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The coupling of catalytically relevant conformational fluctuations in subtilisin BPN′ to solution viscosity revealed by hydrogen isotope exchange and inhibitor binding

Kingman Ng [1,*] and Andreas Rosenberg

Department of Laboratory Medicine and Pathology, and Graduate Program in Biophysical Sciences, University of Minnesota, Minneapolis, MN 55455 (USA)

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Abstract

We have measured the tritium outexchange of subtilisin BPN′. A consistent and rather small group of hydrogens was isolated by their sensitivity to inhibitor binding. The viscosity dependence of exchange from these inhibitor protected hydrogens was then examined in 0.05 *M* MES buffer, pH 6.5 and 10 °C. The viscosity of the reaction medium was varied by added glycerol and ethylene glycol. The exchange rates were corrected to be compared at identical hydroxyl ion and water activity. The salient observation is the strikingly similar viscosity coupling behavior when compared to the deacylation step of ester hydrolysis catalyzed by the same enzyme (Ng and Rosenberg, Biophysical Chemistry, 39 (1991) 57). We have obtained a viscosity coupling constant of 0.68 ± 0.18 for hydrogen exchange in glycerol (cf. 0.65 ± 0.11 for deacylation in glycerol, sucrose, glucose and fructose); 1.67 ± 0.07 for outexchange (cf. 1.92 ± 0.09 for deacylation), in the presence of ethylene glycol. The two reactions are very chemically dissimilar, yet they show very similar viscosity coupling behavior. This together with the well established role of structural fluctuations in hydrogen exchange implies a similar role of structural fluctuations in the deacylation step of subtilisin BPN′ catalyzed ester hydrolysis.

Keywords: Hydrogen exchange; Inhibitor binding; Viscosity coupling; Subtilisin BPN′

1. Introduction

The binding of a ligand to the protein molecule is often the first step of an enzyme catalyzed reaction. A complete structural description of the protein ligand interaction is thus important for identifying the active site of the enzyme and in some cases the dominant kinetic intermediates. Once the active site is defined, the catalytic free energy advantage gained from the binding can then be derived (the Circe principle of W.P. Jencks [1]). Recent advances in site-directed mutagenesis techniques have provided an opportunity to determine the contributions of individual amino acid residues to this free energy advan-

[1] Present address: Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637.

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tage. These kinds of studies have revealed a lot of information on the type of the chemical driving force for enzyme catalysis, for example, hydrogen bond stabilization. The energetic throttle is provided via transition state stabilization. However, such studies generally do not consider the possible effects of the thermodynamic and structural fluctuations present in the protein matrix.

The importance of internal mobility of a protein as manifested in the form of structural fluctuations, which facilitate the protein ligand interaction, has gradually gained recognition. There is a rich variety of protein motions revealed by a number of spectroscopic and diffraction techniques. In general, these experiments have been carried out on systems of simple reactions. Hydrogen isotope exchange kinetics in particular has been very useful in elucidating the fluctuation properties of biological macromolecules. The well established feature of transient exposure of all the exchangeable groups in the native state enables one to look into practically all regions of the protein matrix. The behavior of the chemical exchange reaction and the effects of environment on the exchange rates reveal information concerning the nature of conformational fluctuations associated with these transients. The major drawback is that there is no direct relationship *per se* between the fluctuation properties as revealed by these studies and biological activity such as enzyme catalysis. In this article, we report the use of the hydrogen exchange technique to elucidate the properties of the specific fluctuations relevant to subtilisin BPN' catalysis.

In general, there are two basic experimental approaches to hydrogen isotope exchange studies. One could look at either the exchange kinetics of some specific residues within the protein, notably by NMR, or at the total exchange of the protein. However, the study of single proton exchange reaction provides only a fragmentary view of the whole protein dynamics picture. On the other hand, experimentally one finds that only a portion of the total isotope exchange can be measured under a particular set of conditions. This raises the question of assigning relevant physical meaning to the data. It is thus always better to monitor the total exchange kinetics but the analysis of the simultaneous exchange of many hydrogens is extremely difficult. In this investigation, we have adapted a modified version of the total exchange method [2]. We intend to isolate a specific class of hydrogens by blocking their exchange with inhibitor molecules. The exchange of those trapped hydrogens from the vicinity of the active site would describe fluctuations likely to mirror motion in the active site of the enzyme. These types of studies have been carried out successfully for several simpler protein/ligand systems [3].

In this article, we report the study of tritium outexchange kinetics of the serine protease, subtilisin BPN'. Trace labelling using tritium represents a minimal perturbation of the native protein structure. Our overall goal is to characterize the contribution of solvent coupled structural fluctuations to the exchange rate constants of the protected group in terms of a viscosity coupling constant. For cases where other solvent variables can be separated from viscosity, such viscosity coupling constant describes the extent to which the friction arising from dynamics within the protein matrix is coupled to solution viscosity. For this purpose, we have studied the viscosity dependence of outexchange of the inhibitor protected hydrogens in the presence of glycerol and ethylene glycol. We then compared the viscosity coupling constant of the exchange process with that obtained for subtilisin BPN' catalyzed hydrolysis of thioester [4]. Despite the fact that the two reactions have basically different chemical mechanisms, we observed very similar viscosity dependence for both reactions. Hydrogen exchange is base catalyzed, whereas a water molecule has to be delivered to the subtilisin active site for deacylation. These observations imply a direct role for structural fluctuations in the deacylation step of the subtilisin catalyzed hydrolysis of thioesters.

2. Materials and methods

Crystallized subtilisin BPN' was obtained from Sigma Chemical Company and used without further purification. Phenylboric acid was obtained from Aldrich Chemical Company. Tritiated water

(25 mCi/ml) was obtained from Du Pont, NEN Research Products. Sephadex G-25 of medium grade, was from Pharmacia, Inc. Glycerol and ethylene glycol were all of reagent grade obtained from commercial sources.

The exchange rate was measured by the following four steps:

(1) Typically 150 mg/ml of subtilisin BPN' was prepared in 0.05 *M* Tris base with the pH adjusted to 8.0 by HCl. An aliquot of tritiated water was added to the protein solution and incubated at room temperature for 30 minutes. The total activity in the inexchange sample was from 2.27 to 2.50 mCi/ml.

(2) The excess solvent tritium was then removed by gel filtration of the inexchange solution through a Sephadex G-25 column (1.5 × 12 cm) at 4 °C. The Sephadex column was prequilibrated with the desired buffer with or without the inhibitor, 25 m*M* phenylboric acid. Typical sample volume applied to the column was from 0.5 to 1.0 ml. Buffers used were 0.05 *M* Tris-HCl for samples at pH 8.0; 0.05 *M* sodium phosphate for samples at pH 7.0 and 0.05 *M* hemisodium (2-[*N*-morpholino]ethane-sulfonic acid (MES) for samples at pH 6.0 and 6.5. The pH was measured at room temperature and the quoted pH's refer to these values. This first separation step brings the protein to the desired set of conditions for outexchange.

(3) For cases without phenylboric acid, outexchange continued for a predetermined length of time. An aliquot (0.2 ml) was then removed and solvent isotope that had accumulated was removed by a second gel filtration step. The eluate was partitioned, part for scintillation counting, and part for determining the protein concentration, normally by measuring the optical density (O.D.) at 280 nm using an extinction coefficient of $3.22 \times 10^4\ M^{-1}\ \mathrm{cm}^{-1}$ [5]. The rate profile for the exchange was then constructed by the time dependence of the number of unexchanged isotope labels on the protein, *H*. The quantity $H(t)$ was determined by [6];

$$H(t) = \frac{CPM \times 111}{CPM^0 \times P} \qquad (1)$$

where CPM^0 denotes the number of counts per minute per unit volume in the inexchange sample, *CPM* the number of count per minute per unit volume at each time point, and *P* the protein concentration.

(4) The kinetic influence on the exchange rate by the inhibitor was studied as follows; we have used a reversible inhibitor, phenylboric acid which has a dissociation constant of $2.31 \times 10^{-4}\ M$ [7]. The first separation step was now carried out in the presence of 25 m*M* phenylboric acid. The eluate was incubated with the 25 m*M* phenylboric acid for another 30 minutes at 10.0 °C. An aliquot was then removed and subjected to a second gel filtration. This second separation step removed from the protein the inhibitor and also the labelled solvent molecules that had accumulated during the incubation period. The outexchange reaction can then be carried out as described before. For experiments in the presence of cosolvents, the eluate from the second separation step was mixed with a cosolvent stock solution to give the desired cosolvent content before doing the outexchange. The pH or hydrogen ion activity of the samples after addition of the cosolvents was measured and corrected for changes in the junction potential according to [8] in order to obtain true pH readings. With such correction, we were then able to maintain a true constant hydrogen ion activity in all cosolvent mixtures.

3. Results

3.1 The effect of inhibitor on hydrogen exchange in subtilisin BPN'

The hydrogen exchange from subtilisin BPN' was measured in 0.05 *M* Tris-HCl at pH 8.0, 10 °C, with and without 25 m*M* of the inhibitor, phenylboric acid. The inexchange was carried out in the same buffer but at room temperature for 0.5 to 1 hour in order to achieve a measurable statistical level of labelling. These inexchange conditions also represent a minimum extent of autolysis during the inexchange period. Figure 1 shows the relative amount of intact enzymes as a function of time based on an FPLC analysis. Here "intact" also refers to full enzyme activity.

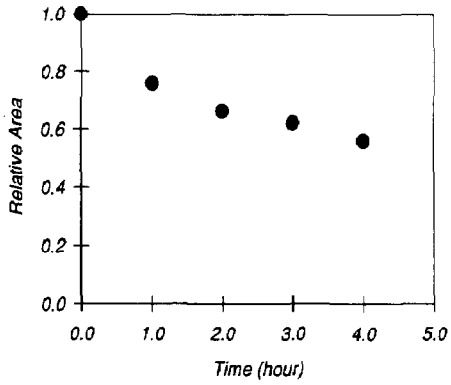

Fig. 1. The relative amount of intact enzyme as a function of time, at 25 °C pH 6.2, based on an FPLC analysis [9] and expressed as the relative area of the peak corresponding to the intact enzyme. The FPLC analysis was done on a Mono S HR 5/5 cation exchange column (Pharmacia) equilibrated with buffer A (10 m*M* phosphate, pH 6.2). The protein sample was loaded onto the column and then eluted by increasing the percentage of buffer B (10 m*M* phosphate, pH 6.2 containing 0.5 *M* NaCl) to 15% in a linear gradient over 30 minutes at a flow rate of 1 ml/min.

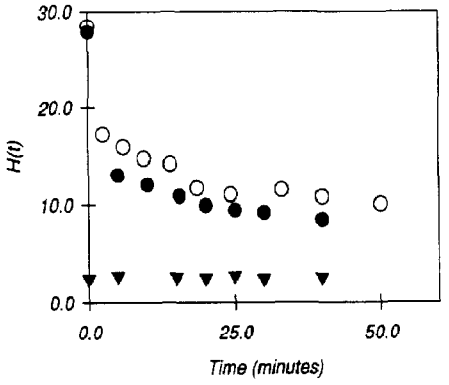

Fig. 2(a). Plots of the outexchange parameter $H(t)$ as a function of time t with (●) and without (○) 25 m*M* phenylboric acid. The outexchange was done in 0.05 *M* Tris-HCl buffered at pH 8.0, 25 °C. Also shown is $H(t)$ as a function of time t after incubation with 25 m*M* phenylboric acid for 24 hours (▼). The zero time for such exchange refers to the point when the inhibitor is removed.

All the peaks from the FPLC analysis were pooled to check for enzyme activity and only one major peak was found to be catalytically active. The zero time refers to the time when the enzyme is first dissolved in solution. As seen from Fig. 1, FPLC analysis shows that about 80–90% of the protein remains intact and fully active at these inexchange conditions.

Figure 2(a) shows the outexchange parameter $H(t)$ as a function of time t with and without the inhibitor. The salient feature here is the clear difference in $H(t)$ between the two cases, although the difference is only a few hydrogens. Such a difference becomes quite steady after the loss of the very fast protons at earlier times. The relatively fast protons could very well be located on the protein surface and contribute little information for our present purpose. The few hydrogens protected in the presence of inhibitor are thus related to the enzyme active site, which we intend to focus on. The blocking of the outexchange of some of the hydrogens is reversible as the inhibitor, phenylboric acid, is a reversible inhibitor. We have observed, as seen in Fig. 2(a), that after 24 hours of incubation with the inhibitor, almost all the isotope label was lost and no noticeable exchange was observed.

For each site in the protein the number of hydrogens remaining unexchanged at time t can be represented as $H(t) = \exp(-kt)$. For a protein with n exchangeable sites the total exchange is simply the sum of individual sites; $H(t) = \Sigma \exp(-k_i t)$. It is known that the rate constants can be distributed over seven or more powers of ten [10]. That is, the present time window for observation (minutes) only permits kinetic examination of a small class of hydrogens of the total rate distribution function. This can be visualized

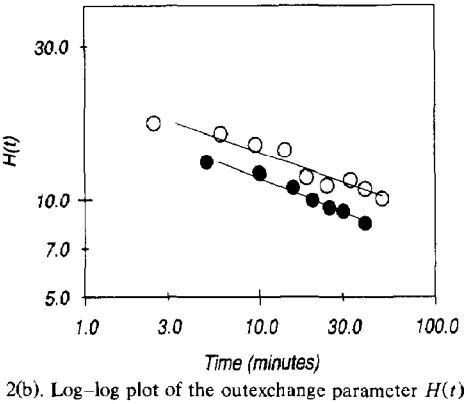

Fig. 2(b). Log–log plot of the outexchange parameter $H(t)$ as a function of time t. Same experimental condition as in (a).

by plotting the data in the form of log $H(t)$ versus log t, as shown in Fig. 2(b). The linearity of such a plot points to a power law distribution of the rate constants within the section of the total distribution curve we observed [11]. It is the nature of the partial labelling process that only sites with similar rate constants contribute to the observed exchange process. To be more specific, the hydrogens we are observing here probably represent a small portion of the total power law distribution function. It is important to note that the slope and linearity of the plot does not change considerably in the presence of inhibitor. The inhibitor concentration is fixed at 25 mM and typical protein concentration after first separation is approximately 1–2 mM. Such ratio of ligand concentration to the inhibitor dissociation constant indicates that 96 to 99% of the enzyme has a ligand depending on the choice of pH [12]. This is crucial for the present investigation so that we are always looking at approximately the same group of hydrogens with and without the inhibitor.

3.2 The outexchange of the protected tritium labeled hydrogens

The tritium labelling technique is of a statistical nature and there could be day-to-day variation in determining the exchange parameter H. It is important to firmly establish that there is indeed a definable group of hydrogens, the exchange of which is protected by the inhibitor in a reversible manner. In order to test for the absence of artifactual variation, we carried out the following control experiment. The outexchange was first followed in the presence of 25 mM phenylboric acid. The sample was then divided into two portions: One had the phenylboric acid removed and outexchange was then followed afterwards. The other half was left unchanged and the outexchange at later times in the presence of phenylboric acid proceeded as before. It is clear from Fig. 3(a) that the outexchange time profile splits at the sample dividing point. There are sites which can now exchange which would have otherwise been blocked in the presence of phenylboric acid.

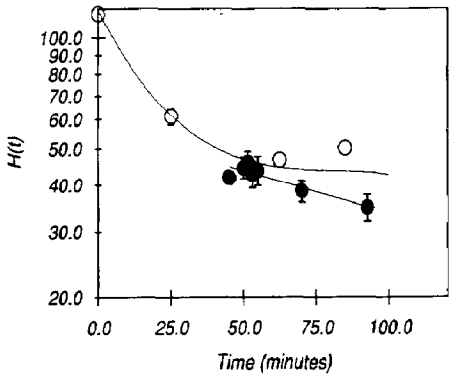

Fig. 3(a). The logarithm of $H(t)$ for the whole exchange experiment done at pH 6.0, 10 °C is plotted versus time t. The outexchange was first followed for 45 minutes in the presence of 25 mM phenylboric acid. The sample was then split into two portions. One portion had the phenylboric acid removed and again the outexchange followed. Further outexchange in the presence of phenylboric acid was followed with the other portion of the reaction mixture. The solid symbol (●) represents exchange without and the open symbol (○) represents exchange with inhibitor.

Having established the presence of a group of hydrogens, the exchange of which is reversibly blocked by the inhibitor, we then proceeded to study the outexchange of these hydrogens. The outexchange from the protected group was started by a second separation step after an incubation in 25 mM phenylboric acid for 30 to 45 minutes. Hydrogen exchange rate is well known to be

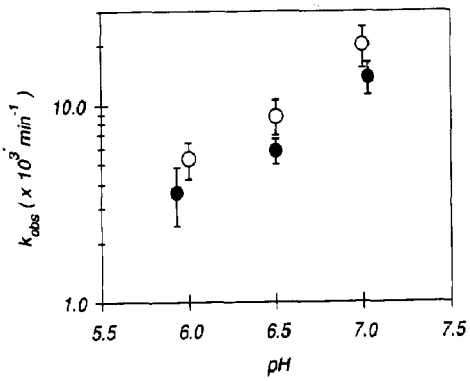

Fig. 3(b). Apparent first-order rate constants ($\times 10^3$ min^{-1}) of outexchange in subtilisin BPN' at three different pH's, 10 °C, with (●) and without (○) 20 wt.% glycerol. The sample was incubated with 25 mM phenylboric acid for 30 to 45 minutes. Outexchange was then followed after gel filtration to remove the inhibitor.

dependent on pH. The apparent rate constant for isotope exchange is comprised of three terms [6]:

$$k_{obs} = k_0 + k_H[H^+] + k_{OH}[OH^-] \quad (2)$$

where k_0 is the rate constant for direct exchange with water, k_H and k_{OH} are the rate constants for the specific acid and base catalyzed exchange respectively. In most cases, the direct exchange contributes very little to k_{obs} so that the outexchange is either acid or base catalyzed. For exchange in proteins, the observed rate usually goes to a minimum around pH 3 to 4. We have chosen to work at pH 6.5, which is also optimal for subtilisin catalysis. Our choice of pH relegates us to a predominately base catalyzed pathway. The rate constant for outexchange is thus pseudo first order and depends on the hydroxyl ion activity. According to eq. (2) and observations of exchange from homopolymeric peptides, a 1-unit change in pH should lead to a 10-fold change in the observed rate constant. In practice this is rarely the case for proteins and the parameter $\delta = \Delta \log k_{obs}/\Delta pH$ is typically found to be about 0.7–0.8. Hilton and Woodward [13] found a value of about 0.6 for the exchange of some individual residues in pancreatic trypsin inhibitor, which could be followed by NMR. We have measured the outexchange of the inhibitor protected hydrogens at three different pH's, i.e. 6.0, 6.5 and 7.0. A single rate constant was adequate to describe the outexchange in all cases which is in accord with the premise that we are looking, due to the choice of experimental conditions, at a rather small group of hydrogens with similar rate constants. The observed rate constants were plotted in Fig. 3(b) as a function of pH. The present data indicate a value of about 0.58 for the δ parameter. It is sufficient for the present purpose that we are on the rising (hydroxyl ion catalyzed) side of the pH exchange curve. We have also determined the exchange rates at three different pH's in the presence of 20% (by weight) glycerol. The rates as shown in Fig. 3(b) although attenuated, follow a rising pattern very similar to the case of plain buffer. Here the δ parameter is 0.55. In addition, we have shown previously that the pK_a for the ester hydrolysis catalyzed by subtilisin BPN' is not shifted by 30% (w/w) glycerol [4].

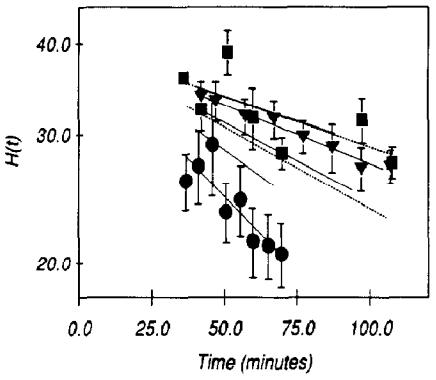

Fig. 4. The logarithm of the number of hydrogens remaining unexchanged, plotted as a function of time during an outexchange experiment carried out at pH 6.5, 10 °C. The buffer, 0.05 *M* MES, was mixed with varying amounts of cosolvent. Solid lines represent experiments with glycerol as cosolvent, 0, 10, 20, 30, 40 wt.%. We have entered the individual data points with error bars only for the experiments with 0 and 40 wt.% of glycerol, (●) and (▼) respectively. The data in the presence of 30 and 50 wt.% ethylene glycol are represented by broken lines. The individual data points with error bars are presented only for the 50 wt.% run (■). The lines represent least square fits to the data points. The error seems to be independent of cosolvent content except in the case of 50 wt.% ethylene glycol, where the scatter, as seen, increased by a factor of 2.

All these observations combined with the premise that we are looking at hydrogens related to the subtilisin active site imply that any major shift in the pH minimum by glycerol is very unlikely. This is probably due to the fact that glycerol is preferentially excluded from the protein hydration sphere [14]. The local concentration of glycerol on the protein surface is approximately one quarter of the bulk concentration.

3.3 The effects of cosolvents

The base catalyzed outexchange from the inhibitor protected group of hydrogens was measured in 0.05 *M* MES buffer, at pH 6.5, 10 °C and in the presence of glycerol and ethylene glycol. The logarithm of $H(t)$ is shown as a function of time t at various cosolvent contents in Fig. 4. Exchange rates were determined as before from the slope of the semi-logarithm plots. If the first time point could not be determined precisely enough, the slope was calculated without the first

point. This did not greatly affect our results, although the deviation of the slope could sometimes reach 30%. The salient feature is the dependence of the outexchange rates on the presence of cosolvents. The observed exchange rate is given by;

$$k_{obs} = k_{OH} a_{OH} \tag{3}$$

where we have now used the hydroxyl ion activity instead of concentration. This expression can be rewritten as

$$k_{obs} = k_{OH} K_W / a_H \tag{4}$$

where K_W is the ion product of water. That is, the second order rate constant is given by:

$$k_{OH} = k_{obs} a_H / K_W \tag{5}$$

Although the experiments were carried out at fixed hydrogen ion activity, the ion product K_W was also changed by the presence of cosolvents. In other words, the cosolvent dependence of the second order rate constant k_{OH} does not necessarily follow the dependence measured for k_{obs}. The correction for a change in K_W can be carried out as follows; if we define the water solution without cosolvents as the standard state, we can set all the activities, a_H, a_{OH} and a_W to unity, where a_W is the water activity, a_H and a_{OH} are defined as before. Furthermore, we can adjust the scale so that the value of K_W is unity instead of 1.01×10^{-14} (298 K, 1 atm). With the above definition for the standard state and scale adjustment, we can express K_W as;

$$K_W = a_H a_{OH} / a_W \tag{6}$$

According to our definitions $K_W = 1$ for the pure water state. Any correction due to the presence of cosolvents can be calculated as a ratio relative to this state. The outexchange was measured at an identical pH for all the samples and the corrected second order rate constant normalized to the same hydrogen ion activity is given by;

$$k_{OH} = k_{obs} (a_H a_{OH} / a_W)^{-1}. \tag{7}$$

The corrected and normalized rate constants are shown in Table 1.

All experiments were performed at fixed, corrected hydrogen activity. As a result, if there is no major correction for the ion product of water, then k_{OH} will have to follow almost the same dependence as k_{obs} on cosolvent contents. This is practically the case with glycerol, as seen in Table 1. However, the case of ethylene glycol is very different, as there is a large correction factor for the ion product of water.

Table 1

The cosolvent dependence of the experimental apparent first-order rate constant (k_{obs}, in min^{-1}), the correction factor of K_W and the corrected second order rate constant (normalized to the same hydrogen ion activity) for tritium outexchange of inhibitor protected hydrogens in Subtilisin BPN' determined in 0.05 *M* MES buffer, at pH 6.5, 10 °C. The correction factor for the change of the ion product in glycerol is from [15]; while that for ethylene glycol is from [16]

Cosolvent	η/η_0	k_{obs} ($\times 10^3$ min^{-1})	$\frac{a_H a_{OH}}{a_w}$	k_{OH} ($\times 10^3$ min^{-1})
Buffer	1.00	8.76 ± 1.81	1.00	8.76 ± 1.81
Glycerol				
10 wt.%	1.33	6.50 ± 0.87	0.98	6.63 ± 0.88
20 wt.%	1.84	4.79 ± 0.46	0.99	4.84 ± 0.46
30 wt.%	2.67	3.09 ± 0.44	1.02	3.03 ± 0.43
40 wt.%	4.11	3.67 ± 0.25	1.05	3.49 ± 0.24
Ethylene glycol				
30 wt.%	2.40	4.96 ± 1.78	2.63	1.89 ± 0.68
50 wt.%	4.30	3.14 ± 1.44	4.03	0.78 ± 0.36

4. Discussion

The question we intend to address in this investigation concerns the effects of solvents on functionally important protein dynamics (coined functionally important motion, FIM, by Frauenfelder and coworkers [17]). The coupling of solvent dynamic properties and the interior dynamics of the protein is crucial in formulating an appropriate model for both protein function and the reaction mechanism [18,19]. We are essentially interested in elucidating how the energy generated through fluctuations of the medium is dissipated/coupled in the protein, and in particular among the functionally important motions. Here we intend to develop an approach using viscosity coupling to link the internal conforma-

tional fluctuations of the protein as revealed by hydrogen isotope exchange kinetics to FIM as defined by sensitivity to inhibitor binding.

There are two features of the inhibitor, phenylboric acid, which make it particularly suitable for the present investigation. The inhibition process has been shown to be very fast [12]. It was reported that the time course of the inhibited subtilisin reactions, at low substrate concentration, was strictly first-order. If the binding or debinding of the inhibition was slow, deviations from first-order kinetics would have been observed. The rapid equilibration allows us to study the rate constants as influenced by protein structural movement *per se*, instead of observing the effects of cosolvents on the inhibitor on-off rate. Another factor worthy of mention here is the geometry of inhibitor binding at the active site of subtilisin. The geometry of the phenylboric acid inhibitor at the active site of subtilisin BPN' has been determined [20]. There is a covalent bond between O_γ of the catalytic serine 221 and the boron atom of the inhibitor. Furthermore, the boron atom is coordinated tetrahedrally. The imidazole side chain of histidine 64 is, at the closest approach, more than 4 Å from the boron atom and cannot be covalently bonded to the inhibitor. The histidine 64 side chain, however, helps to stabilize the complex by a hydrogen bond with the boric acid hydroxyl group occupying the leaving group site. This structure is equivalent to the tetrahedral intermediate proposed for the enzymatic hydrolysis of the substrate peptide or ester bond. All these observations suggest that the inhibitor behaves as a substrate-like binding agent. This is crucial for selecting hydrogens related to the subtilisin active site. In addition, any structural fluctuations associated with the outexchange of these hydrogens can be directly linked to the catalytic process.

Hydrogen isotope exchange kinetics has proven to be useful in elucidating structural fluctuations in macromolecules. Besides the hydrogens on the protein surface, there is a large portion of exchangeable hydrogens in the protein interior. It has been well established that the transient exposure of the exchangeable groups in the native state is facilitated by structural fluctuation. These fluctuations are possibly subject to the influence of the Brownian motion of solvent molecules and could have effects on reactions taking place inside the proteins. It has been suggested that enzyme catalysis in general is triggered by solvent induced structural fluctuations [21]. Thus a full description of rate phenomena in proteins should include protein structural dynamics, solvent dynamics and their possible interactions. A straightforward approach to address this question is to study the effect of solvent viscosity on reactions where one of the reactants is a part of the protein matrix. The construction of a possible frictional force term and the search for underlying mechanisms from the measured viscosity dependence has, however, always been found to be difficult.

In principle, one can fit the viscosity dependence to any mathematical formulation. It has been observed in a number of experiments (4,22–26] that the reaction rates generally follow a power law dependence on the solvent dynamic viscosity (η). A coupling or scaling constant can be defined as;

$$\kappa = \delta \log K(X)/\delta \log(\eta/\eta_0), \tag{8}$$

where $K(X)$ is the rate constant for the reaction rate observed to be a function of the cosolvent contents, X. The constant, κ, measures the rate attenuation in the presence of $X\%$ by weight of cosolvent. The rates in a solution of cosolvent content $X\%$ by weight and viscosity η are always compared to the corresponding rate in a solution of viscosity η_0 containing no cosolvent. The main difficulty in interpreting the results is in distinguishing between the thermodynamic (chemical activity) and dynamic (viscosity) factors imposed on the protein by the solvent. Other possible solvent effects can be minimized by using several different solvents. The coupling constant (κ) obtained, if independent of the solvent, can be assigned physical meaning [27]. Reactions with κ close to 1 are those associated with fluctuations tightly coupled to the solvent, while reactions with κ close to zero are characterized by very little coupling [28,29]. There are basically two types of solvent coupled fluctuations. The first type are the large scale fluctuations, for example hinge bending motions of two domains. These

motions, although of large amplitude, are relatively slow and are thus as a rule irrelevant to the vibrational motion along the reaction coordinate. The other type of fluctuations are governed by a harmonic barrier and the reaction rates linked to such motions are expected to follow Kramers' equation [30]. On the other hand, solvent independent modes are also possible for exchange of thermal energy among the internal degrees of freedom in a protein matrix via local structural mobile defects [31]. In summary, the types of structural fluctuations associated with the rate phenomena are subject to different coupling to the bulk solvent as manifested in the viscosity coupling constant, κ. Based on this premise, the present work aims to clarify the contribution of protein structural fluctuations to the deacylation step of subtilisin catalyzed hydrolysis by comparing the κ obtained for deacylation to that of the exchange of the inhibitor protected hydrogens. If the viscosity coupling behavior is similar for the two reactions, the types of fluctuations involved will be similar and *vice versa*.

Two general mechanisms have been proposed to describe how the encounter between the hydroxyl ion as catalyst and the exchanging site occurs [6]; one is via local unfolding of the protein (EX_2 mechanism) and the other is a penetration of the catalyst into the protein (reverse EX_2 mechanism). The EX_2 mechanism exposes the exchange site to bulk solvent where the exchange then takes place. On the other hand, in the reverse EX_2 mechanism the exchange occurs via conformational rearrangements that allow access to the exchange site by diffusion processes without disrupting the protein structure. Both mechanisms can be formally represented by the schematic equation

$$\mathrm{NA} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{A} \xrightarrow{k_3} \text{exchange}, \qquad (9)$$

where NA is the non-accessible state and A is the accessible state where exchange takes place. The apparent rate constant can then, for conditions $k_2 \gg k_3$, be expressed as

$$k_{app} = K_{eq} k_3$$

The equilibrium (K_{eq}) and rate constant (k_3) involved have different meaning for the two mechanisms. The K_{eq} for the EX_2 mechanism represents the thermal or partial thermal unfolding equilibrium, whereas k_3 is very similar to the rate constant observed for the exchanging group in bulk solution. For the reverse EX_2 mechanism, K_{eq} refers to the distribution of water and catalyst ion between the bulk and protein phase, and k_3 is no longer similar to that observed with small peptides in the bulk solution. On the other hand, it is more likely to be related to the Kramers type of rate expression in the high viscosity limit, as the exchange reaction is now taking place in the protein matrix. It is thus clear that the two exchange pathways predict different viscosity coupling behavior [25,32]. For the EX_2 mechanism, the effects of viscosity, if any, on the rate constant k_3 can be adequately determined using data from experiments with small peptides and amino acids. In other words, the cosolvent effect we see represents a shift in the conformational equilibrium represented by K_{eq}. The cosolvent effect presents itself often in the form of a power law and appears mostly in cases of slowly exchanging hydrogens. For the reverse EX_2 mechanism, the effect we expect to see is on the protein phase *per se*. It is not likely that the cosolvent introduces a conformational change in the present case. There is no direct evidence for these changes induced at the concentration of glycerol we use. The observed viscosity dependence then points to a possible coupling of internal structural movement to external viscosity. The crucial point is, however, not whether EX_2 or reverse EX_2 is the correct model. Both models presented above depend explicitly on structural fluctuations as providing the means for the transient solvent contact [6]. The question is, if by comparing the viscosity coupling behavior of deacylation to similar properties observed for the exchange of inhibitor protected hydrogens, can we imply a similar role for conformational fluctuations in enzyme catalysis?

We shall now address the apparent viscosity dependence of our data. Because of the rather short inexchange time and the experimental observation window, we have emphasized in the result section that we are observing a consistent and rather small group of hydrogens. The appar-

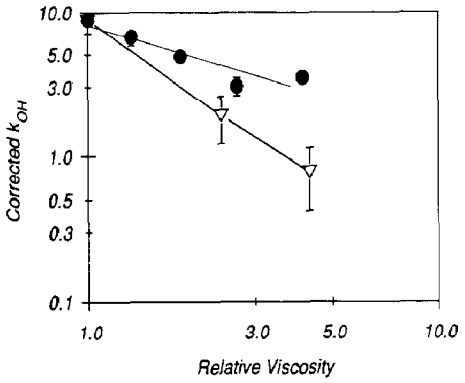

Fig. 5. Plots of logarithm of the corrected exchange rate k_{OH} (see eq. 7 in the text) as a function of logarithm of (η/η_0). The outexchange was followed in 0.05 *M* MES buffer, buffer containing glycerol (•) and ethylene glycol (▽) at pH 6.5,1 0 °C after incubation with 25 m*M* phenylboric acid for 30 minutes.

ent viscosity dependence of the exchange rates and catalytic turnover can be compared by the viscosity coupling constant κ, defined by eq. (8). The logarithm of the corrected exchange rate k_{OH} is shown as a function of $\log(\eta/\eta_0)$ in two different solvents in Fig. 5. The values of the viscosity coupling constant κ for the two cosolvents used can be obtained from a least-square analysis of the slope of the log–log plot and are listed in Table 2. We have also in Table 2 listed the κ's from our recent viscosity study of the same enzyme system, the subtilisin catalyzed hydrolysis of a thioester [4].

The salient feature of the contents of Table 2 is the similarity of the viscosity coupling constants we have determined for hydrogen exchange to similar constants observed for the deacylation step of the subtilisin catalyzed ester hydrolysis. All the mechanisms proposed for hydrogen exchange, depend on transient solvent contact facilitated by structural fluctuations. The significance of similar fluctuations in enzyme catalysis is, however, not clearly established. Different cosolvent properties are expected to exert recognizably different effects on the process under investigation depending on the nature of coupling between the protein and solvent. We have in this investigation, among a number of possible cosolvent effects, made corrections for the effect of a change in the chemical activity of hydrogen ions, hydroxyl ions and water. This allows us to assign the observed effects to change in viscosity. Also in the case of subtilisin catalyzed hydrolysis of thioester, we reached the conclusion that the results can be adequately explained by microviscosity effects on the unimolecular deacylation step. Although the two reactions can be formally described by the schematic eq. (9), the chemical mechanisms are basically different. The hydrogen isotope exchange reaction is catalyzed by hydroxyl ion resulting in a negatively charged intermediate [6]. On the other hand, deacylation involves a series of events. First, a water molecule has to be delivered to the enzyme active site and then activated by the imidazole via general base catalysis. This leads to the formation of the tetrahedral intermediate, which in turn breaks down by expulsion of the enzyme via imidazolium catalyzed protona-

Table 2

The viscosity coupling constant κ observed in two different reactions of subtilisin BPN': (i) Base catalyzed outexchange of inhibitor protected hydrogens, at pH 6.5, 10 °C; and (ii) acylation and deacylation limited hydrolysis of peptide bonds at pH 8.6, 7.0 and 25 °C. κ's of hydrogen exchange are from this work, while κ's of peptide hydrolysis are from the previous work [4].

Protein reaction	pH	Temperature (°C)	Cosolvent	κ
Base catalyzed outexchange				
inhibitor protected hydrogens	6.5	10	glycerol	0.68±0.18
			ethylene glycol	1.67±0.07
Peptide hydrolysis				
acylation limited	8.6	25	glycerol, ethylene glycol, sucrose, glucose, fructose	0
deacylation limited	7.0	25	glycerol, sucrose, glucose, fructose	0.65±0.11
			ethylene glycol	1.92±0.09

tion of the serine O_γ, leaving the acid product [33]. Despite the chemical dissimilarity, we are still seeing a very good correlation between the two reactions in terms of the viscosity coupling constant κ, to the extent that we can even compare the data for each individual solvent. Such good correlation of viscosity coupling behavior between the two reactions allows us to conclude that the coupling constant we observed in the deacylation step of subtilisin BPN' catalyzed hydrolysis of thioester has real physical meaning. The κ is with high probability expressing a frictional force along the reaction coordinate due to the structural movement of the protein matrix.

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