Solvent Isotope Effects on the Onset of Inhibition of Porcine Pepsin by Pepstatin†

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ABSTRACT: Pepstatin is a slow and tight-binding inhibitor of pepsin. Preincubating enzyme and inhibitor in H_2O and in D_2O in the absence of substrate generates an *inverse* solvent isotope effect of $^Dk = 0.69 \pm 0.06$ on the apparent first-order rate constant for the decay in enzymatic activity. Proton inventory analysis of the inverse isotope effect suggests a single transition-state proton with a fractionation factor of 1.41 ± 0.05. In contrast, combining enzyme with inhibitor and substrate (Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe) simultaneously along with observing the decay in enzymatic activity during catalytic turnovers generates a *normal* solvent isotope effect of $^Dk = 1.25 \pm 0.09$. Proton inventory analysis of the normal isotope effect suggests a single reactant-state proton with a fractionation factor of 1.46 ± 0.03. These two experimental designs are often considered equivalent, but the differences in isotopic data require that the pathway for onset of pepstatin inhibition in the absence of substrate must be different from the pathway in the presence of substrate. In the former, the inhibitor can only bind to free enzyme; in the latter, the inhibitor is hindered from binding to free enzyme because of competition with substrate but can bind to intermediate forms of enzyme generated during catalytic turnovers, downstream from enzyme–product complexes.

Porcine pepsin displays a normal solvent isotope effect on the maximal velocity, with no isotope effect on V/K, for hydrolysis of Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe (referred to below as a "fast" substrate because $V/K > 10^7$ M^{-1} s^{-1}); moreover, this normal isotope effect on catalysis has a linear proton inventory consistent with a single proton transfer (Rebholz & Northrop, 1991). In contrast, "slow" substrates (*i.e.*, $V/K = 10^4$–10^6 M^{-1} s^{-1}) such as Leu-Leu-Ala-Leu-Phe-*p*-nitro-Phe-Arg-Leu (Rebholz & Northrop, 1991) or *N*-(trifluoroacetyl)-Leu-Phe (Hunkapillar & Richards, 1972) express much larger isotope effects on both V and V/K, which in added contrast display curved proton inventories suggestive of more than one proton transfer in the transition state. To account for these differences, Rebholz and Northrop postulated that pepsin obeys an isomechanism in which the enzyme finishes proteolysis in a different form than when it started and that the isotope effect with the fast substrate arises from an isotopically-sensitive step within the isomerization of free enzyme. Because isomechanisms may express characteristic patterns of dead-end inhibition (Rebholz & Northrop, 1994), the kinetics of inhibition of pepsin by pepstatin were examined in search of confirmatory evidence for the pepsin isomechanism. Assays were conducted as a function of varied concentrations of substrate in the presence and absence of inhibitor. However, when reciprocal rate constants for the onset of inhibition were plotted against reciprocal concentrations of substrate of H_2O and compared to similar data obtained in D_2O, the lines crossed (unpublished results). A normal solvent isotope effect at high concentrations of substrate crossed over to an inverse isotope effect at low concentrations, an observation which could not be accounted for by a single origin whose expression was concentration-dependent. Consequently, the study of pepstatin inhibition kinetics as a function of varied concentrations of substrate was abandoned in favor of contrasting the presence and absence of substrate on the solvent isotope effects on the onsets of inhibition. Proton inventories were performed and are here analyzed in a new way. Normally, a single proton inventory is performed and analyzed to determine the number of protons involved and whether the isotope effect originates in a ground state or a transition state. In the present study, four proton inventories [two published previously, Rebholz and Northrop (1991)] from one enzyme-catalyzed reaction are examined and compared. Numbers and origins are again determined but for a different purpose: that of establishing similarities and differences between inventories in order to identify and locate separate steps within a more complex kinetic mechanism. The necessary precision is more stringent in the former because the data often stand alone; in the latter, the similarities and differences are supplemented with other data.

EXPERIMENTAL METHODS

Materials. Procine pepsin and pepstatin were purchased from Sigma, Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe from Bachem, CA, and D_2O from Cambridge Isotope Laboratories. Concentrations of pepstation were calculated from weighings and using a formula weight of 685.9. Concentrations of pepsin were estimated from titrations with pepstatin.

Kinetics of Pepstatin Binding during Preincubation. An OLIS-Cary 14 spectrophotometer interfaced to a computer was used to assay pepsin after intermittent preincubation with pepstatin. Preincubations were performed in 1 cm semi-microquartz cuvettes in a volume of 1 mL at pL 4.0 (*i.e.*, pH or pD) buffered with 0.04 M formate at an ionic strength of 0.1 M achieved by addition of KCl. Incubation mixtures contained 12 nM pepsin and from 28.8 to 146 nM pepstatin. The temperature of cuvettes was maintained at 25 ± 0.1 °C with a specially-crafted thermojacketed cuvette holder connected to a circulating bath. As a function of varied incubation times, substrate (Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe) was added to each cuvette to a final concentration of 200 μM, and the initial velocities of remaining enzymatic activity were measured by monitoring the cleavage of the *p*-nitropheny-

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lalanine–norleucine bond at 310 nm as described by Inouye and Fruton (1967).

Kinetics of Pepstatin Binding during Catalysis. An OLIS U.S.A. stopped-flow system attached to the OLIS-Cary 14 was used to observe the onset of inhibition during catalytic turnover. One syringe of the mixing system contained 267 μM substrate and from 58.4 to 583 nM pepstatin; the other contained 24 nM pepsin. Both were buffered at pL 4.0 at 25 ± 0.1 °C with 0.04 M formate at an ionic strength of 0.1 M achieved by addition of KCl. Reactants were mixed, and the cleavage of the *p*-nitrophenylalanine–norleucine bond again was monitored at 310 nm for upwards of 60 s (longer times resulted in signal drift due to diffusion of unmixed reactants) with the most significant data collected in less than 15 s. Data for protein inventory analysis were obtained at 120 μM substrate and 6 nM pepsin in D_2O at 0, 21.62, 42.40, 62.33, 81.50, and 99.40 g-atom % deuterium, estimated by volumes of mixing.

Data Processing. Kinetic data were analyzed using RA-GASSEK (regression and graphical analysis for steady-state enzyme kinetics), a computer program employing the nonlinear regression routine of Duggleby (1984). Initial velocities were obtained from normal progress curves by fitting digitized absorbencies as a function of time to the integrated form of the Michaelis–Menten equation described by Duggleby (1985), shown in eq 1:

$$t = \frac{Y_t - Y_0}{v_0} + \frac{2v_0/t_{1/2} - (Y_\infty - Y_0)}{v_0[1 - \ln(4)]}\left(\frac{Y_t - Y_0}{Y_\infty - Y_0} + \ln\left(1 - \frac{Y_t - Y_0}{Y_\infty - Y_0}\right)\right) \quad (1)$$

where v_0 is the initial velocity and Y_0, Y_t, and Y_∞ are the absorbencies at time zero, time t, and infinite time, respectively. Initial velocities obtained following first-order decay of enzymatic activity during preincubation experiments were fit to eq 2:

$$Y_t = Y_0 e^{-kt} \quad (2)$$

where Y_0 and Y_t represent the initial and residual activity, respectively. Progress curves for slow, tight binding of pepstatin during catalytic turnovers also were fit to eq 2, but with Y_0 and Y_t representing absorbencies at 310 nm.

To evaluate proton inventories arising from multiple transition states, first-order rate constants were fit to Kresge formalism of the so-called Gross–Butler equation shown in eq 3 (Kresge, 1964):

$$k_n = k_H(1 - n + n\phi^T)^P \quad (3)$$

where n is the fraction of deuterium in the waters, ϕ^T is the deuterium fractionation factor of the transtion state, k_H is the rate constant for the onset of inhibition in H_2O, k_n represents observed rate constants in mixtures of D_2O and H_2O, and P is the number of protons contributing to the isotope effect. Similarly, the parallel equation for inventories arising from multiple reactant states is shown in eq 4:

$$k_n = \frac{k_H}{(1 - n + n\phi^R)^P} \quad (4)$$

where ϕ^R is the deuterium fractionation factor of the reactant state. Attempts to fit data to the complete Kresge equation with ground and transition states resulted in fractionation factors not significantly different from a value of 1.

RESULTS

The slow onset of inhibition of pepsin by pepstatin in the absence of substrate was monitored at different concentrations of excess inhibitor. All experiments displayed a first-order decay in enzymatic activity. Shown in Figure 1 are results obtained with 12 nM enzyme and 28.8 nM inhibitor, where the fitted first-order rate constant[1] is $k_H = 0.035 \pm 0.002$ s^{-1}. The first-order rate constant is larger in D_2O than in H_2O, giving an inverse solvent isotope effect of $^Dk = 0.69 \pm 0.06$. Shown in Figure 2 is the effect of varying the concentration of pepstatin on both the first-order rate constant for the onset of inhibition and the solvent isotope effect. The rate constant increases as a linear function of increased concentrations of inhibitor (intercept = –0.003 ± 0.01; slope = 0.00276 ± 0.00017), but the solvent isotope effect is independent of the concentration of inhibitor.

The slow onset of inhibition of pepsin by pepstatin during catalytic turnover also displayed first-order kinetics. Figure

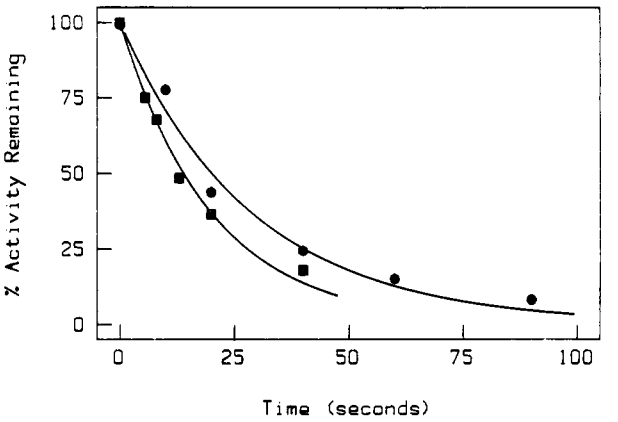


FIGURE 1: Time-dependent inhibition of pepsin by pepstatin during preincubation. The decay in enzymatic activity was measured in H_2O (●) and D_2O (■). The lines were drawn from fittings to a first-order decay represented by eq 2.

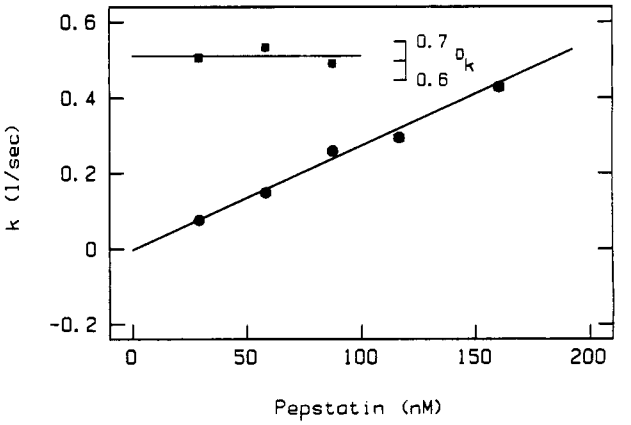


FIGURE 2: Kinetics of the onset of inhibition of preincubated pepsin as a function of the concentration of pepstatin. Apparent first-order rate constants (k) were determined from experiments similar to those shown in Figure 1 using higher concentrations of pepstatin and were fit to a straight line (●). Isotope effects (■) at low and moderate pepstatin concentrations were also determined and compared to a horizontal line. The accelerated rate of decay in D_2O precluded reliable estimates of rate constants at high concentrations of inhibitor.

[1] The data shown represent the inhibitor in least excess, where the onset of inhibition might be expected to deviate from a simple first-order decay due to a significant decrease in the concentration of free inhibitor as the enzyme–inhibitor complex accumulates. However, no systematic trend in the residuals of fittings to multiple experiments was detected, although the data points at the far right in Figure 1 could, by themselves, be interpreted as indicating a falling off of inhibitor potency. In addition, the linearity of Figure 2 indicates that if the estimates of the first-order rate constants from Figure 1 were underestimated, then the underestimate must be small and within the experimental error. Apparently, the fitted rate constant is most sensitive to the initial loss in enzymatic activity where $[I]_{free}$ has changed the least.

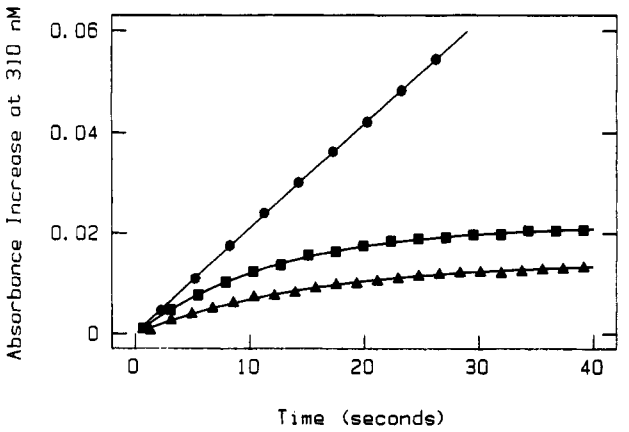


FIGURE 3: Time-dependent inhibition of pepsin by pepstatin during catalytic turnover. Progress curves of pepsin catalysis in the presence of pepstatin were obtained in H_2O (▲) and D_2O (■) and fit to eq 2. In the absence of inhibitor (●), the data were fit to eq 1. The uninhibited line is essentially linear within the region shown but decays according to a normal progress curve at longer times.

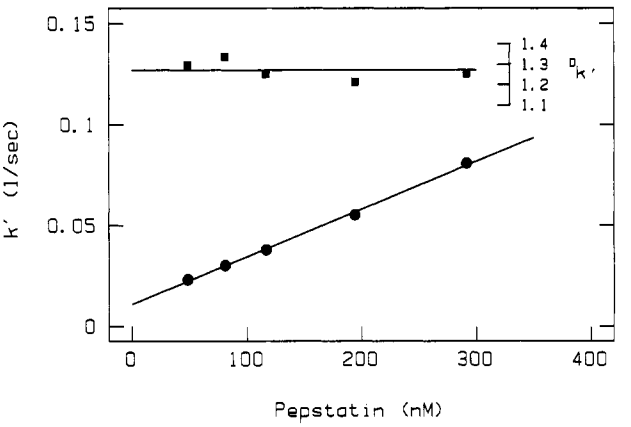


FIGURE 4: Kinetics of the onset of inhibition of catalytic pepsin as a function of the concentration of pepstatin. Apparent first-order rate constants (k') were determined from experiments similar to those shown in Figure 3 using higher concentrations of pepstatin (●) and fitted to a straight line. Isotope effects (■) were also determined and compared to a horizontal line.

3 contrasts a linear increase in absorbance as a function of time during hydrolysis of excess (*i.e.*, $27K_m$) Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe with nonlinear increases in absorbance when pepstatin is present. The latter curves were fit to a first-order decay without correction for changes in substrate concentrations during the time course, resulting in $k_H = 0.081 \pm 0.001$ s^{-1}. The decay of the rate of absorbance increase is slower in D_2O than in H_2O, giving a normal solvent isotope effect of $^Dk = 1.25 \pm 0.09$. Figure 4 shows that variation of the concentration of pepstatin during catalytic turnover similarly causes a linear increase in the apparent first-order rate constants (intercept = 0.0125 ± 0.0004; slope = 0.000219 ± 0.000005) while the solvent isotope effect on the onset of inhibition is again independent of the concentration of inhibitor (inset Figure 4: intercept = 1.251 ± 0.019; slope = –0.235 ± 0.374).

First-order rate constants for onsets of inhibition also were measured in mixed isotopic waters and examined by the proton inventory method of Schowen (1978). Data obtained during preincubation experiments were fit to eq 3 giving $P = 0.43 \pm 0.38$ protons and are shown in Figure 5. An attempted fit to eq 4 did not converge, but a minimal variance ($\sigma^2 = 0.44$) was obtained as P approached infinity. Because P must be a whole number, the data also were fit to eq 3 with P fixed at 1 and 2 protons. The former regressed to less variance (σ^2 = 0.36 and 0.40, respectively) and gives $k_H = 0.036 \pm 0.001$ s^{-1} and $\phi^T = 1.41 \pm 0.05$ for one proton. Similarly, data obtained during catalytic turnover were fit to eq 4 giving $P = 0.60 \pm 0.39$ protons and are shown in Figure 6. An attempted fit to eq 3 did not converge, but a minimal variance ($\sigma^2 = 3.6$) was obtained as P approached infinity. Regressions on eq 4 converged to less variance with P fixed at 1 than at 2 ($\sigma^2 = 2.76$ and 3.06, respectively) and gives $k_H = 0.064 \pm 0.001$ s^{-1} and $\phi^R = 1.46 \pm 0.03$ for one proton.

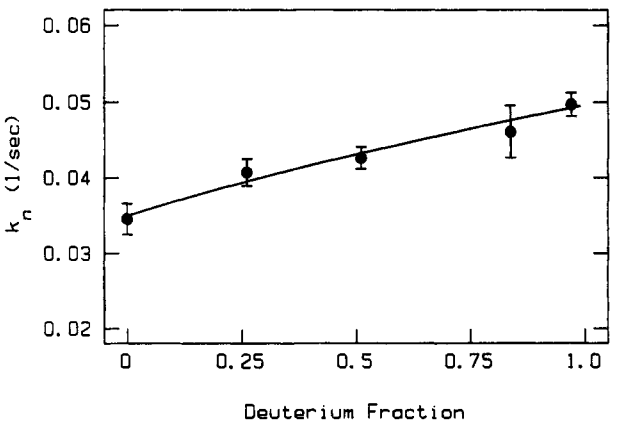


FIGURE 5: Transition-state proton inventory of solvent isotope effects determined during preincubation. Experiments similar to those shown in Figure 1 were repeated in mixed isotopic waters. The line was drawn from a fit to eq 3, where $P = 0.43$ protons.

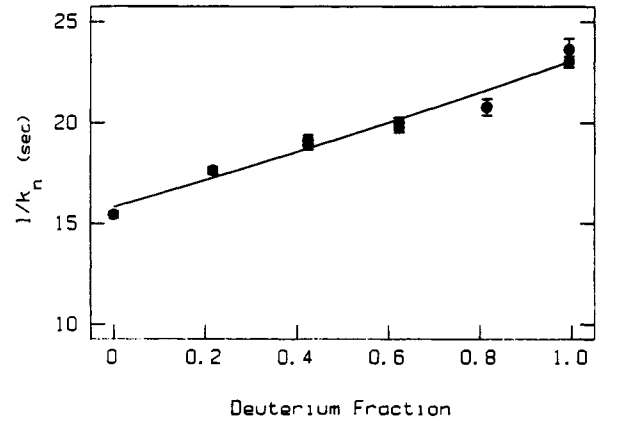


FIGURE 6: Reactant-state proton inventory of solvent isotope effects determined during catalytic turnovers. Experiments similar to those shown in Figure 2 were repeated in mixed isotopic waters. The line was drawn from a fit to eq 4, where $P = 0.6$ protons.

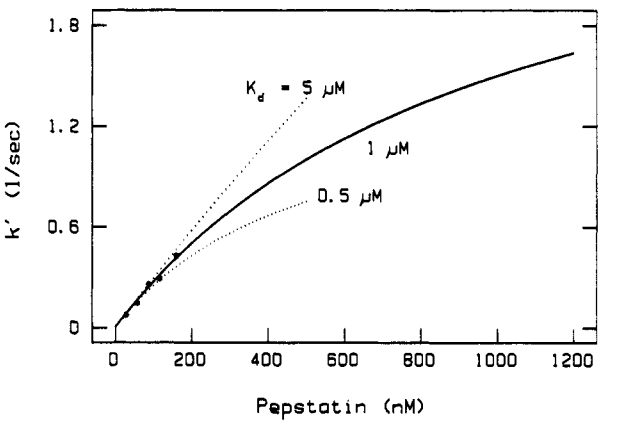


FIGURE 7: Simulated slow onsets of inhibition. The solid line was drawn from eq 5 using the following values for rate constants: $k_1 = 10^8$ M^{-1} s^{-1}, $k_2 = 100$ s^{-1}, $k_3 = 3$ s^{-1}, and $k_4 = 0.000136$ s^{-1}. The dotted lines were drawn with k_2 either increased to 500 s^{-1} or decreased to 50 s^{-1}. Data points are the reciprocals of those shown in Figure 3.

DICUSSION

The apparent first-order kinetics for the onset of inhibition of pepsin by pepstatin shown in Figure 1 confirm the presence of a slow step associated with the binding of the inhibitor to the enzyme. Three general mechanisms may be considered to account for slow onsets of inhibition (Frieden, 1970; Cha, 1975; Duggleby *et al.*, 1982; Morrison & Walsh, 1987):

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} FI \qquad \text{(mechanism A)}$$

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} FI \qquad \text{(mechanism B)}$$

$$E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} F + I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} FI \qquad \text{(mechanism C)}$$

In mechanism A, enzyme and inhibitor combine in a slow step; in mechanism B, enzyme and inhibitor first combine followed by a separate slow step, EI → FI; and in mechanism C, enzyme and inhibitor combine after a separate slow step, E → F. Plots of apparent rate constants versus concentrations of inhibitor for these three mechanisms generate functions consistent with mechanism A, a straight line, mechanism B, a normal rectangular hyperbola, and mechanism C, an inverse rectangular hyperbola. The linear plot in Figure 2 of rate constants for onsets of inhibition of pepsin versus pepstatin is consistent with mechanism A and inconsistent with mechanism C but can be reconciled with mechanism B (see below and Figure 7). However, mechanism A (generally considered unlikely) is inconsistent with the observation that the apparent rate constants for onsets of inhibition of pepsin by pepstatin are larger in the presence of substrate than in the absence of substrate (*i.e.*, k_H = 0.081 s^{-1} versus 0.035 s^{-1}, respectively, with [pepsin] = 12 nM and [pepstatin] = 28.8 nM; similar differences were observed at higher concentrations of inhibitor). If the diffusion-controlled combination of enzyme and inhibitor were rate-limiting in and of itself, adding substrate could only decrease the onset of inhibition by competing with inhibitor for free enzyme, which was not observed. It follows that a separate slow step must either precede or follow the binding of pepstatin to free pepsin.

The onset of inhibition for mechanism B is governed by eq 5 (Frieden, 1970; Cha, 1975; Duggleby *et al.*, 1982; Morrison & Walsh, 1987):

$$k_{obs} = \frac{k_4(1 + [I]/K_1)}{1 + [I]/K_d} \qquad (5)$$

where $K_d = k_2/k_1$ is the dissociation of the collision complex, EI, and $K_i = k_2k_4/[k_1(k_3 + k_4)]$ is the dissociation constant of the tightened complex, FI. Equation 5 yields a rectangular hyperbola with an intercept equal to k_4 and a horizontal asymptote equal to $k_3 + k_4$. However, with $K_d > [I] > K_i$, eq 5 reduces to:

$$k_{obs} = k_4 + k_4[I]/K_i \qquad (6)$$

which is an equation for a straight line. Figure 7 illustrates simulations of eq 5 on the basis of the K_i for pepstatin of 4.57 × 10^{-11} M (Workman & Burkitt, 1979) and the assumption of a diffusion-controlled combination of enzyme and inhibitor, $k_1 = 10^8$ M^{-1} s^{-1}. In order to obtain a rectangular hyperbola which passes through the data points, K_d must be near 1 μM and not outside the range of 0.5–5 μM. This value is consistent with the results of Rich and Bernatowicz (1982) who found that pepstatin analogs which did not display slow binding had values of $K_d > 0.01$ μM. The curve in Figure 7 shows that the apparent linearity of the data points is not inconsistent with the hyperbolic function of eq 5 at [pepstatin] ≪ K_d and therefore mechanism B is not excluded by the data in Figure 2.

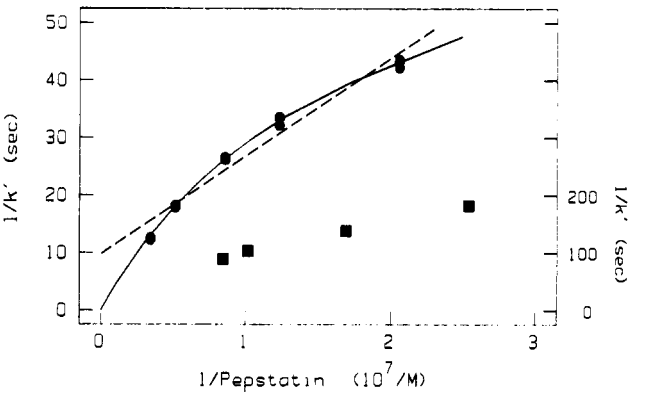


FIGURE 8: Double-reciprocal replot of onsets of inhibition versus pepstatin. Data (●, left axis) are from Figure 4, obtained with Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe, the fast substrate, at 27K_m; data (■, right axis) are from Figure 3B of Rich and Sun (1980), obtained with Phe-Gly-His-*p*-nitro-Phe-Phe-Ala-Phe-OMe, a slower substrate, at 9K_m. The solid line was drawn from reciprocals of the linear fit of Figure 4; the dashed line was drawn from a linear fit to the reciprocal data points.

The rate of the onset of inhibition of pepsin by pepstatin during catalytic turnover in the presence of saturating Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe is much faster than expected. Not only are the observed rate constants greater at similar concentrations of pepstatin in the presence versus absence of substrate as noted above but correction for substrate binding by multiplying observed values of k' by $(1 + [S]/K_m)$, the usual correction for slow-binding competitive inhibitors, results in an even larger disparity. Similarly, the onsets of inhibition are much faster in the presence of the fast substrate at greater saturation than has been reported earlier with a slower substrate at lower saturation (see legend to Figure 8); the latter should result in higher steady-state concentrations of free enzyme, giving greater access to a simple competitive inhibitor. The faster reactivity of the inhibitor with a faster, more saturating substrate indicates that pepstatin binds to a second form of pepsin when substrate is present that is different from the free form of pepsin. Consistent with this interpretation are the differences in isotope effects and proton inventories on the rate constants for onsets of inhibition in the presence and absence of substrate.

It is significant that the rate constants in Figure 4 are a linear function of the concentration of pepstatin because this result is in conflict with the finding of Rich and Sun (1980) who also examined the kinetics of the onset of pepstatin inhibition of pepsin during catalytic turnovers. They plotted *reciprocal* rate constants for the onset of inhibition against *reciprocal* concentrations of pepstatin and reported a linear relationship. Their data are reproduced in Figure 8 and contrasted against a reciprocal plot of the data from Figure 4. Rich and Sun (1980) interpreted their linear relationship as indicative of saturation kinetics for a competitive inhibitor binding to free enzyme according to mechanism B and eq 5 in which $k_4 \ll k_3$, slope = k_2/k_1k_3, and intercept = $1/k_3$; values for K_d of pepstatin and other inhibitory analogs were then corrected for substrate using $(1 + [S]/K_m)$. However, because manual mixing was employed in their experiments,

the concentration range of pepstatin had to be severely restricted to within the narrow range of 39–118 nM. Consequently, the resulting data appear nearly linear in both a normal or double-reciprocal plot. By contrast, the use of a rapid mechanical mixing device makes possible the study of a wider range of concentrations of pepstatin, and this results in data which are clearly hyperbolic in the double-reciprocal plot of Figure 8.

Solvent isotope effects on the onsets of inhibition during preincubation provide considerably more mechanistic information than do their time courses alone, which have not previously been explored. To illustrate, if mechanism A were operative during preincubation, little or no isotope effect should be expressed on the onsets because simple binding of ligands to the active sites of enzymes is altered very little by isotopic substitutions (LeReau *et al.*, 1989). However, if a solvent isotope effect were present as a result of the cumulative effect of the displacement of a large number of water molecules during binding (Rand, 1992), then multiple protons should be expressed in the transition-state proton inventory. Therefore, the observation of a significant isotope effect on the onset of pepstatin inhibition of pepsin, originating from a single proton, provides independent evidence to rule out mechanism A. Similarly, mechanism C should generate isotope effects which are concentration-dependent, varying from an equilibrium isotope effect to a kinetic isotope effect. This is because k' approximates $k_1 + k_2$ of mechanism C at low and k_1 at high inhibitor concentrations (Duggleby *et al.*, 1982). Hence, the observed concentration-independent isotope effect in Figure 2 provides independent evidence to eliminate mechanism C.

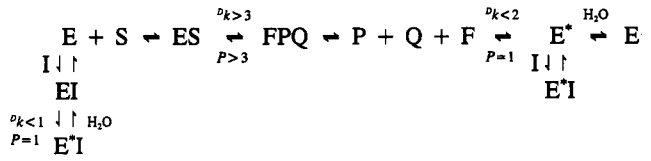
An obvious candidate for the slow step in the remaining mechanism B is the dissociation of the tightly-bound water molecule which presumably functions as the second substrate during peptide hydrolysis. Rich and Sun (1980) have postulated that the displacement of this active site water molecule by the 3(*S*)-hydroxyl group of the statine residue of pepstatin is responsible for very tight binding of pepstatin to pepsin, on the basis of comparative kinetics of structural analogs including dideoxypepstatin. A similar slow displacement of water was postulated to explain the slow onset of inhibition of thermolysin by phosphonamidate and phosphanate tripeptide analogs (Bartlett & Marlowe, 1987). This postulate is neither supported nor refuted by the solvent isotope effects on the onsets of inhibition. Nevertheless, because the binding of a water molecule is necessary to complete a turnover, the simplest explanation presumes that a water molecule accounts for the difference between E and E* as shown in mechanism D below, during both preincubation and catalytic turnover. At present, none of our data (including unpublished results) address the question of whether E*I is the same or different under these two conditions. Also, it should be noted that a slow displacement of water as the only cause of the slow step in pepstatin binding makes it difficult to reconcile with an inverse solvent isotope effect with origins in a transition state. To illustrate, consider the following dissociation of H_2O from a hydrogen-bonded complex with a carbonyl group:

$$—C=O\cdots H_a—O—H_b \rightarrow —C=O + H_a—O—H_b \quad (7)$$

Deuterium in position a would be expected to contribute to an isotope effect on the rate of dissociation of water in either one of two ways: as a primary kinetic isotope effect reflecting a pseudotransfer of the H_a from —C=O to —OH_b or as an equilibrium isotope effect reflecting a difference between the fractionation factors of bound and free water. The former requires that the isotope effect be normal, while the latter requires that the isotope effect originate in the reactant state, neither of which is observed. Deuterium in position b, however, may generate an inverse isotope effect and with origins in a transition state because —OD is twice as strong a base as —OH (Thornton & Thornton, 1970). On the other hand, no other explanation has been offered for the slow step in pepstatin binding, and none is suggested by the new isotopic data. Nevertheless, the new proton inventories on the onsets of inhibition (assuming they withstand further refinements in precision) set limits that exclude some alternative explanations, such as the displacement of multiple water molecules by pepstatin, because such explanations require an inventory arising from more protons than are consistent with the inventories. This new form of an isotope effect holds a potential for elucidating precisely this kind of a new question.

Solvent isotope effects on the onsets of inhibition during catalysis ought to be identical to those of preincubation if the inhibitor were a simple competitive inhibitor binding only to the free enzyme. Contrasting Figures 1 and 3 with Figures 2 and 4 shows that pepstatin cannot be a simple competitive inhibitor of pepsin. Another pathway for the onset of inhibition must be operative when catalysis is occurring. An obvious candidate is the transfer of the bound water to the substrate during normal hydrolysis of a peptide bond followed by the necessary dissociation of products, leaving behind a dehydrated form of pepsin to which pepstatin can bind. The isotope effect could then arise from a change in the steady-state concentration of the dehydrated form of enzyme, downstream from the enzyme–product complexes. Because the kinetic isotope effect expressed on the hydrolysis of Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe must also be downstream from product formation (Rebholz & Northrop, 1991), the simplest mechanistic explanation is that both isotope effects have the same origin. Consistent with this hypothesis are (1) both are less than 2 (*i.e.*, $^{D}V = 1.51$ and $^{D}k = 1.25$), (2) both arise from a single proton transfer, and (3) both are different from the isotope effect originating in proteolysis (*i.e.*, $^{D}(V/K) = 3.9$, $P > 3$). Furthermore, if both isotope effects have the same origin, then because both are normal, it follows that the isotopically-sensitive step in the isomerization segment must come *before* the binding of pepstatin; if it came after, deuterium would cause an increase in the form of enzyme to which pepstatin binds, which would increase the rate of formation of the enzyme–inhibitor complex, causing an apparent inverse isotope effect on the rate of onset of inhibition. Consistent with the identity of origins and the assigned order of steps is the observed $\phi^R = 1.46$. This is an exceptionally large fractionation factor to be attributed to an equilibrium isotope effect between a reactant and the solvent (Quinn & Sutton, 1991). However, a kinetic isotope effect which changed the distribution of enzyme forms during multiple catalytic turnovers could be as large as a normal intrinsic kinetic isotope effect on catalysis. Moreover, such an isotope effect would generate proton inventories consistent with the reactant form of the Kresge equation, despite its true origins in a transition state because the steady-state distribution would mimic the Kresge equilibrium distribution. The isotope effect on turnover with the fast substrate, $^{D}V = 1.51 \pm 0.02$, is a close match to the apparent reactant-state fractionation factor during turnover, $\phi^R = 1.46 \pm 0.03$, consistent with the hypothesis that both isotope effects have the same origin.

The proposed kinetic isomechanism for pepsin with three separate isotopically-sensitive steps is summarized in mechanism D, together with the proposed pathways for binding pepstatin in the presence and absence of substrate. Enzyme



Mechanism D

in form E represents hydrated pepsin with Asp-32 unprotonated (Fruton, 1976) and Asp-215 protonated (Kitson & Knowles, 1971). Form E* is a dehydrated pepsin in the same protonated state. Form F is dehydrated pepsin in the reverse protonated state generated by the general-acid/general-base chemical mechanism postulated by Dunn and Fink (1984) and suggested as the isomerized form of free enzyme in the isomechanism of Rebholz and Northrop (1991). The isotope effect on rate of hydrolysis of slow substrates originates in the conversion of the substrate to bound products, ES → FPQ. The isotope effect on both the rate of hydrolysis of the fast substrate and the onset of inhibition by pepstatin during catalysis originates in the reprotonation of the active site aspartic acid residues, F → E*, in a process reminiscent of that proposed by Fisher *et al.* (1986) for reprotonation of active site thiols of proline racemase. This isotope effect on fast substrate hydrolysis generates a change in the steady-state distribution of dehydrated enzyme (E*) which in turn *appears* as a reactant-state fractionation factor in the proton inventory, attributed to the equivalency: $^{D}V = [E^*]_{H_2O}/[E^*]_{D_2O} = \phi^R \approx 1.5$. This equivalency requires that the reprotonation step be the primary rate-limiting step of catalysis with its isotope effect approaching an intrinsic value. The onset of inhibition is relatively slow because [E*] is low, it being the product of the rate-limiting step. The isotope effect on the onset of inhibition in the absence of substrate is the release of water from the pepsin–pepstatin complex, EI → E*I. This release is unusually slow, causing a slow onset of tight binding, because the active site water molecule must traverse the hydrophobic pocket filled with pepstatin.

Mechanism D can account for a variety of otherwise contradictory evidence supporting either acyl or amino covalent intermediates in the chemical mechanism of porcine pepsin (Dunn & Fink, 1984). For example, transpeptidation reactions such as the formation of Tyr-Tyr from Cbz-Glu-Tyr (Neumann *et al.*, 1959) or Leu-Leu from Leu-Tyr (Takahashi *et al.*, 1974) could be catalyzed without the aid of covalent intermediates if the apparent irreversibility of peptide hydrolysis in 55 M water rested in the isomerizations steps, F → E* → E. A large free energy drop must accompany the isomerization, and this same free energy must be available to the dehydrated form of enzyme (E*) for extracting a molecule of water from adjacent carboxyl and amino termini of a pair of peptides, making E* kinetically competent to synthesize a new peptide bond. Also, mechanism D may account for some differences between *in vitro* and *in vivo* inhibitory activities of transition-state analogs. Analogs more closely resembling the substrate must compete with substrate for the E form of enzyme, while those more closely resembling products might bind more readily to the E* or F forms. The kinetics of the onset of inhibition *in vivo* depend heavily on the steady-state concentration of isoforms, which in specific cases is unknown. However, because the activity of intracellular water is reduced, rehydration of an aspartic protinease may be slower inside a cell than in laboratory buffer. Such a possibility may have useful implications in the search for inhibitors of therapeutically important aspartic proteases such as renin and the HIV protease.

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