

Follow the Protons: A Low-Barrier Hydrogen Bond Unifies the Mechanisms of the Aspartic Proteases

DEXTER B. NORTHPROP*

*Division of Pharmaceutical Sciences, School of Pharmacy,
University of Wisconsin–Madison, Madison, Wisconsin 53705*

Received March 19, 2001

ABSTRACT

Seven proton transfers in five steps participate in a catalytic turnover of an aspartic protease. The Rosetta Stone for elucidating their role is a low-barrier hydrogen bond that holds the two aspartic carboxyls in a coplanar conformation. The proton of this bond shuttles between oxygens during chemical steps via hydrogen tunneling, unlike in previous proposals where it was transferred to substrate. After the release of products, both carboxyls are protonated and the bond is missing. Re-forming the bond is a significant step within a kinetic isomechanism. The bond also explains—at long last—the extremely low pK in pH profiles.

Introduction

Aspartic proteases (also called aspartic endopeptidases, EC 3.4.23.x) have the longest history in enzymology, and the most perplexing. Rennet and later chymosin have been used in the making of cheese for thousands of years.¹ However, pepsin is usually considered the first enzyme in this family, was the first to be recognized as an active principle (i.e., gastric juice dissolves meat, in 1783)² and given a name (in 1825),² and was only the second to be extracted (in 1836)² or crystallized (in 1930).³ The second enzyme in the family is nepenthesin, detected in insect-eating plants by Darwin (in 1875),⁴ thereby making this the oldest family as well. Extracts of pepsin had to be acidified to regain full activity, with different acids giving different results. Sørensen⁵ noted that if activities were plotted against hydrogen ion concentrations, the results were similar. To solve his scaling problem, he employed a logarithmic abscissa—and invented pH (in 1909). The identifying characteristic of what were the acid proteinases is their wide bell-shaped pH profiles with acidic optima, and this fundamental characteristic has never been accounted for, despite being featured in this, the primal report of pH-dependent enzymatic activity.

Dexter B. Northrop received a B.A. degree in chemistry in 1962 from St. Olaf College, and a Ph.D. in biochemistry in 1969 from Western Reserve University, guided by the late Harland G. Wood. He was a postdoctoral fellow with Bert Vallee at Harvard and with W. Wallace Cleland at Wisconsin. He joined the faculty there in 1971. He has served on the editorial boards of *Archives in Biochemistry and Biophysics*, *Biochemistry*, and the *Journal of Biological Chemistry*. His research interests include theoretical and experimental studies of the effects of isotopes and of high pressure on enzyme kinetics. He descends from Joseph Northrop, who settled in New Milford, Connecticut, in 1639, as does the late John Howard Northrop, who first crystallized pepsin and demonstrated that its catalytic properties derived from the protein. Accounting for its pH profile, however, eluded the 1946 Nobel Laureate.

That long uncertain reach across history is without precedent and, moreover, is accompanied by other seemingly unrelated uncertainties. Frustration peaked with transpeptidation reactions involving *both* halves of peptide substrates, implying covalent acyl *and* amino enzyme intermediates, whose parallel existence within a single enzymatic mechanism seemed unlikely.⁶ In the past two decades, new uncertainties have appeared, notably anomalous isotope effects, driven by the clinical significance of the HIV proteases within an intense search for mechanism-based inhibitors. Then, with the singular report of a low-barrier hydrogen bond (LBHB) by computational chemists Piana and Carloni,⁷ *all* of the various uncertainties resolved within a unifying hypothesis. What follows is the essence of this remarkable resolution. Because of space limitations, not every datum can be reinterpreted, so interested readers are directed to the excellent review by Meek⁸ plus references therein and invited to read with new eyes.

The Low-Barrier Hydrogen Bond

Piana and Carloni⁷ performed ab initio molecular dynamics simulations on HIV-1 protease, focusing on the conformational flexibility of the active site carboxyls, Asp 25 and Asp 25'. They found the monoprotonated form to be the most stable, with a LBHB between the O δ 1 atoms that compensates for their negative charge repulsion and holds them within an interatomic distance of 2.5 ± 0.1 Å. Moreover, as shown in Figure 1, this LBHB along with peptide dipoles holds the carboxyls in a coplanar conformation with a water molecule between—and hydrogen bonded to—both O δ 2 atoms. The resulting symmetrical 10-atom cyclized structure thus provides a scaffold to impart proximity, orientation, and nucleophilicity to the water molecule.

LBHBs are an exciting new feature in our understanding of enzymatic catalysis.⁹ Their dominant characteristics are a reliance upon matched pK s of the electronegative atoms sharing the hydrogen, low fractionation factors when deuterium replaces protium, and downfield chemical shifts of 18–22 ppm for the hydrogen in proton NMR spectra. The first characteristic is satisfied with a pairing of carboxyls, and the second will be discussed below (see eq 1), while the third has not been observed directly in aspartic proteases, presumably because of autolysis by free enzymes. However, the inhibited complex between HIV-1 protease and pepstatin has been examined by proton NMR, with negative results.¹⁰ Consistent with that finding are additional simulations by Piana et al.,¹¹ showing both carboxyls protonated, with no LBHB, as the most stable form of the complex. ¹³C NMR spectra of HIV-1 protease specifically labeled in the Asp 25 and Asp 25' carboxyls show a single peak with free enzyme and two peaks with the complex.¹⁰ These are consistent, respectively, with a

* Address correspondence to the author at the School of Pharmacy, University of Wisconsin–Madison, 777 Highland Ave., Madison, WI 53705-2222. Phone: (608) 263-2519. FAX: (608) 262-3397. E-mail: dbnorthrop@pharmacy.wisc.edu.

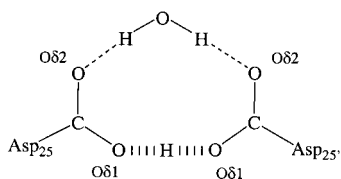


FIGURE 1. Coplanar catalytic carboxyls of HIV-1 protease with a low-barrier hydrogen bond between the O δ 1 oxygens and normal hydrogen bonds between a water molecule and the O δ 2 oxygens; after Piana and Carloni.⁷

LBHB in free enzyme causing both carboxyls to appear ionized, and different environments in the complex causing the two protonated carboxyls to express different chemical shifts.¹¹

pH Kinetics

For nearly a century, two acidic groups were thought to be involved in the catalytic activity of pepsin, one protonated and one not, with pKs approaching extremes of 4 and 1, respectively. The first was obviously a carboxyl, but the second seemed much too acidic to be so as well. When a phosphoric acid residue was found in porcine pepsin A a half century ago, it became a candidate for the low pK, but dephosphorylation had no effect on activity. When both groups were deemed carboxyls, different environments seemed necessary, so one was attributed to the hydrophobic active site of the enzyme and the other to peptide substrates. But esterification of carboxyl groups of substrates had no effect either, thereby leaving the origin of the low pK unresolved for another 30 years.

The response of LBHBs to D₂O finally solves this oldest mystery in enzymology. Hunkapillar and Richards¹² observed bell-shaped pH profiles for hydrolysis of *N*-trifluoroacetyl-L-phenylalanine by pepsin that narrowed in D₂O; i.e., the lower pK shifted right *but the upper pK shifted left*. Normally, D₂O shifts all pKs to the right by about half a unit because of the small fractionation factor of H₃O⁺.¹³ A reanalysis of the pH profiles of k_{cat} from Hunkapillar and Richards is presented in Figure 2. The fit to the BELL pH equation of data obtained in H₂O converged with pKs of 2.9 ± 0.1 and 4.5 ± 0.1 , while those in D₂O converged to the default separation of only 0.6 unit, the minimum statistically possible. The fitted curve in Figure 2B is the same for separated pKs of 3.5 and 4.1, or identical pKs of 3.8 ± 0.1 , or anything in between. LBHBs cause opposite shifts in the pKs of the conjugate bases participating in the bond. Both protonation and deprotonation of the LBHB complex are more difficult, which shifts apparent pKs outward, much more so in H₂O than in D₂O.⁹

Hunkapillar and Richards estimated a ${}^{\text{D}_2\text{O}}k_{\text{cat}} \approx 3$ by comparing the maximal values of their fitted curves. However, the more appropriate comparison is between the pH-independent maxima,¹³ represented by the horizontal bars in Figure 2. That ratio yields ${}^{\text{D}_2\text{O}}k_{\text{cat}} = 1.8 \pm 0.4$ for pKs separated by 0.6 unit, and lower values if the fractionation factor is greater than $\phi = 0.3$. Hence, the actual kinetic isotope effect is modest, and most of the

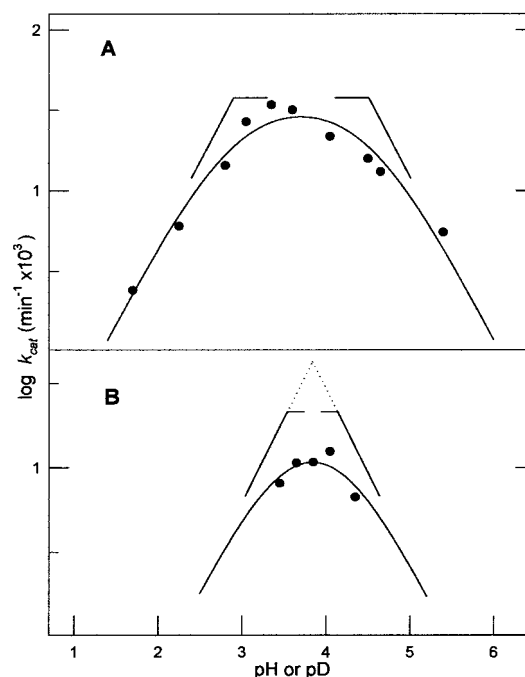


FIGURE 2. Kinetics of pepsin acting on *N*-trifluoroacetyl-L-phenylalanine as a function of pH (A) and pD (B). The fitted pH-independent kinetic constants are $k_{\text{cat}} = 0.038 \pm 0.006 \text{ min}^{-1}$ in H₂O and $k_{\text{cat}} = 0.022 \pm 0.002 \text{ min}^{-1}$ in D₂O. The dotted line indicates the range of carboxyl pKs consistent with the fitted line in D₂O. (Similar shapes of profiles were obtained using values of $k_{\text{cat}}/K_{\text{M}}$.)

rate difference has a *thermodynamic* origin: weakening of the LBHB causes a large decrease in enzyme poised for catalysis.

The narrowness of these pH profiles in D₂O suggests that this pH-dependent step is fully expressed. In contrast, pH profiles of HIV-1 protease show both pKs of $k_{\text{cat}}/K_{\text{M}}$ farther apart and both shifted to the right in D₂O, with only the lower pK expressed in profiles of k_{cat} .¹⁴ These contrasts require that the pH-dependent step being expressed in this HIV-1 protease study is different from the one in the pepsin study, that it comes later in the kinetic mechanism than the loss of the LBHB, and that it is not fully expressed, which causes apparent pKs to be shifted outward even farther.

The Kinetic Isomechanism

The artificial peptide Leu-Ser-Nph*Nle-Ala-Leu-OMe was synthesized as a chromophoric substrate (i.e., Nph is *p*-nitro-phenylalanine) for commercial assays of chymosin, but it is an excellent substrate for pepsin as well. Values of k_{cat} are pH-independent and orders of magnitude greater than pH-dependent values of earlier synthetic peptide substrates.¹⁵ This “fast substrate” also displays a small solvent isotope effect of ${}^{\text{D}_2\text{O}}k_{\text{cat}} = 1.51 \pm 0.02$, coupled to no effect on $k_{\text{cat}}/K_{\text{M}}$, which meant that this isotopically sensitive step came *after* the first irreversible step, the release of a product. A kinetic isomechanism was therefore proposed, with a substrate form of enzyme, E, having a proton on one active site carboxyl, and a product form, F, having it on the other. Such an iso step would

Table 1. Product Inhibition Kinetics of HIV-1 Protease¹⁸

substrate/product	K_{is} (mM)	K_{ii} (mM)	$D_2O K_{is}$	$D_2O K_{ii}$
Ac-Ser-Glu-Asp-Tyr*Phe-Leu-Asp-Gly-NH ₂				
Phe-Leu-Asp-Gly-NH ₂	3.8 ± 0.5	12 ± 0.2	1.1 ± 0.4	3.0 ± 0.9
Ac-Ser-Glu-Asp-Tyr	3.5 ± 0.4	12 ± 0.8	4.2 ± 1.6	3.8 ± 1.3
Ac-Ser-Glu-Asp-Tyr*Phe-Leu-Asp-Gly-NH ₂				
Ac-Arg-Ala-Ser-Glu-Asp-Tyr (pH 6)	7 ± 2	8 ± 2	4 ± 2	4 ± 1
Ac-Ser-Glu-Asp-Tyr*Phe-Val-Val-NH ₂				
Ac-Arg-Ala-Ser-Glu-Asp-Tyr (pH 4.5)	12	4	1.2 ± 0.4	1.0 ± 0.8
Ac-Arg-Ala-Ser-Glu-Asp-Tyr (pH 3.5)	—	0.23		

provide a proton transfer that expresses a kinetic isotope effect on k_{cat} but not on k_{cat}/K_M .

The case for an isomechanism was confirmed by isotope effects on the slow onset of inhibition by pepstatin. A normal $D_2O k = 1.25 \pm 0.09$ occurs when catalysis is in progress, but a surprising inverse $D_2O k = 0.69 \pm 0.09$ occurs during preincubation experiments with free pepsin.¹⁶ Proton inventories on both are linear, suggesting single proton transfers, and unlike inventories on substrate capture (see below). Hence, during catalysis the inhibitor binds to an iso form of enzyme different from that which binds substrate, whereas in preincubations it binds to the same form by a two-step process, $E + I \rightleftharpoons EI \rightleftharpoons FI$. The slow second step was previously assigned to the release of a tightly bound water molecule, but it was not clear why D_2O was bound less tightly. Now, the small fractionation factor of a LBHB in free aspartic protease, E, versus no LBHB in the tightened pepstatin complex, FI, provides a resolution of this uncertainty: the F form of enzyme is favored in D_2O , which accounts for the inverse effect.

Product Inhibition Mechanisms

In ordered uni bi kinetic mechanisms, inhibition by the first product is noncompetitive and that by the second is competitive; however, if free enzyme isomerizes, then both are noncompetitive. Accordingly, on the basis of graphical analysis of product inhibition patterns, pepsin was deemed to obey an ordered mechanism with the scissile carboxyl product released first. However, Rebholz¹⁷ reanalyzed the published competitive inhibition patterns using regression analysis and found several with small but statistically significant intercept effects; i.e., $K_{is} < K_{ii} < \infty$. Later, in HIV-1 protease kinetic studies, Rodriguez and Meek¹⁸ found unambiguous noncompetitive patterns along with solvent isotope effects on some product inhibition constants. The scissile amino product Phe-Leu-Asp-Gly-NH₂ in Table 1, for example, is decidedly noncompetitive, with $K_{is} \approx K_{ii}$, and shows a large solvent isotope effect on K_{ii} . Solvent isotope effects on ligand binding are rare and

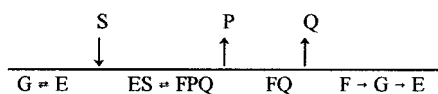


FIGURE 3. Line diagram of a two-step iso uni bi ordered kinetic mechanism. The first iso step, $F \rightarrow G$, deprotonation of the carboxyl with the lower pK , is effectively irreversible at the pH optimum and contributes only to k_{cat} . The second iso step, $G \rightleftharpoons E$, is shown as reversible in the beginning of the mechanism, as it would appear in k_{cat}/K_M , and irreversible at the end of the mechanism, as it would appear in k_{cat} .

usually small.¹⁹ However, K_{ii} has a kinetic component in addition to binding. At saturating $[S]$, inhibitors bind to transient product forms of enzyme, such as F or FQ in Figure 3. In D_2O , the conversion of F to E is slower, causing $[F]$ to increase, which enhances inhibitor binding. Better binding generates a lower inhibition constant and appears as a normal isotope effect, i.e., $K_H/K_D > 1$. Hence, this solvent isotope effect on K_{ii} has a kinetic origin, due to changes in the distribution of product forms of enzyme, and is not an effect on “binding” as such.

Similar results are found with scissile carboxyl products in Table 1, only now both inhibition constants show large normal solvent isotope effects. K_{is} measures binding at low $[S]$, which means free enzyme forms are at equilibrium and any solvent isotope effect must have a thermodynamic origin. $K_H/K_D > 1$ at low $[S]$ favors binding in D_2O , which means Ac-Ser-Glu-Asp-Tyr binds to enzyme without the LBHB. Returning to the scissile amino product, its $D_2O K_{is} \approx 1$ means that Phe-Leu-Asp-Gly-NH₂ has no favoritism; it binds equally well with or without the LBHB.

For the alternate scissile carboxyl product, Ac-Arg-Ala-Ser-Glu-Asp-Tyr at pH 6, K_{is} and K_{ii} are similar and both display solvent isotope effects, but at pH 4.5 K_{ii} is lower while K_{is} is higher and neither displays isotope effects. Moreover, at pH 3.5, the pattern becomes uncompetitive—with a much lower K_{ii} . It follows that in order to explain $K_{ii} < K_{is}$ in the absence of solvent isotope effects, there must be an additional isomer of free enzyme, G in Figure 3, that lacks a LBHB. Because transpeptidation data (see below) show the F form to be diprotonated, it predominates at the lower pH and the additional form G must be monoprotonated. At low $[S]$ and moderate pH, inhibitors may bind to either G or E, or both, which are at equilibrium, with G favored in D_2O ; at high $[S]$, they bind to F and G, which are in steady-state transit, with F favored in D_2O .

For the alternate scissile carboxyl product, Ac-Arg-Ala-Ser-Glu-Asp-Tyr, at pH 6, its K_{is} and K_{ii} values are similar, and both display solvent isotope effects but at pH 4.5 K_{ii} is lower while K_{is} is higher and neither displays isotope effects. Moreover, at pH 3.5, the pattern becomes uncompetitive—with a lower K_{ii} than at pH 6. It follows that this product does not bind to E but will bind to either G or F, with a strong preference for F (and probably FQ).

The Non-Covalent Transpeptidation Mechanism

When Tyr-Tyr was produced from Cbz-Glu-Tyr and pepsin, Neumann et al.²⁰ proposed an amino enzyme covalent intermediate. But when both [³H]Leu-[³H]Leu

and [^{14}C]Leu-[^{14}C]Leu were produced from [^{14}C]Leu-Tyr-[^3H]Leu⁶—in an unequal ratio—the construction of a plausible covalent chemical mechanism became difficult. Both acyl and amino enzymes seemed necessary but unlikely, and the lack of stoichiometry was unsettling.

The kinetic isomechanism presents a new pathway for peptide bond synthesis with no need for covalent intermediates nor stoichiometry. A lag is present in the progress curve of acyl transpeptidation catalyzed by pepsin acting on Leu-Ser-Nph*Nle-Ala-Leu-OMe in the presence of excess Leu-Leu to form Leu-Ser-Nph*Leu-Leu.²¹ Most importantly, the lag is tightly coupled to bursts in the progress curves of the proteolytic products, Leu-Ser-Nph and Nle-Ala-Leu-OMe, and neither lags nor bursts are expected if a covalent intermediate is involved. That this transpeptidation unequivocally requires an isomechanism was established by the measurement of an inverse solvent isotope effect of $D_2O k = 0.40 \pm 0.09$ on the transpeptidation lag, coupled to normal effects of $D_2O k \approx 2$ on the proteolytic bursts. It follows that some or all of the scissile carboxyl product, Leu-Ser-Nph, dissociates and must accumulate before participating in transpeptidation. Dissociation and rebinding allows for the joining of diverse acyl and amino groups without regard to stoichiometry. The coupled isotope effects require that a step following Leu-Ser-Nph dissociation is slower in D_2O . As in the product inhibition studies, D_2O causes [F] to increase, so more enzyme binds Leu-Ser-Nph and catalyzes transpeptidation. Evidence that F is diprotonated comes from penicillopepsin transpeptidations, which display a pH optimum about 1 unit below the optimum for proteolysis.²² (Under different conditions, one or the other proteolytic product may dissociate poorly from F, and thereby dominate transpeptidation from an FQ complex. For example, pepsin acting on Ala-Ala-Nph-Nph at pH 5.5 generates varying amounts of products, with Ala-Ala-Nph > Nph-Nph-Nph > Ala-Ala-Nph-Nph-Nph > Nph-Nph > Nph > Nph-Nph-Nph-Nph.²³ Moreover, running transpeptidation reactions in the presence of added free [^3H]Nph failed to produce any labeled products. Obviously, very little scissile amino product, Nph, dissociates from pepsin and rebinds under these conditions.)

The noncovalent pathway requires that most of the free energy drop takes place in the iso segment and not in the chemical segment. This proviso is needed because sometimes transpeptidation products equal or exceed proteolytic products, as in the example immediately above. That disparity requires the energy level of F to approximate that of ES, as illustrated in Figure 4. Peptides binding to F must readily flow back through the chemical segment and be joined together in a new peptide bond. A LBHB at the end of that flow provides the energy for synthesis. For example, the formation of a LBHB in ketosteroid isomerase has been estimated to contribute over 7 kcal/mol to the stabilization of an intermediate.²⁴ This is more than enough to drive the synthesis of peptide bonds, which have heats of formation of approximately 2 kcal/mol.²⁵ Also, peptide bond hydrolysis in organic solvents approaches an equilibrium near one; therefore,

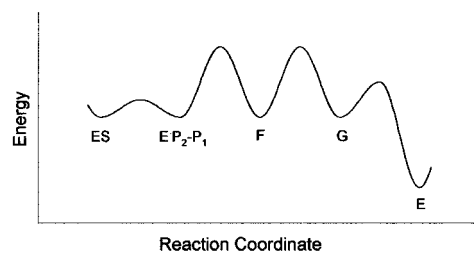


FIGURE 4. Activation energy diagram for proteolysis and transpeptidation catalyzed by pepsin. Enzyme is saturated with substrate S, and the reaction effectively begins with ES. Transpeptidation can occur by rebinding of products P₁ and P₂ (or an added peptide P', not shown) to enzyme form F. Product complex FP₁-P₂ can regenerate ES (or synthesize new P₁-P') coupled to formation of a LBHB. Enzyme forms G and E cannot support transpeptidation because the former is kinetically incompetent, i.e., it is short-lived, and the latter is energetically incompetent, i.e., it must lose its LBHB.

the energetics of Figure 4 within a hydrophobic active site and coupled to the formation of a LBHB are plausible.

Solvent Isotope Effects and Proton Inventories

Fractionation factors of exchangeable enzyme protons (except for thiols) are usually similar to that of water.¹³ But this is certainly not the case for a LBHB which is disfavored in D_2O by as much as a factor of 4.²⁶ Consequently, substrate capture, which employs free enzyme as its sole reactant state,²⁷ must express a large normal solvent isotope effect regardless of its energy profile, so long as a LBHB is lost before the first irreversible step. The fractionation factor for the formation of the LBHB in free enzyme, E, relative to solvent, S, is given by

$$\phi^E = \frac{[E-D]/[E-H]}{[S-D]/[S-H]} = 0.3-0.5 \quad (1)$$

The fractionation factor for protonation of one carboxyl group in the G form of free enzyme is assumed to be $\phi^G = 1$. Hence, with E as the reference reactant state, the operative factor for $G \rightleftharpoons E \rightarrow (ES)$ is $\phi^G/\phi^E = 1/(0.3-0.5) = 2-3$. With the energy level of E fixed as a constant, the presence of the LBHB lowers the energy level of G in D_2O , as illustrated by the first dashed line in the activation energy diagram of Figure 5. This reduces the rate of capture proportionately.

However, $D_2O(k_{cat}/K_m) \approx 2-3$ was not observed with pepsin acting on the fast substrate. On the contrary, this line of inquiry began when a normal solvent isotope effect on k_{cat} was measured and contrasted to $D_2O(k_{cat}/K_m) = 0.84 \pm 0.21$.¹⁵ It follows that if a measured $D_2O(k_{cat}/K_m)$ for an aspartic protease approaches a null value, then there must be a second, inverse effect on a transition state offsetting the mandatory normal effect arising from a LBHB in the reactant state. Indeed, a pH-independent $D_2O(k_{cat}/K_m) = 0.85 \pm 0.09$ was measured for HIV-1 protease acting on Ac-Ser-Glu-Asp-Pro-Val-Val-NH₂—but never published for lack of an explanation.¹⁸ The absence of any effect of pH on this value identifies its origin as something other than a chemical step involving proton transfers. Inverse solvent isotope effects on substrate capture are not uncommon

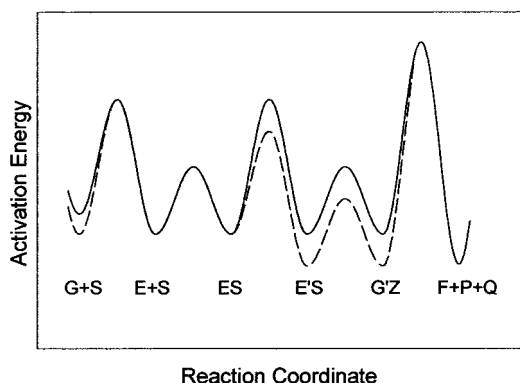


FIGURE 5. Activation energy diagram for substrate capture by an aspartic protease. Steps $ES \rightarrow F^T \rightarrow G'Z$ and $G'Z \rightarrow F^TQ \rightarrow FQ \rightarrow FQ + P \rightarrow F + Q$ were combined for simplicity. The solid line represents the energy profile in H_2O and was calculated using the following relative rate constants: $k_1 = 20$, $k_2 = 10$, $k_3 = 100$, $k_4 = 100$, $k_5 = 10$, $k_6 = 10$, $k_7 = 100$, $k_8 = 100$, $k_9 = 1.5$. The dashed line represents energies in D_2O and was calculated by using the following changed rate constants: $k_1 = 10$, $k_5 = 30$, $k_9 = 0.5$. Hence, the first change generates ${}^{D_2}O(k_{cat}/K_m) = 2$; adding the second gives ${}^{D_2}O(k_{cat}/K_m) = 0.8$; and adding the third gives ${}^{D_2}O(k_{cat}/K_m) = 1.7$ (or 1.01 if $k_9 = 1$). An ${}^{15}N$ equilibrium isotope effect of ${}^{15}K_{eq} = 0.95$ between $E'S$ and $G'Z$ is nearly fully expressed in a calculated ${}^{15}(k_{cat}/K_m) = 0.96$.

Table 2. Proton Inventories on k_{cat} for Porcine Pepsin

form of eq 2	k_H	ϕ^T	ϕ^R	σ
numerator only; $p = 3^{15}$	135 ± 6	0.76 ± 0.27		8.66
denominator only; $p = 1$	138 ± 3		2.19 ± 0.09	4.03
numerator and denominator; $p = 1$	138 ± 2	1.61 ± 0.30	3.35 ± 0.58	2.41

and have been attributed to restrictions on torsional motions of exchangeable protons as enzymes “crunch” down on substrates.^{28,29} Crystallographic studies on several aspartic proteases have identified a “flap closing” associated with binding of substrates and inhibitors.¹ Therefore, equating enzyme crunch with flap closing, the activation energy diagram in Figure 5 includes inverse kinetic and equilibrium isotope effects on the conformational change represented by $ES \rightleftharpoons E'S$ and indicated by the second dashed line. (The loss of a LBHB could itself generate an inverse solvent isotope effect, but the results of Hunkapillar and Richards show that the LBHB-dependent step is clearly pH-dependent and accompanied by a normal effect.)

Conclusive evidence for this combination of offsetting isotope effects are regression analyses presented in Table 2 for fitting proton inventories of pepsin to the equation of Kresge:³⁰

$$k_n = k_H \frac{(1 - n + n\phi^T)^p}{(1 - n + n\phi^R)^p} \quad (2)$$

where k_n represents apparent rate constants in mixtures of D_2O and H_2O , k_H is an apparent rate constant in H_2O , n is the fraction of deuterium in mixed isotopic waters, ϕ^T is the fractionation factor for a transition state, p is the number of protons in flight, and ϕ^R is the fractionation factor for a reactant state. Data were obtained with the control substrate, Lys-Lys-Ala-Lys-Phe* Nph -Arg-Leu, which

generated ${}^{D_2}O k_{cat} = 2.02 \pm 0.15$.¹⁵ Originally, there was no reason to consider reactant-state fractionation factors, so data were fitted to the numerator of eq 2 only, with three protons in flight, as shown in the first entry to Table 2. The fitted line was less curved than the data would suggest.¹⁵ Given the advent of the LBHB, data were subsequently refitted to the denominator of eq 2 with a single proton, and a much better fit was obtained. Finally, data were fitted to single protons in both the numerator and the denominator using the full equation, which generates a curved inventory that passes through the center of the data points, accompanied by a significant decrease in σ . The third fit in Table 2 provides clear evidence for a low fractionation factor in the reactant state, with $\phi^{ES} = 1/\phi^R = 1/(3.35 \pm 0.58) = 0.30 \pm 0.05$, and an inverse kinetic solvent isotope effect on a transition state, ${}^Dk = 1/\phi^T = 1/(1.61 \pm 0.30) = 0.62 \pm 0.12$. The initial reactant state for k_{cat} is ES , so ϕ^R may originate in an *equilibrium* isotope effect, which would mean the formation and loss of the LBHB can occur within the enzyme–substrate complex, i.e., $GS \rightleftharpoons ES$. Alternatively, ϕ^R may originate in a *kinetic* isotope effect downstream from the virtual transition state, i.e., $F \rightarrow G$ or $G \rightarrow E$, that modulates the steady-state transit of $[ES]$. It also follows that if an inverse effect is associated with flap closing, then a normal effect will be expressed subsequently on flap opening, as illustrated in Figure 5 by a lower reactant state for $G'Z$ in D_2O . Hence, there are three newly identified but subtle origins for solvent isotope effects in aspartic proteases, in addition to the obvious ones arising from proton transfers. No wonder individual data sets were perplexing!

The Chemical Mechanism

Previous proposals for chemical mechanisms have one carboxyl protonated, acting as a general acid to donate its proton to the substrate scissile carbonyl, and the other carboxyl unprotonated, acting as a general base to accept a proton from a water molecule.⁸ But the LBHB ties up the carboxyl proton and rules these proposals out. Piana and Carloni's model in Figure 1 not only has the LBHB holding the carboxyls in a coplanar configuration, but also has the water molecule held in position by hydrogen bonds to $\delta O2$ oxygens—opposite the LBHB. The water must be near the scissile bond, and that places the LBHB proton out of the reach of the substrate.

Starting with this cyclized structure as enzyme form E , Figure 6 shows a proposed chemical mechanism consistent with pH kinetics and some curved proton inventories.¹⁴ After substrate binding and flap closing, $E \rightleftharpoons ES \rightleftharpoons E'S$, a counterclockwise movement of electrons within the cycle and extending through the scissile carbonyl moves two protons clockwise and generates a tetrahedral intermediate bound to a diprotonated F' form of enzyme. The next step, a clockwise movement of electrons around the cycle, moves two protons counterclockwise and generates the zwitterion intermediate bound to a monoprotonated G' form of enzyme. Finally, collapse of the zwitterion

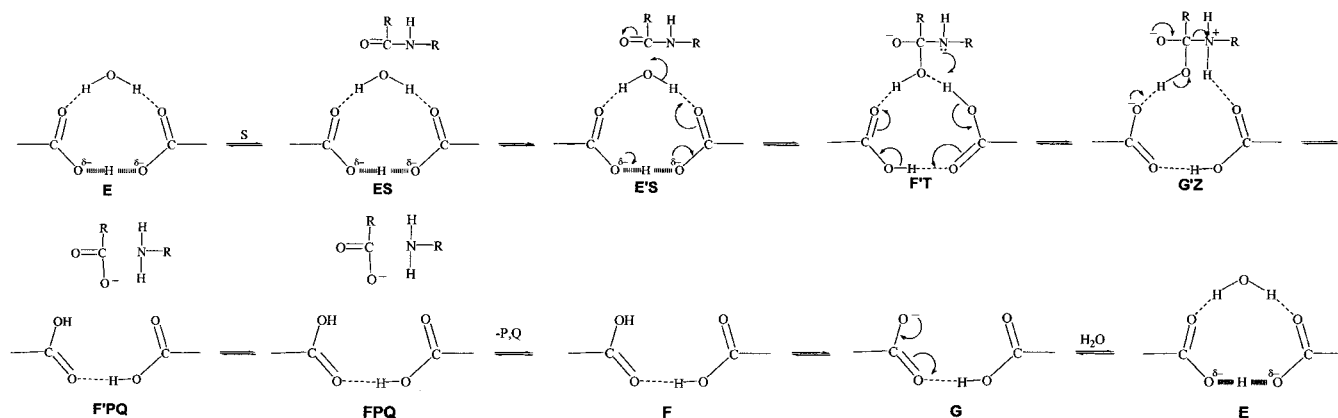


FIGURE 6. Chemical and kinetic isomechanism of an aspartic protease. The order of product release is not designated, and release of products is shown as a single step.

cleaves the scissile bond, destroys the coplanarity of the carboxyls, and leaves the enzyme in the F' form.

That completes the chemistry with regard to the substrate, but not for the enzyme. Flap opening and product dissociations release free enzyme in the diprotonated F form, the form that catalyzes transpeptidation reactions. To complete a turnover, this form must be deprotonated, rehydrated, and allowed to restructure the 10-atom cyclic structure with the LBHB. These three steps (shown as two) constitute the isomerization segment of the kinetic isomechanism, the first and third of which should express normal solvent isotope effects with linear inventories. Both effects could contribute to increased [F] during turnovers in D_2O , which could generate an inverse isotope effect on the onset of pepstatin binding—if the inhibitor bound only to F. But pepstatin also binds to E.¹⁶ Hence, the observed normal isotope effect is almost certainly due to most of the pepstatin binding to G, followed by an isotopically sensitive protonation as binding tightens in a GI \rightarrow FI step.

Energetics and Hydrogen Tunneling

Regarding how LBHBs provide rate accelerations, Cleland⁹ writes: "The principle here is simple. A weak hydrogen bond in the ground state becomes a LBHB in the transition-state or in a transient intermediate. The energy released in forming the LBHB is used to help the reaction that forms it, thus lowering the activation barrier for the reaction." In stark contrast, the chemical mechanism in Figure 6 runs contrary to Cleland's principle, because a LBHB is *lost* along the way. From an enthalpic point of view, this should *raise* the activation barrier—and slow the rate. This energy deficit could be offset by a loss in entropy as the tightly structured coplanar carboxyls, trapped water molecule, and tertiary carbonyl give way, but it is difficult to see how the one could do more than offset the other and thus provide any rate acceleration.

A way out of this quandary is provided by Gerritzen and Limbach,³¹ who characterized a hydrogen-bonded complex between acetic acid and methanol in tetrahydrofuran, illustrated in Figure 7, that bears an uncanny resemblance to the complex of Piana and Carloni. In a

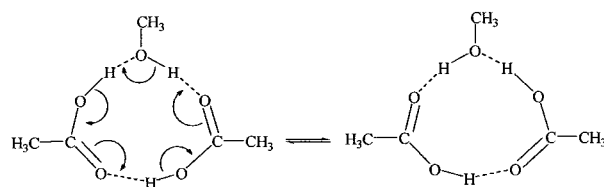


FIGURE 7. Synchronous intermolecular cyclic proton transfers between methanol and acetic acid in tetrahydrofuran; after Gerritzen and Limbach.³¹

nonpolar environment—not unlike the active site of pepsin—a pair of carboxyls and a hydroxyl group spontaneously form a cyclic 10-atom complex. Within this complex, single and double bonds rapidly exchange positions, reminiscent of aromaticity, coupled to exchanges between covalent and noncovalent hydrogen bonds. Hence, the cyclic exchange includes rapid but formal proton transfers. As measured by proton NMR, $k \approx 3000 \text{ s}^{-1}$ at 298 K, with deuterium isotope effects of $k_{HHH}/k_{HHD} = 2.2$ for single substitutions and $k_{HHH}/k_{DDD} = (2.3)^3$ for three substitutions. These data mean that the rule of the geometric mean is obeyed: there are no isotope effects on isotope effects and no coupled motions, so each transfer occurs independently. Importantly, energies of activation for H and D are essentially identical, leaving differences in the Arrhenius pre-exponentials as the dominant if not sole origin of kinetic isotope effects. That means the cyclic exchange proceeds by means of reactant-state quantum mechanical hydrogen tunneling. This nonclassical behavior was recently confirmed by *ab initio* calculations on a similar complex, leading to the conclusion that "concerted nonclassical transfer of two or three protons along hydrogen-bond bridges is an effective proton-transfer mechanism" with "clear biological implications".³²

The Gerritzen and Limbach model also has reduced interatomic distances between oxygen atoms and thereby provides a compelling chemical precedent for steps three and four of the mechanism in Figure 6. This precedent suggests that the purpose of the LBHB in aspartic proteases is to facilitate the formation of a similar cyclic complex in which the electron density is dispersed to accomplish rate acceleration by means of reactant-state

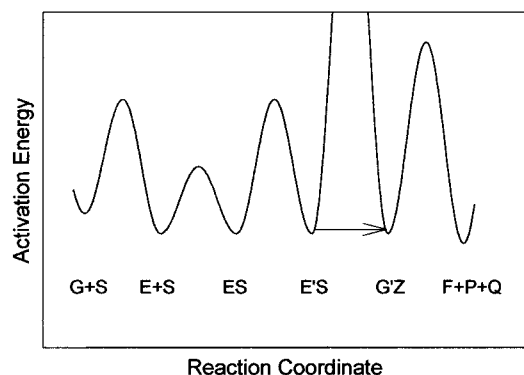


FIGURE 8. Activation energy diagram for substrate capture with hydrogen tunneling. Steps $E'S \rightarrow F'T \rightarrow G'Z$ and $G'Z \rightarrow F'PQ \rightarrow FPQ \rightarrow FQ + P \rightarrow F + Q$ again were combined, only now the former is represented with an insurmountable barrier. Rate acceleration is accomplished by means of ground-state hydrogen tunneling, as indicated by the horizontal arrow.

tunneling, illustrated by the arrow in Figure 8. This process should put the enzyme substrate complex, $E'S$, in equilibrium with the zwitterion complex, $G'Z$, while $F'T$ may or may not be a discreet intermediate. Evidence for this equilibrium can be found in the inverse ^{15}N isotope effect of $^{15}(k_{\text{cat}}/K_M) = 0.995 \pm 0.002$ for HIV-1 protease acting on a fast substrate.³³ Moreover, this inverse effect remains the same in D_2O , while, in contrast, a normal effect of $^{15}(k_{\text{cat}}/K_M) = 1.004 \pm 0.004$ with a slow substrate increases to $^{15}(k_{\text{cat}}/K_M) = 1.020 \pm 0.004$ in D_2O .³³ The latter identifies peptide bond-breaking as being concerted with a proton transfer in the fifth step. With that assignment, the quest to follow *all* of the protons is complete.

Conclusion

Aspartic proteases hydrolyze peptide bonds by using cyclic proton transfers and reactant-state hydrogen tunneling, but then they must be “reloaded” (cleared of products, deprotonated, and rehydrated) and “cocked” (LBHB reformed), and that is the hard part with good substrates. This mechanistic hypothesis is timely because evidence of hydrogen tunneling for other enzymes is accumulating³⁴ and isomechanisms have entered the mainstream.³⁵ Given the recent discovery that β -secretase, the Alzheimer’s amyloid precursor protein-cleaving enzyme, is an aspartic protease,³⁶ we are assured that a keen interest in the mechanism of this family of enzymes will continue. Obviously, a search for mechanism-based inhibitors has a greater chance of success when based on a correct mechanism. Success with transition-state analogue inhibitors rests on tighter binding of substrates within a stabilized transition state, but if rate acceleration is accomplished by some other means, then this design should fail. Looking to new designs, one might expect to achieve tighter binding by developing inhibitors that do not break the short, strong LBHB.³⁷

The author is greatly indebted to Thomas D. Meek for sharing unpublished kinetic data and for engaging in discussions containing precious eureka moments filled with pure joy. Previous work was supported by NIH Grant GM-46695.

References

- (1) Davies, D. R. The structure and function of the aspartic proteinases. *Annu. Rev. Biophys. Biophys. Chem.* **1990**, *19*, 189–215.
- (2) Gillespie, A. L. *The Natural History of Digestion*; W. Scott: London, 1898.
- (3) Northrop, J. H. Crystalline pepsin I. Isolation and tests for purity. *J. Gen. Physiol.* **1930**, *13*, 739–766.
- (4) Darwin, C. *Insectivorous Plants*; John Murry: London, 1875; p 88.
- (5) Sørensen, S. P. L. Enzymstudien II. Mitteilung. Über die Messung und die Bedeutung der Wasserstoffionen-konzentration bei enzymatischen Prozessen. *Biochem. Z.* **1909**, *21*, 201–304.
- (6) Neumann, H.; Knowles, J. R. Acyl- and amino-transfer routes in pepsin-catalyzed reactions. *J. Am. Chem. Soc.* **1975**, *97*, 3557–3559.
- (7) Piana, S.; Carloni, P. Conformational flexibility of the catalytic aspartate in HIV-1 protease: an *ab initio* study on the free enzyme. *Proteins: Struct., Funct. Genet.* **2000**, *39*, 26–36.
- (8) Meek, T. D. Catalytic mechanisms of the aspartic proteinases. In *Comprehensive Biological Catalysis: a Mechanistic Reference*; Sinnott, M., Ed.; Academic Press: San Diego, 1998; Chapter 8.
- (9) Cleland, W. W. Low barrier hydrogen bonds in enzymatic catalysis. *Arch. Biochem. Biophys.* **2000**, *382*, 1–5.
- (10) Smith, R.; Brereton, I. M.; Chai, R. Y.; Kent, S. B. Ionization states of the catalytic residues in HIV-1 protease. *Nature Struct. Biol.* **1996**, *3*, 946–950.
- (11) Piana, S.; Sebastiani, D.; Carloni, P.; Parrinello, M. An *ab initio* molecular dynamics-based assignment of the protonation state of pepstatin A/HIV-1 protease cleavage site. *J. Am. Chem. Soc.* **2001**, *123*, 8730–8737.
- (12) Hunkapillar, M. W.; Richards, J. H. Studies on the catalytic mechanism of pepsin using a new synthetic substrate. *Biochemistry* **1972**, *11*, 2829–2839.
- (13) Quinn, D. M.; Sutton, L. D. Theoretical basis and mechanistic utility of solvent isotope effects. In *Enzyme Mechanism from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, FL, 1991; pp 73–126.
- (14) Hyland, L. J.; Tomaszek, T. A., Jr.; Meek, T. D. Human immunodeficiency virus HIV-1 protease. 2. Use of pH rate studies and solvent kinetic isotope effects to elucidate details of chemical mechanism. *Biochemistry* **1991**, *30*, 8454–8463.
- (15) Rebholz, K. L.; Northrop, D. B. Slow step after bond-breaking by porcine pepsin identified using solvent deuterium isotope effects. *Biochem. Biophys. Res. Commun.* **1991**, *176*, 65–69.
- (16) Cho, Y. K.; Rebholz, K. L.; Northrop, D. B. Solvent isotope effects on the onset of inhibition of porcine pepsin by pepstatin. *Biochemistry* **1994**, *33*, 9637–9642.
- (17) Rebholz, K. L. Enzymatic iso mechanisms: alanine racemase, fumarase, and aspartic proteinases. Ph.D. Thesis, University of Wisconsin—Madison, 1993.
- (18) Rodriguez, E. J.; Meek, T. D. Unpublished results.
- (19) Northrop, D. B. Uses of Isotope Effects in the Study of Enzymes. In *Methods: A Companion to Methods in Enzymology*; Cornish-Bowden, A., Ed.; Academic Press: New York, 2001; Vol. 24, pp 117–124.
- (20) Neumann, H.; Levin, H.; Berger, A.; Katchalski, E. Pepsin-catalyzed transpeptidation of the amino-transfer type. *Biochem. J.* **1959**, *73*, 33–41.
- (21) Cho, Y.-K.; Northrop, D. B. Transpeptidation by porcine pepsin catalyzed by a noncovalent intermediate unique to its isomechanism. *J. Biol. Chem.* **1999**, *273*, 24305–24308.
- (22) Hofmann, T.; Blum, M.; Cunningham, A. Studies on the mechanism of action of penicillopepsin. *Adv. Exp. Med. Biol.* **1991**, *608*, 243–254.
- (23) Balbaa, M.; Blum, M.; Hofmann, T. Mechanism of pepsin-catalyzed amino-transpeptidation reactions. *Int. J. Biochem.* **1994**, *26*, 35–42.
- (24) Zhao, Q.; Abeygunawardana, C.; Gittis, A. G.; Mildvan, A. S. Hydrogen bonding at the active site of 5–3-ketosteroid isomerase. *Biochemistry* **1997**, *36*, 14616–14626.
- (25) Mihalyi, E. *Application of Proteolytic Enzymes to Protein Structure Studies*; CRC Press: Cleveland, OH, 1972; pp 39–101.
- (26) Kreevoy, M. M.; Liang, T. M. Structure and isotopic fractionation factors of complexes, $A_1HA_2^{-1}$. *J. Am. Chem. Soc.* **1980**, *102*, 3315–3322.
- (27) Northrop, D. B. On the meaning of K_m and V_{max}/K_m in enzyme kinetics. *J. Chem. Ed.* **1998**, *75*, 1153–1157.
- (28) Cleland, W. W. The use of isotope effects in the detailed analysis of catalytic mechanisms of enzymes. *Bioorg. Chem.* **1987**, *15*, 283–302.
- (29) Cho, Y. K.; Northrop, D. B. Effects of high pressure on solvent isotope effects of yeast alcohol dehydrogenase. *Biophys. J.* **2000**, *79*, 1621–1628.

- (30) Kresge, A. J. Solvent isotope effect in H₂O–D₂O mixtures. *Pure Appl. Chem.* **1964**, *8*, 243–258.
- (31) Gerritzen, D.; Limbach, H. H. Kinetic isotope effects and tunneling in cyclic double and triple proton-transfer between acetic acid and methanol in tetrahydrofuran studied by dynamic H-1 and H-2 NMR spectroscopy. *J. Am. Chem. Soc.* **1984**, *106*, 869–879.
- (32) Fernandez-Ramos, A.; Smedarchina, Z.; Rodriguez-Otero, J. Double proton transfer in the complex of acetic acid with methanol: Theory versus experiment. *J. Chem. Phys.* **2001**, *114*, 1567–1574.
- (33) Rodriguez, E. J.; Angeles, T. S.; Meek, T. D. Use of nitrogen-15 kinetic isotope effects to elucidate details of the chemical mechanism of human immunodeficiency virus 1 protease. *Biochemistry* **1993**, *32*, 12380–12385.
- (34) Sutcliffe, M. J.; Nigel S.; Scrutton, N. S. Enzyme catalysis: over-the-barrier or through-the-barrier? *Trends Biochem. Sci.* **2000**, *25*, 405–408.
- (35) Rebolz, K. L.; Northrop, D. B. Kinetics of iso mechanisms. *Methods Enzymol.* **1995**, *249*, 211–240.
- (36) Vassar, R.; Bennett, B. D.; Babu-Khan, S.; Kahn, S.; Mendiaz, E. A.; Denis, P.; Teplow, D. B.; Ross, S.; Amarante, P.; Loeloff, R.; Luo, Y.; Fisher, S.; Fuller, J.; Edenson, S.; Lile, J.; Jarosinski, M. A.; Biere, A. L.; Curran, E.; Burgess, T.; Louis, J. C.; Collins F.; Treanor, J.; Rogers, G.; Citron, M. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **1999**, *286*, 735–741.
- (37) A provisional patent application to this effect has been submitted. By using pepstatin as the prototype in the synthesis of tight-binding inhibitors, medicinal chemists may have been trapped in a false minimum.

AR000184M