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SLOW STEP AFTER BOND-BREAKING BY PORCINE PEPSIN IDENTIFIED USING SOLVENT DEUTERIUM ISOTOPE EFFECTS[1]

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SUMMARY: The relatively fast artificial substrate Leu-Ser-ρ-nitro-Phe-Nle-Ala-Leu-OMe generates a solvent isotope effect of 1.51 ± 0.02 only on the maximal velocity of peptide hydrolysis catalyzed by porcine pepsin (EC 3.4.23.1). The absence of an isotope effect on V/K places the isotopically-sensitive step after peptide bond cleavage and the release of the first product. Reprotonation of the active site aspartic carboxyls is proposed as the most likely interpretation of this observation. Structural and kinetic similarities between pepsin and other aspartic proteinases, including the therapeutically important targets HIV protease and renin, suggest a similar slow reprotonation step after catalysis. This mechanistic feature has important implications regarding inhibitor design; if most of the enzymes are present in a product-release form during steady-state turnover, then perhaps inhibitors should be designed as product analogs instead of substrate analogs. © 1991 Academic Press, Inc.

The prevailing paradigm for the kinetics and energetics of pepsin and other aspartic proteinases consists of a relatively fast step for substrate binding, a slow step for the chemical transformation of substrate to product, and then another fast step for product(s) release. The large barrier for the chemical transformation step dictates that the binding of substrates will be in rapid-equilibrium. The evidence usually cited in support of rapid-equilibrium kinetics for pepsin--and by extension, to the applicability of this model to aspartic proteinases in general--is the finding of Fruton (1) that K_m is numerically equivalent to the dissociation constant, K_s. However, the relevance of $K_m = K_s$ to rapid-equilibrium kinetics only holds for a simple three-step kinetic mechanism. As soon as more steps are introduced, the complexity of K_m makes it possible for it to be numerically equivalent to K_s under non-rapid-equilibrium conditions as well. In the absence of evidence to the contrary, Occam's razor dictates that the simple three-step kinetic mechanism be invoked. We now present contrary evidence and propose a more complex kinetic mechanism.

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MATERIALS AND METHODS

Porcine pepsin was purchased from Sigma and used without further purification. Leu-Ser-ρ-nitro-Phe-Nle-Ala-Leu-OMe (**I**) was purchased from Bachem and used without further purification. Lys-Lys-Ala-Lys-Phe-ρ-nitro-Phe-Arg-Leu (**II**) was provided by Professor Daniel H. Rich. Buffers were 0.040 M formate with ionic strength adjusted to 0.10 M with KCl. Percent deuterium in the reaction mixtures was determined by the method of Northrop, *et al.* (2), and pD of the solution is reported as the pH meter reading plus 0.4. Kinetic data were collected on an OLIS modified CARY-14, 15, or 16 interfaced to a computer. The cleavage of the ρ-nitro-Phe-Nle or the Phe-ρ-nitro-Phe bond was monitored at 310 nm using extinction coefficients determined at each pL (*i.e.*, pH or pD). Temperature was kept at 25 $\pm$ 0.1 °C with a specially crafted thermojacketed cell holder. Kinetic data were analyzed using RAGASSEK (Regression and Graphical Analysis for Steady-State Enzyme Kinetics) employing the non-linear regression routine of Duggleby (3).

RESULTS

Maximal velocities (V) and values for V/K were determined for peptide **I** in H_2O and D_2O at varying values of pL. Plots of pL versus log(V) and log(V/K) are flat in H_2O and D_2O from pH 3.0 to 5.5 (not shown). There is a solvent deuterium isotope effect on V but no significant effect on V/K as listed in Table 1. In contrast, when peptide **II** is used, there is a solvent isotope effect on both V and V/K. The pH profile for peptide **II** published by Pohl and Dunn (4) shows that V is pH independent from pH 3 to 5 while the plot of log(V/K) versus pH increases with a slope of 1.8. Proton inventories were conducted at pL 4.96 by determining kinetic constants in 0, 49.5, and 98.9 percent D_2O. Non-linear regression analysis of the proton inventory data solving for the number of protons in the Gross-Butler equation (5) resulted in a value of 1.00 $\pm$ 0.38 protons for ^{D}V of peptide **I** while a curved inventory was observed for ^{D}V of peptide **II** as shown in Figure 1.

DISCUSSION

These two synthetic substrates show four differences in kinetic behavior with porcine pepsin: pH dependent versus pH independent V/K, presence versus absence of $^{D}(V/K)$, size of ^{D}V, and curved versus linear proton inventories. These differences appear indicative of a shift in the rate-limiting step. For peptide **II**, the isotopically-sensitive peptide bond-breaking step probably provides the greatest barrier[3] to both V and V/K; for peptide **I**, V/K is limited by an isotopically-insensitive step, while V is now limited by some heretofore unidentified but clearly isotopically-sensitive step occurring *after* the reaction segment comprising V/K. As shown in Table 1, the value for V/K of peptide **I** is approximately three times that for peptide **II**, while their values for V are identical, consistent with this interpretation.

V contains the rate constants for all the segments in an enzymatic reaction after substrate addition while V/K contains only the rate constants through the first irreversible step. As a result, the larger isotope effect is more commonly expressed on V/K due to slow steps downstream which often suppress the effect on V (7). Both V and V/K for porcine pepsin include the bond-breaking

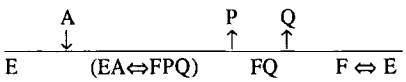
[3]The definition of a rate-limiting step as detected by isotope effects on V and V/K has been disputed, but for the purposes addressed in this discussion, the definitions of Ray (6) will suffice.

TABLE 1. Solvent Deuterium Isotope Effects on Porcine Pepsin

Substrate	V/E_t (sec^{-1})	V/K ($M^{-1}sec^{-1}$)	DV	D(V/K)
Peptide **I**	133 ± 2	(2.7 ± 0.2)x10^7	1.51 ± 0.02	0.84 ± 0.21
Peptide **II**	136 ± 9	(8.2 ± 2.8)x10^6	2.02 ± 0.15	3.9 ± 1.4

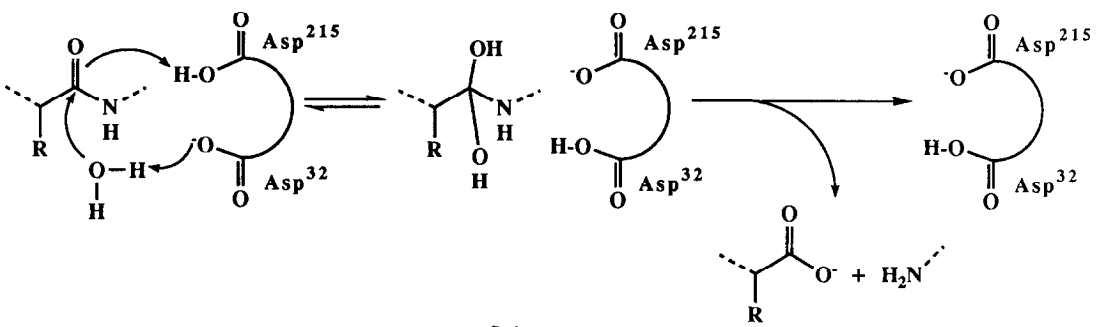
steps in the reaction, and if an isotope effect on bond-breaking were being expressed, the effect would be expected to be seen in both V and V/K. Indeed, solvent isotope effects for the much slower substrates N-trifluoroacetyl-L-Phe (8) and Gly-Gly-Gly-ρ-nitro-Phe-Phe-OMe (9) show equivalent isotope effects on both V and V/K of 3 and 2, respectively. The presence of an effect only on V of peptide **I** requires that this isotope effect does not arise from the bond-breaking steps but rather originates from a later step in the reaction that contributes to V but not to V/K.

We propose that such a step could be a reprotonation of the enzyme. After both products are released, the enzyme may be in a different form, F, that goes through a reprotonation to return to form E as shown in Scheme I.



Scheme I

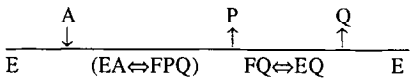
In this model, the catalytically-active aspartic carboxyl groups in the active site may be left in a wrong state of protonation that is unable to support a second catalytic turnover. Such a protonation change has been proposed by Dunn and Fink (10), involving a general base-catalyzed attack by water on the carbonyl group of the sissile bond, as illustrated in Scheme II.



Scheme II

In the catalytically active form of pepsin, residue Asp^{32} is protonated and Asp^{215} is not; after turnover, residue Asp^{215} is the one that is protonated while Asp^{32} is not. A proton transfer from Asp^{215} to Asp^{32} (not shown) is then required before another turnover may occur. This change in the state of protonation is strikingly reminiscent of the rate-limiting reprotonation of proline racemase described by Albery and Knowles (11). Two catalytic thiols are thought to participate in the racemization, only one of which is protonated in the catalytically active form; the other removes a proton from one side of proline while the former donates a proton to the opposite side of proline. The enzyme mirrors the chiral change in the substrate and is left with the proton on the second thiol and thus is unable to support a second racemization. A molecule of water (or buffer) must then act as a go-between to transport the proton back to the original thiol, a process that is slower than the racemization.

An alternative kinetic explanation is that the release of the second product cannot occur until after the proton dependent step, as shown in Scheme III.



Scheme III

In either case, the partially rate-limiting step that expresses the solvent isotope effect comes *after* the conversion of substrate to products. This requirement supports a steady-state kinetic model for porcine pepsin and argues against the rapid-equilibrium model commonly invoked when K_m approximates K_s (1). If V/K is determined by one segment (E→FQ) of the reaction and V is determined by a different segment (FQ→E), it is obvious that their ratio may be smaller, equal to, or larger than K_s.

Because an isotope effect on V but none on V/K was also reported for recombinant human renal renin (12), a rate-limiting reprotonation may be a common feature among the aspartic proteinases acting on natural substrates, and this steady-state model may have important implications for the design of inhibitors of other aspartic proteinases. If the proton dependent step occurs before the release of the second product, then FQ is accumulating during steady-state and inhibitors designed as analogs to P may be more effective *in vivo* than analogs of A. Similarly, if the proton dependent step occurs after the release of the second product, then form F is accumulating and inhibitors may be more effective if designed as analogs to Q. The reason being that analogs of A are by definition competitive inhibitors, and blockage of an enzyme by competitive inhibitors must ultimately be overcome by the ever increasing concentrations of substrate. Termed "metabolic resistance" by Duggleby and Christopherson (13), this process has been proposed for the *in vivo* failure of the experimental antineoplastic drug, *N*-phosphonacetyl-L-aspartate, a competitive inhibitor of aspartyl transcarbamylase. These new designs may lead to novel inhibitors for therapeutically important aspartic proteinases, such as HIV aspartic protease as well as renin.

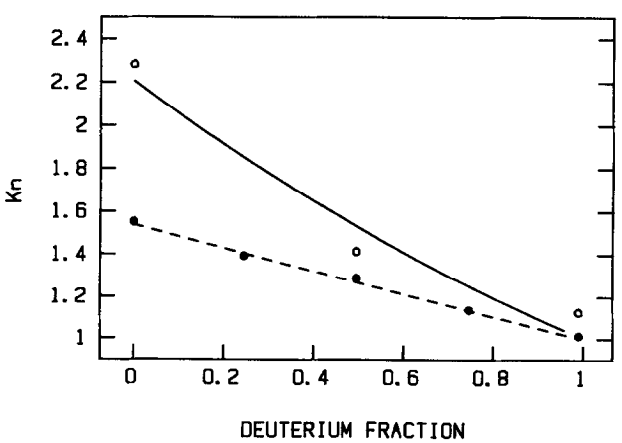


FIGURE 1. Plots of the proton inventories for peptide **I** (•) and peptide **II** (o). The solid line was calculated from a 3-proton model; the dashed line was calculated from a 1-proton model based on a fit of 15 data points; only the averages of these points are shown.

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