.05 M phosphate buffer (pH 7.0) at 25 °C

k_{cat} $(\times 10^{-3})(s^{-1})$	K_m (mM)
1.27 ± 0.09	0.13 ± 0.01

stant of the Michaelis-Menten complex. Many kinetic studies have demonstrated that while K_m (app) of specific amide substrates is a true binding constant, K_m (app) of specific ester substrates is a combination of an equilibrium constant and rate constants. Furthermore, k_{cat} of specific amide substrates is controlled by the acylation step, k_2, while k_{cat} of specific ester substrates is controlled by the deacylation, k_3 [12,30]. For the amide substrate used in this work, the ratio of k_3 (deacylation) to k_2 (acylation) has been reported to be 33 [31]. Based on the above discussion, the ratio of k_{cat} values of ester to amide substrate used for the present study should reflect the same ratio of k_3 to k_2 as found for the amide hydrolysis. This is because we have the same peptide sequence for both substrates. The acyl-enzyme intermediate is thus the same for both cases. The ratio of the k_{cat} for ester to that of amide is 25 and thus in fairly good agreement with the value reported by Wells et al. [31].

The similar K_m values obtained for the two substrates indicate that the binding properties are very similar in both cases. This is in agreement with the observation by Farmers and Hageman [19] that subtilisin BPN′ does not seem to have a secondary hydrophobic site capable of binding the thiobenzyl group, in contrast to α-chymotrypsin. The ratio of the two K_m values is given by eq. 2 as,

$$K_m(a)/K_m(d) = \{K_s(a)/K_s(d)\} \cdot \{k_2(d)/k_3\}$$

where a and d refer to acylation- and deacylation-controlled reaction, respectively. As $k_2(d)/k_3 \gg 1$, then $K_m(a)/K_m(d) \gg 1$ unless $K_s(a)/K_s(d) \ll 1$. $K_s(d)$ is probably $\gg K_s(a)$ because $k_2(a) \ll k_2(d)$ as $k_2(a) \ll k_3$ and $k_3 \ll k_2(d)$. In other words, the larger $K_s(d)$ is probably due to a much greater $k_2(d)$. It thus seems safe to conclude that our results on the present two substrates, together with other kinetic evidence are compatible with the enzyme turnover rate limited either by acylation (for amide hydrolysis) or deacylation (for thiobenzyl ester hydrolysis).

Figs 1 and 2 show the effects of a number of cosolvents on the K_m (app) and k_{cat} for the hydrolysis of N-suc-AAPF-pNA by subtilisin BPN′. The K_m values are roughly the same for all the cosolvents tested, except glycerol and ethylene glycol. The absence of any observed viscosity effects on K_m could be because the present reaction, which has a bimolecular rate of the order of

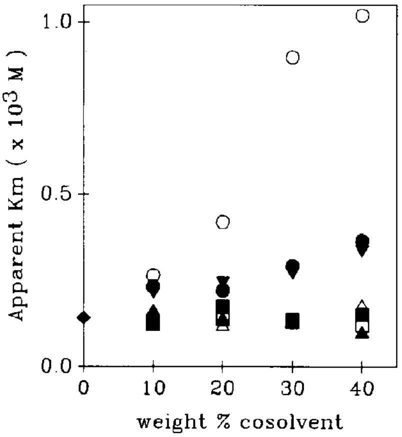


Fig. 1. Plot of apparent K_m ($\times 10^3$ M) as a function of cosolvent weight contents (% w/w) for subtilisin BPN′-catalyzed hydrolysis of N-suc-AAPF-pNA. The K_m values were determined at 25°C, in 0.1 M Tris-HCl buffer (pH 8.6) (◆), and buffer containing glycerol (●), ethylene glycol (○), sucrose (△), glucose (▲), fructose (□), glucose + fructose (■) and glycerol + glycine (▼).

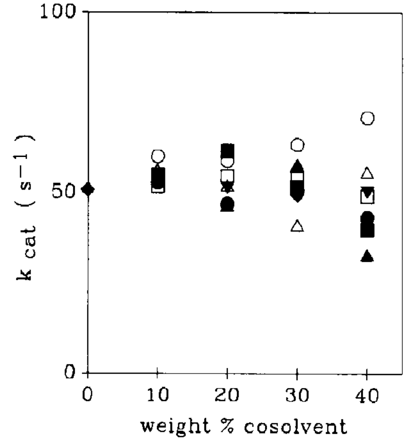


Fig. 2. Plot of k_{cat} (s^{-1}) as a function of cosolvent weight contents (% w/w) for subtilisin BPN′-catalyzed hydrolysis of N-suc-AAPF-pNA. The k_{cat} values were determined at 25°C, in 0.1 M Tris-HCl buffer (pH 8.6) (◆), and buffer containing glycerol (●), ethylene glycol (○), sucrose (△), glucose (▲), fructose (□), glucose + fructose (■) and glycerol + glycine (▼).

10^6, is not diffusion controlled by reactants' transport. There is a mild increase in the case of glycerol, up to about 2.5-fold at 40% (w/w). There is, however, 7-fold increase at the same weight content of ethylene glycol. One possible cause could be that there is a non-specific effect by glycerol and ethylene glycol, for example, on the productive binding volume at the active site. During the acylation step the P1 side chain (according to the terminology defined in ref. 32) moves deeper into the hydrophobic cleft as the substrate advances from the Michaelis-Menten complex to the tetrahedral transition state complex [33,34]. It has been shown that the optimal productive binding volume is about 160 ± 30 Å^3 [35]. The lower substrate affinity observed in the presence of glycerol and ethylene glycol probably indicates that the productive binding volume is smaller as part of the volume may be now occupied by these solvent molecules.

There is very little dependence of k_{cat} on the cosolvent contents. It is thus not useful to plot the apparent second-order rate constant, which is the ratio of k_{cat} to K_m, vs cosolvent contents because of the quite large nonspecific effects on K_m by glycerol and ethylene glycol. We have also observed the same k_{cat} in 20% (w/w) Ficoll 400, which gives a much larger viscosity than all the other solvents used. These observations on k_{cat}, together with those on K_m argue strongly that the present reaction is chemically limited. The applicability of viscosity analysis is worth further comment here. The large quantities of viscogenic solute required to modify the viscosity can cause changes in the enzyme structure. Such effects on enzyme structures would also manifest in any observed rate dependence on cosolvent contents. It is not likely in this case because k_{cat} is not sensitive to any of the cosolvents tested. On the other hand, the absence of viscosity effect could be due to a number of internal compensating mechanisms for the Kramers' type of friction, for example, proton activity, water activity and dielectric constant.

The pH dependence of k_{cat}/K_m for the present substrate hydrolysis shows a sigmoidal increase from pH 6 to 8 that reflects the titration of the catalytic His 64 with $pK_a = 7.1 \pm 0.1$ [36]. The pH profile remains relatively flat over the range of pH 8 to 10 and declines thereafter [37]. It is important to note that we are working at pH 8.6 and not in the transition region. Typical pH shifts within our working range of cosolvent contents are not more than 0.5 units after electrode correction according to Gelsema et al. [13]. Although such a shift appears to be quite large, it is still not in the transition region of the pH curve. The change in proton activity induced by cosolvents is not expected to have any effect on the observed enzyme turnover rates in our working pH range.

Another possible static cosolvent effect is the change in water activity of the bulk medium. As we are looking at the acylation step in the case of amide hydrolysis (as opposed to the deacylation step in ester hydrolysis), nucleophilic attack by water instead of Ser 221 on the scissile bond is very unlikely. The change in water activity is thus not expected to play any kinetic role in the acylation step.

It is well known that cosolvents like glycerol and ethylene glycol change the dielectric constant of the solution. The dependence of dielectric constant on glycerol and ethylene glycol is almost identical [38]. We are not aware of any published dielectric constant dependence on sucrose. However, it has been reported by Akerlöf [38] that the dependence on cane sugar is very similar to that on glycerol and ethylene glycol. They were all reported to decrease the dielectric constant of the solution. In order to test for possible effects by dielectric constant, we have performed measurements in glycerol with the dielectric constant of the medium compensated by glycine. The required concentration of glycine for each glycerol content is determined by the equation [39];

$$D(\text{glycine}) = D(\text{water}) + 22.6C \qquad (3)$$

The results in glycerol with glycine are also shown in figs 1 and 2. It is clear that there is no effect on the enzyme turnover by dielectric constant of the bulk medium. It can thus be concluded that there is no viscosity effect, whether in terms of reactant transport or Kramers' type of friction, on the acylation step of subtilisin BPN′ catalyzed hydrolysis of amide.

In contrast to the amide hydrolysis, the thiobenzyl ester reaction is clearly dependent on cosolvent contents. Figs 3 and 4 depict the K_m (app) and k_{cat} dependence on cosolvent contents. All the K_m values remain about the same for all the cosolvents tested, although they are somewhat higher than in buffer. On the other hand, k_{cat} is largely attenuated by 40% (w/w) cosolvents, except in the case of the polymer poly(ethylene glycol) (mol. wt. = 8000; PEG 8000). These observations argue strongly that the predominant effect of increasing viscosity is not manifested in the diffusion encounter processes. One would observe an increase in K_m with constant k_{cat} for enzyme reactions to be diffusion controlled. The present results in PEG 8000 are very similar to the case of cinnamoylchymotrypsin observed by Gerig and McLeod [40]. These authors found that the deacylation kinetics are not affected in 0.93 M polyvinylpyrrolidine, which has a viscosity almost 200-times greater than that of pure buffer. The fact that k_{cat} is insensitive to PEG 8000 strongly supports the contention that it is the microviscosity that we are observing. The possibility of the observed cosolvent effects on k_{cat} being due to enzyme structure changes is also unlikely because of the absence of such an effect for amide reaction, unless it is very specific to the deacylation step. We have performed control experiments, similar to those described for amide hydrolysis, to investigate other possible ways of rate attenuation by static effects of the cosolvent.

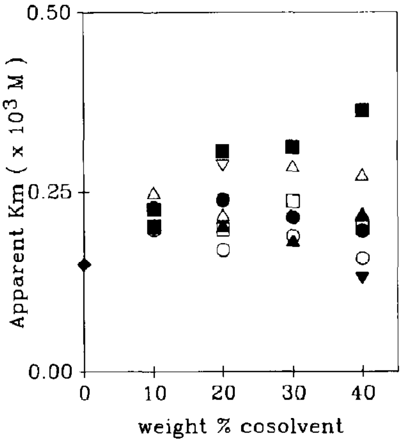


Fig. 3. Plot of apparent K_m($\times 10^3$ M) as a function of cosolvent weight contents (% w/w) for subtilisin BPN′-catalyzed hydrolysis of N-suc-AAPF-SBzl. The K_m values were determined at 25°C, in 0.05 M phosphate buffer (pH 7.0) (◆), and buffer containing glycerol (●), ethylene glycol (○), sucrose (△), glucose (▲), fructose (□), glucose + fructose (■), poly(ethylene glycol) (▽) and glycerol + glycine (▼).

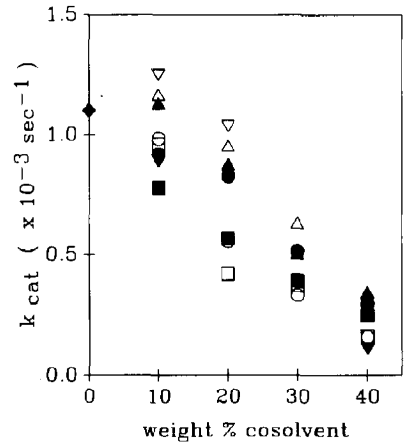


Fig. 4. Plot of k_{cat} ($\times 10^{-3}$ s^{-1}) as a function of cosolvent weight contents (% w/w) for subtilisin BPN′-catalyzed hydrolysis of N-suc-AAPF-SBzl. The k_{cat} values were determined at 25°C, in 0.05 M phosphate buffer (pH 7.0) (◆), and buffer containing glycerol (●), ethylene glycol (○), sucrose (△), glucose (▲), fructose (□), glucose + fructose (■), poly(ethylene glycol) (▽), and glycerol + glycine (▼).

As the measurements were carried out in 0.05 M phosphate buffer (pH 7.0), cosolvents like glycerol could easily affect the rates by either; (a) changing the proton activity or (b) shifting the pK_a of some ionizable groups on the protein molecule that are responsible for the catalytic hydrolysis. Fig. 5 shows our measured pH profile for the hydrolysis of N-suc-AAPF-SBzl in 0.05 M phosphate buffer, 10 and 30% (w/w) glycerol, in the pH range 6–7.5. K_m is relatively insensitive to proton activity for all three cases. There is, however, a mild dependence of k_{cat} on pH, about 1.5–2-times at most. It is important to note that the pK_a values for all the pH titration curves shown in fig. 5 are about the same. The presence of glycerol, up to 30% by weight or equivalently 4.7 M, does not introduce considerable shift in the pK_a of the ionization process responsible for the ester hydrolysis. The effects on k_{cat} by all the cosolvents are much larger and thus cannot be

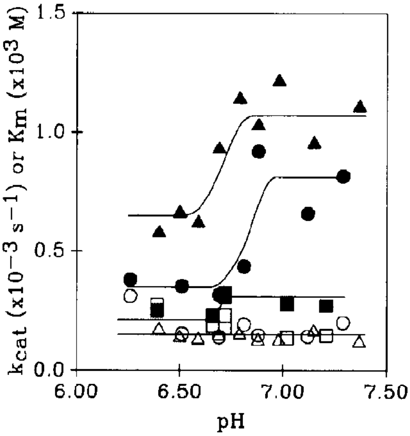


Fig. 5. Plot of k_{cat} ($\times 10^{-3}$ s^{-1}) and apparent K_m ($\times 10^3$ M) as a function of proton activity (pH) for subtilisin BPN′-catalyzed hydrolysis of *N*-suc-AAPF-SBzl in 0.05 M phosphate buffer (▲, △), and buffer containing 10% (w/w) (●, ○) and 30% (w/w) (■, □) glycerol, at 25°C. The solid and open symbols denote k_{cat} and K_m, respectively.

explained in terms of proton activity or pK_a shift alone.

The effects by dielectric constant of the bulk medium can be addressed directly using KCl. The required concentration can be determined from the equation [39];

$$D(\text{KCl}) = D(\text{water}) - 10C \qquad (4)$$

There are, however, possible effects by ionic strength when as much as 1 M KCl is added. In fact, we have observed an almost 4-fold decrease in k_{cat} at 1.14 M KCl but K_m remained constant and tended to be smaller when compared to buffer and cosolvent containing buffer (Ng and Rosenberg, unpublished observations). The 1.14 M KCl solution corresponds to the same dielectric constant as a 40% (w/w) glycerol solution. As a result, we have performed the same control experiments as described above for amide hydrolysis in order to resolve the effects by dielectric constant. The two tested cases in 10 and 40% (w/w) glycerol give approximately the same results as in buffer, but the values are slightly lower as shown in figs 3 and 4. It is possible that the rather high ($\sim$ 0.5 M) concentration of glycine used in 40% (w/w) glycerol could also contribute to viscosity.

One other possible effect that needs to be considered is the change in water activity. The fact that k_{cat} is insensitive to PEG 8000 argues against the present observed rate attenuations being solely due to change in water activity. On the other hand, the alteration in rate constants resulting from water activity change is likely to be within 10%, our standard experimental error. This can be reached from the following argument. Gibbs free energy is a function of the logarithm of the water activity. This, together with the fact that the logarithm of the relative rate change is a function of the free energy change, suggests that the relative rate and water activity change will have the same dependence on cosolvent contents. The 'relative' state here refers to the buffer system with no cosolvent. Such rate scaling with cosolvent is valid only, of course, if the change in rate constants is merely due to water activity change. The water activity data in the presence of PEG indicate very mild changes [41] although large and extended water-PEG network structures have been suggested [42].

Based on the above discussion of control experiments that are designed to test several possible cosolvent effects besides microviscosity, none of these alone could explain the rather large attenuation of bimolecular rate constants (k_{cat}/K_m) by cosolvents. These, together with the similar behavior in all the solvents tested, strongly argue for microviscosity. The major correction factor in the present case seems to be in the change of proton activity. We are, however, at this point only aware of such glass/calomel electrode correction reading for proton activity in glycerol, ethylene glycol and sucrose [13]. However, there is no reason to have vastly different pH corrections in the case of other sugars like glucose and fructose, as compared to sucrose. For reactions involving the formation of activated complex, the bimolecular rate constant is related to viscosity by [6];

$$k_b = A\eta^{-\kappa} \exp(E_a/kT) \qquad (5)$$

where E_a is the activation energy, A represents an empirical constant and κ denotes the viscosity coupling constant. Fig. 6 shows a replot of the logarithm of corrected (k_{cat}/K_m) vs $\log(\eta/\eta_0)$. The viscosity coupling constant is found to be

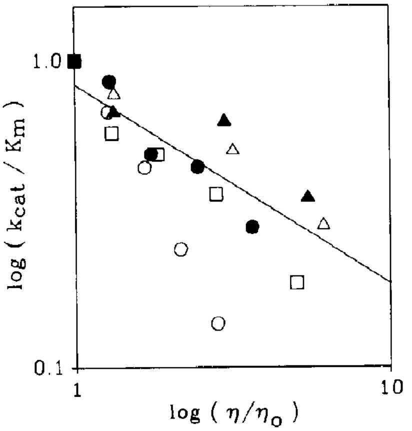


Fig. 6. Plot of log (k_{cat}/K_m) determined at 25 ° C, in 0.05 M phosphate buffer (pH 7.0) (■), and buffer containing glycerol (●), ethylene glycol (○), sucrose (△), glucose (▲), fructose (□), as a function of log(η/η_0). The ratio of k_{cat} to K_m is corrected for proton activity by comparing the values in the presence of cosolvents to buffer systems with the same proton activity. The values plotted here are thus not true absolute measured data but are relative numbers instead. The slope of such a plot represents the viscosity coupling constant and is 0.65 ± 0.11 for the present case. Note that the two data points at 30 and 40% (w/w) ethylene glycol are not included for calculating the viscosity coupling constant.

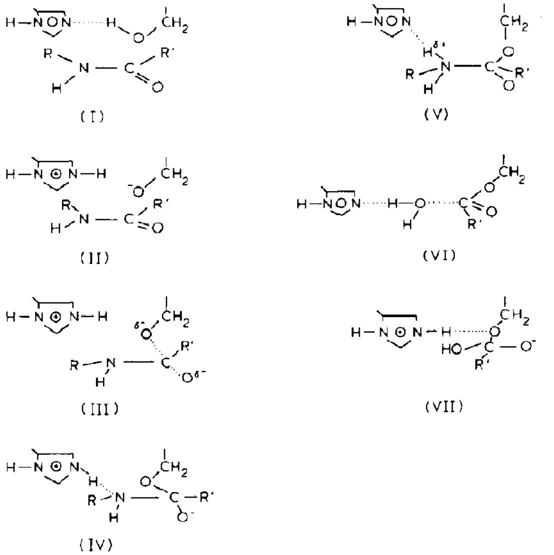
0.65 ± 0.11. This probably indicates that both local or decoupled and global or coupled dynamics are responsible for the reaction [43,44]. As seen in fig. 6, the case of ethylene glycol needs to be handled with more precaution. The data with ethylene glycol alone give a coupling constant greater than unity. The value of 1 is the limiting case and the rates cannot decrease faster than η^{-1} [44]. A coupling constant greater than unity is likely to reflect not only the influence of viscosity, but also the concentration dependence of additional effects. For example, Somogyi et al. [9] have observed a discontinuity in the acid-catalyzed proton exchange rate from Trp 63 of lysozyme in the presence of 30% (v/v) ethylene glycol. These authors argued that such observations can be rationalized in terms of the different thermal stability of proteins based on the data of free energy of transfer of indole side chain to ethylene glycol [45,46]. In addition, Rosenberg et al. [47] have reported a very different fluorescence quenching pattern in ethylene glycol which cannot be attributed to viscosity effects.

4. Discussion

Serine proteases are among the most widely studied enzyme systems. Although they exist as two families, the 'trypsin-like' and the 'subtilisin-like', they all have a similar catalytic site characterized by the serine, histidine and aspartate triad. These enzymes impart total rate enhancements of at least 10^9–10^{10}-times that of the nonenzymatic hydrolysis of amide bonds. An enzyme is believed to bind the transition state more strongly, greatly increasing its concentration and thus accelerating the reaction. Electrostatic free energy associated with the changes of charges of the reacting system has been shown to be important to achieve such differential binding properties of the enzyme [48]. A recent calculation on subtilisin based entirely upon electrostatic interaction [49] was found to agree with site-directed mutagenesis experiments [24,31] to within 1 kcal/mol. It is thus the different energetics in enzyme and solution environments that contribute to catalysis.

The question is, however, how is this difference in energetics achieved by enzymes? And to what extent do the protein and solvent dynamics contribute to such differential energetic properties? We shall discuss our results in light of a possible dynamic role for fluctuations of the environment. The term 'environment' used here has two different meanings. First, it refers to the immediate environment in which the reaction takes place. In other words, it corresponds to the enzyme active site. Second, it refers to the surrounding environment with which the reacting system is in contact. The relation between the two types of environments has important implications in regard to the reaction dynamics. The reacting system could be strongly coupled to internal vibrational modes, as manifested in the form of a fluctuating force along the reaction coordinate. When the system is in contact with a fluctuating medium like a viscous solvent, not only will the vibrations be influenced, but also an additional frictional force along the coordinate will arise. The effect of such frictional force is analogous to barrier recrossing as described by Kramers [6].

The chemical mechanism of subtilisin-catalyzed hydrolysis can be broken down to several proton-transfer steps as in other serine proteases [50] (see scheme 1);



Scheme 1.

In order to describe the proton-transfer reaction, the most obvious reaction coordinate would be the proton coordinate itself. However, the proton motion will be strongly coupled to internal or intramolecular vibrational modes in the reaction system. It may also be coupled to the environment via electrostatic forces, provided by polar solvent molecules or charged groups in the active site of an enzyme molecule. As a result, two other coordinates are also important for the proton-transfer probability. One is related to the separation between the acceptor and donor atom. The barrier height for proton transfer can be very dependent on such coordinate Q. Fluctuations in Q will provide a dynamical modulation of the transfer probability flux towards the product side along the reaction coordinate. Another important coordinate is a collective solvent coordinate s. It refers to solvent orientation polarization or alignment of polar solvent molecules.

Recently, Borgis and Hynes [51] have developed a model of proton transfer reactions based on these coordinates. These authors found that the numerical calculation for a simple model of symmetric intramolecular proton transfer, $AH^+ + A \rightarrow A + H^+A$, occurring in water can be well described by two fairly simple analytical formulas, which correspond to low- and high-frequency Q vibrations. The low-frequency Q vibrations correspond to energy comparable to kT. This condition can be satisfied at room temperature by systems with the A species involved in hydrogen bonds. However, when the As are farther apart and hydrogen bonding is negligible, the frequency of the Q vibration will be set by other stronger bonds in which A atoms participate. For example, if A is an oxygen atom, bonds such as CO or NO exist and then the frequency will tend to be higher. As also noted by these authors, these ideas could be highly relevant in enzyme systems although no direct application on enzymatic proton transfer has been carried out. Two arguments strongly support such a relevant link to enzyme systems. First, the proton-transfer barrier is very sensitive to the distance between the donor and acceptor site. The 'bring-together' approach facilitated by the enzyme structure could lead to rate enhancements not possible in the solution counterpart of intermolecular proton transfer. Second, the strong electrostatic coupling of the proton to the environment could be greatly increased by nearby preoriented polar ionic groups in an enzyme active site. We shall in the following attempt to test the usefulness of the Borgis-Hynes model based upon our present experimental findings.

We can now address the question of whether the present results can be rationalized in terms of a model based on fluctuations of the environment where the reaction takes place. The principal observations from the present work can be summarized as follows;

(1) The observed K_m values for both amide and ester hydrolysis are relatively insensitive to all the cosolvents tested, up to 40% by weight;

(2) The observed k_{cat} values for amide hydrolysis are independent of all the cosolvents tested, regardless of the nature of the cosolvent. The absence of dependence has been shown not to be due to a compensation by several cosolvent properties, for example, viscosity, proton activity and dielectric properties.

(3) The observed k_{cat} values for ester hydroly-

sis are attenuated by 40% (w/w) cosolvents. The rate attenuation cannot be explained by proton activity, shifts of pK_a, water activity or dielectric properties alone. A detailed correction procedure gives a viscosity coupling constant of approx. 0.7.

The absence of cosolvent effects on K_m and k_{cat} values for the amide reaction strongly suggests that the enzyme turnover rate could largely be determined by the binding properties provided by the preset environment of the enzyme active site. As this reaction is controlled by acylation, the rate throttle lies in the proton transfer occurring in either structure (I) or (IV) of scheme 1. Note that the acceptor nitrogen atom in either case is bonded to another carbon atom, which thus satisfies the conditions for the high-frequency Q regime of the Borgis and Hynes model. The consequence is that the activation energy is just the activation free energy for the solvent and is not determined by the barrier height of the proton coordinate. The solvent in this case is made up of the comparatively immobile dipoles and ionic groups within the enzyme. In other words, the differential energetics is basically achieved by transition state stabilization. This free energy is larger the less polar the solvent and the larger the distance over which the proton is transferred.

The absence of cosolvent effects on K_m values on the one hand, and largely attenuated k_{cat} values on the other, indicate that the enzyme turnover for ester hydrolysis does not depend on the binding properties as in the amide case. As deacylation is now the rate-limiting step, it should then be dependent on the proton-transfer step in structure (VI) or (VII) of scheme 1. There is, however, a difference between the two structures. For structure (VI), the proton is delivered to the histidine nitrogen by water after a hydrogen bond is formed. This falls into the low-frequency Q regime of the Borgis and Hynes model, while in the case of structure (VII), it is the breakdown of the tetrahedral intermediate by proton transfer from histidine to the serine oxygen. The acceptor oxygen atom is farther apart from the histidine and is bonded to the carbonyl carbon of the substrate. It thus satisfies the high-frequency Q criteria of the Borgis and Hynes model. Such a structural difference leads to different observed kinetic properties. As already described above, the high-frequency Q regime corresponds to activation purely controlled by activation of the solvent. On the other hand, the case for low-frequency Q is more complex. Although one still cannot identify the proton barrier height, the observed activation energy now contains one more term besides the solvent activation. The additional activation energy can be thought of as the cost of a fluctuation in Q for proton transfer to occur. A closer look at the chemical mechanism reveals that since water has to be bound to the active site, at least transiently, this process is likely to be facilitated by protein structural fluctuations. A good analogy would be the fluctuation in the form of transient hydration described for hydrogen exchange kinetics [9]. If the fluctuation of a protein matrix is essential to the activation process, we should be able to observe a viscosity coupling to the external fluctuating medium [52]. Our present experimental findings thus strongly favor the fluctuation picture. In addition, the fact that the present viscosity coupling constant is very similar to that observed by Somogyi et al. [9] for hydrogen exchange dynamics probably indicates that a very similar class of fluctuation is being utilized by subtilisin to control movements along the reaction coordinate in the hydrolysis processes. It is very likely that we are observing structure (VI) instead of (VII) for the ester reaction. In essence, this is because of the presence of this specific structure, which according to the Borgis and Hynes model, leads to an extra activation term in the form of fluctuation and thus the observed viscosity coupling.

The validity of Kramers' approach in describing viscosity dependence of enzyme reactions could have interesting implications on catalytic strategy adapted by enzymes. The bond making and breaking involved in the chemical step has an intrinsically sharp and high barrier. As a result, one would expect to find the Kramers' picture inappropriate. The enzyme is thus probably not just making the reaction barrier lower but also diffusive. One possible advantage of this could be that the enzyme substrate complex then becomes more or less trapped in the vicinity of the barrier top regime. In essence, this may greatly improve the efficiency of fluctuation-assisted barrier climbing.

More work along this line is certainly needed to support this statement.

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