Arazoformyl Dipeptide Substrates for Thermolysin. Confirmation of a Reverse Protonation Catalytic Mechanism†

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ABSTRACT: Cleavage by thermolysin of *N*-(4-methoxyphenylazoformyl)-L-leucyl-L-leucine plus some congeneric peptides provides a highly sensitive new kinetic assay for proteolytic activity. The pH dependence of Michaelis−Menten parameters k_{cat} and K_m establishes kinetically a reverse protonation catalytic mechanism for this metalloprotease [Mock, W. L., & Aksamawati, M. (1994) *Biochem. J. 302*, 57−68]. An acidified water molecule (pK_a of 5, seen in K_m) becomes displaced by substrate carboxamide from the *hyper*cationic Zn^{2+} of the enzyme, yielding potent Lewis acid activation of the peptide linkage for subsequent hydrolysis. Conversion to product is induced by the side chain of enzymic residue His 231 (pK_a of 8, seen in k_{cat}), which provides general base catalysis for addition of H_2O to the zinc-activated scissile carboxamide of the bound substrate. A previously described "superactivation" through chemical modification of the enzyme with acetylphenylalanyl-*N*-hydroxysuccinimide is nonexistent in the case of the new substrates, which indicates that their binding to thermolysin is largely productive, unlike normal peptides. Correct assignment of kinetically observed pK_a values to active site residues, along with recognition of a predominantly nonproductive binding mode for ordinary substrates and thermolysin, forces reinterpretation of previous mechanistic formulations for the enzyme.

The zinc-containing metalloproteases are some of the more intensively studied enzymes, but their chemical mode of action is still under debate. In an effort to resolve the presently enigmatic mechanism of catalysis used by this family of proteolytic enzymes, a new type of peptide-surrogate substrate has been devised for thermolysin, a prototypical extracellular zinc *endoprotease* of bacterial origin. Hitherto, the best-available substrates for examination of steady-state kinetics with this enzyme have been *N*-furanacryloyl tripeptides. Although highly active enzymically, they only allow spectrophotometric monitoring of amide cleavage over a limited range of substrate concentrations, due to a relatively small net absorption change accompanying hydrolysis and a high base line. We find that incorporation of an "arazoformyl" residue into the P_1-moiety[1] (acyl portion of the scissile linkage), results in an intensely colored substrate ($\epsilon_{350} = 19\,600$ cm^{-1} M^{-1}, with absorption tailing into the visible region, $\epsilon_{400} \approx 3000$ cm^{-1} M^{-1}). In the presence of thermolysin such peptide analogues exhibit moderate activity, with a specific practical advantage accruing from a very large decrease in spectral extinction that attends carbamoyl cleavage. As shown in Figure 1, the intense azo chromophore breaks apart completely upon substrate hydrolysis, yielding uncolored products, and in consequence this fragmentation provides a highly sensitive assay for catalytic activity. Employment of these new substrates in the subsequently described investigation has provided a unique insight into the catalytic mechanism of thermolysin.

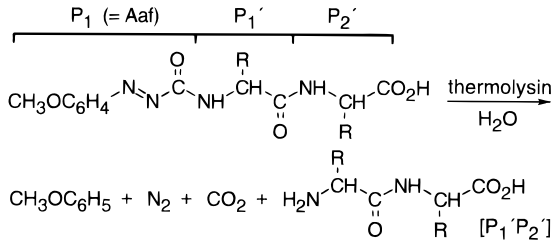

FIGURE 1: Reaction of arazoformyl dipeptides catalyzed by thermolysin. Substrates examined: Aaf-Leu-Leu-OH, Aaf-Leu-Ala-OH, Aaf-Leu-Phe-OH, Aaf-Leu-Gly-OH, Aaf-Leu-D-Ala-OH, and Aaf-Leu-NH_2.

MATERIALS AND METHODS

Enzyme and Buffer Solutions Employed. Thermolysin (EC 3.4.24.2, ×3 recrystallized) was supplied by Calbiochem. Enzyme concentrations in 2.5 M sodium bromide and 0.01 M calcium chloride stock solution were estimated using $A^{1\%}_{1cm,280} = 17.65$ and a molecular mass of 34 600 Da. Buffers employed in this work for kinetic analysis were (0.05 M each) as follows: piperazine, pH 4.5−6.5; Pipes, pH 6.5−7.7; Popso, pH 7.7−8.8; aminopropanesulfonic acid, pH 8.8−9.5. All were shown to give negligible kinetic perturbations with thermolysin. Enzyme investigations have been done with solutions 0.01 M in calcium chloride.

Arazoformyl Dipeptide Substrates. The following is an illustrative preparative procedure yielding Aaf-Leu-Leu-OH (synthetic intermediates have been fully characterized spectroscopically). 4-Methoxyphenylhydrazine was recovered

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[1] Abbreviations: Pipes, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; Popso, piperazine-*N*,*N*′-bis[2-hydroxypropanesulfonic acid]; Aaf, *N*-(4-methoxyphenylazoformyl); Fua, *N*-(3-[2-furyl]acryloyl); Gly^P, aminomethylphosphonic acid residue (analogue of glycine); Xaa, generic amino acid residue. The designations used for the amino acid residues (P) of the substrate and to name the subsites (S) of the active site are that of Schechter and Berger (1967). Amino acid residues are of L-configuration unless otherwise indicated.

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from its hydrochloride by repeated methylene chloride extraction from 10% aqueous alkali, followed by drying (KOH pellets) and rotary evaporation of the CH_2Cl_2. To a solution of 46 g (0.2 mol) of diphenyl carbonate in 50 mL of refluxing benzene was added 19.3 g (0.14 mol) of 4-methoxyphenylhydrazine in 100 mL of benzene dropwise over 0.5 h, and the resulting solution was refluxed under nitrogen for an additional 6 h. Subsequent concentration by rotary evaporation yielded a precipitate, which was collected. Additional product was obtained upon dilution of the filtrate with hexane. Recrystallization of the solids from benzene and hexane gave 28.0 g (76%) of white crystals of phenyl 4-methoxyphenylhydrazoformate ($CH_3OC_6H_4NHNHCO_2$-C_6H_5), mp 110−112 °C, IR ($CHCl_3$) 1750 cm^{-1}. For coupling of this material to a dipeptide, the noncrystalline tetramethylammonium salt of L-leucyl-L-leucine was secured by dissolving 3.1 g (12 mmol) of leucylleucine in 1 equiv of methanolic tetramethylammonium hydroxide, followed by rotary evaporation. The residual oil was taken up in 40 mL of dimethylformamide and heated to 95 °C under a nitrogen atmosphere, while 2.6 g (10 mmol) of phenyl 4-methoxyphenylhydrazoformate was added to the stirred solution. After continued heating for 3.5 h, the red solution was cooled to 15 °C and was acidified with 1 N HCl to a pH of 2−3, as indicated by pH paper. The resulting solution was diluted with 300 mL of ethyl acetate and was then extracted with water (4 × 300 mL). The solid residue obtained by rotary evaporation of the dried (with anhydrous Na_2SO_4) ethyl acetate extract was recrystallized from ethanol and ether to give 2.36 g (58%) of white crystals of *N*-(4-methoxyphenylhydrazoformyl)-L-leucyl-L-leucine, mp 132−134 °C. To a stirred solution of 2.2 g (5.4 mmol) of this material in 125 mL of water containing 1 equiv each of sodium hydroxide and sodium acetate was added portionwise an aqueous solution of 1.16 g (5.4 mmol) of sodium periodate, yielding a deep orange solution within a few minutes. After 20 min the mixture was acidified to a pH of 2−3 as indicated by pH paper, whereupon an orange solid precipitated. Collection and recrystallization from methylene chloride and ether gave 1.93 g (88%) of orange crystals of *N*-(4-methoxyphenylazoformyl)-L-leucyl-L-leucine (Aaf-Leu-Leu-OH), mp 154−156 °C (dec), UV (H_2O) 350 nm (ϵ = 19 600 cm^{-1} M^{-1}). Anal. Calcd for $C_{20}H_{30}N_4O_5$: C, 59.10; H, 7.44; N, 13.78. Found: C, 58.74; H, 7.39; N, 13.64. Other substrates were prepared similarly: Aaf-Leu-Ala-OH (as dicyclohexylamine salt), mp 163−164 °C (dec); Aaf-Leu-Phe-OH, mp 155−158 °C (dec); Aaf-Leu-Gly-OH (as dicyclohexylamine salt), mp 159−161 °C (dec); Aaf-Leu-NH_2, mp 146−148 °C (dec); Aaf-Leu-D-Ala-OH (as dicyclohexylamine salt), mp 175−177 °C (dec). Correct CHN analyses have been obtained for each of the latter substances.

Kinetic Analysis. The catalytic pH dependence (Michaelis−Menten k_{cat}, K_m) for arazoformyl dipeptides with thermolysin was determined at 25.0 (±0.1) °C in buffers previously listed, with spectrophotometric monitoring (350−400 nm, 0.01−5 cm path length) and the method of initial rates. Stock solutions of the arazoformyl dipeptide substrates were shielded from light to avoid *cis*−*trans* isomerization of the azo linkage (Kanstrup & Buchardt, 1991). Enzyme concentration was maintained well below substrate concentration for kinetic parameter determinations in all cases, except at low pH where substrates were relatively inactive and K_m values were especially low, in which case a quadratic modification of the Michaelis−Menten equation had to be used in some cases to allow for reactant depletion in kinetic measurements. The limiting kinetic parameters were obtained as a function of pH by a nonlinear least-squares fit of data to the appropriate equations as given in the figure legends. For superactivation of the enzyme, thermolysin was treated in a prescribed manner (Blumberg & Vallee, 1975) with 2 mM *N*-acetyl-L-phenylalanyl-*N*-hydroxysuccinimide in buffered solution (Popso, pH 8, 30 min duration). Assay with Fua-Gly-Leu-NH_2 at a pH of 7 (Pipes buffer) indicated an activity enhancement factor of 8.3-fold relative to the unmodified enzyme. All pH values in this paper are calibrated pH meter readings uncorrected for ionic strength effects. Tolerances listed are standard errors from least-squares analysis.

RESULTS

Substrate Design. The present report entails a new rendering of the Morihara−Tsuzuki series of six oligopeptide substrates (Morihara & Tsuzuki, 1970) for the metalloenzyme thermolysin (Figure 1). This *endoprotease* has a notable specificity regarding the nature of the side chain of the amino acid residue occupying position P_2' within the peptides that it cleaves, the latter being symbolized as ($H_2N\cdots$)$P_1\cdot P_1'P_2'$-($\cdots CO_2H$), with "·" indicating the scissile peptide linkage. For this enzyme investigation the P_2' residue was systematically replaced across the series, becoming C-terminal L-Leu-OH, L-Ala-OH, L-Phe-OH, Gly-OH, D-Ala-OH, or simply NH_2, with the reactivity consequence of generating a sizable variation in velocity of catalytic hydrolysis. Within the series examined the internal residue P_1' has been kept the same, always L-Leu. Amide bond cleavage occurs between P_1' and P_1 residues, and as previously indicated (Figure 1) our *N*-terminus P_1 residue is the *p*-anisylazoformyl group CH_3-$OC_6H_4N{=}NCO-$ (acronym: Aaf), which upon release rapidly fragments into anisole, molecular nitrogen, and carbonate (Thiele, 1895), probably by a polar mechanism. Under steady-state assay conditions, enzymic hydrolysis of these substrates proceeds to completion and appears uncomplicated; no kinetic or spectroscopic evidence has been found for accumulation of an intermediate in the course of the catalytic reaction (the latter possibility, on a shorter time scale, may be the subject of another investigation). Structure−reactivity correlations obtained with these substrates provide the key to comprehension of thermolysin's mechanism.

Enzyme Kinetics, [S] ≪ K_m. Thermolysin provides normal Michaelis−Menten catalytic behavior, but the kinetic parameters are pH-dependent. The specificity constant k_{cat}/K_m is a quotient that corresponds to the second-order rate constant for peptide hydrolysis under conditions of high dilution, where most of the enzyme remains disengaged from substrate in the steady state. This kinetic parameter has special significance, for pH-induced perturbations in its magnitude commonly yield true pK_a values for active-site functional groups on the enzyme, so long as those entities are involved in substrate binding or in the first committed step of catalysis. The k_{cat}/K_m *versus* pH profile for several of the arazoformyl substrates is presented on log scale in Figure 2. As shown previously for thermolysin cleavage of normal peptides (Feder & Schuck, 1970; Pangburn & Walsh, 1975; Kunugi et al., 1982; Izquierdo & Stein, 1992; Mock & Aksamawati, 1994), the pH dependence of this kinetic parameter is bell-shaped, with acidic- and alkaline-limb pK_a

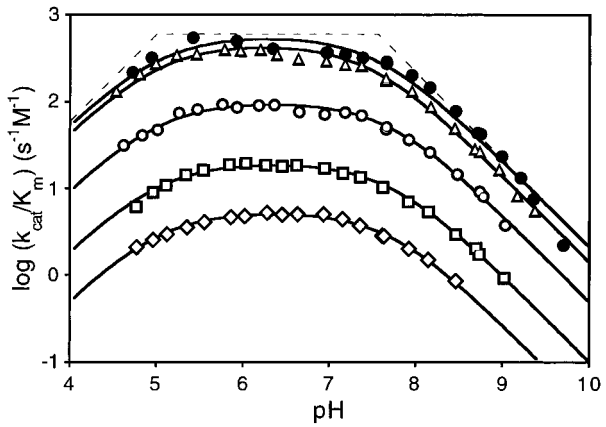


FIGURE 2: Dixon plots of log k_{cat}/K_m *versus* pH for arazoformyl−dipeptide substrates of thermolysin. Curves shown are nonlinear least-squares (log) fits to the equation $(k_{cat}/K_m)_{apparent} = (k_{cat}/K_m)_{lim}/\{(1 + [H]/K_{a1})(1 + K_{a2}/[H])\}$, with the acidic-limb p$K_{a1}$ fixed at 5.0. The latter is a consensus value, adopted due to complications from extremely low K_m values for some substrates on the one hand, and to uncertainty caused by inability to sample kinetics at pH values of sufficiently greater acidity due to buffer limitations and to substrate carboxylate protonation. Fitted parameters for $(k_{cat}/K_m)_{lim}$ and for the second acid dissociation constant are as follows: Aaf·Leu-Leu-OH (filled circles) 580 (±50) s^{-1} M^{-1}, pK_{a2} = 7.56 (±0.06); Aaf·Leu-Ala-OH (triangles) 470 (±20) s^{-1} M^{-1}, pK_{a2} = 7.48 (±0.03); Aaf·Leu-Phe-OH (open circles) 100 (±3) s^{-1} M^{-1}, pK_{a2} = 7.69 (±0.02); Aaf·Leu-Gly-OH (squares) 19.8 (±0.1) s^{-1} M^{-1}, pK_{a2} = 7.71 (±0.02); Aaf·Leu-NH_2 (diamonds) 5.4 (±0.1) s^{-1} M^{-1}, pK_{a2} = 7.71 (±0.02); Aaf·Leu-D-Ala-OH (not shown) $(k_{cat}/K_m)_{lim}$ ~0.1 s^{-1} M^{-1} (single-point estimate). Dashed line asymptotes (slopes +1, 0, −1) are shown for Aaf-Leu-Leu-OH; breaks correspond to pK_a values.

values bracketing a region of maximum activity. In the present instance, analysis of those kinetic inflections yields pK_a values of ~5.0 and ~7.7, respectively. The actual quantities derived in each case by least-squares curve fitting are given for the substrates in the legend to Figure 2, along with a calculated $(k_{cat}/K_m)_{lim}$, the extrapolated maximum value for the specificity constant occurring at intermediate pH for each. Among peptides Aaf·Leu-Xaa-OH that have exclusively the standard L-configuration, this measure of hydrolytic susceptibility varies systematically over a 100-fold range, according to the nature of the P_2' residue. The sixth member of the substrate series, Aaf-Leu-D-Ala-OH, was cleaved much more slowly than its L-congeners; an estimate of its rate constant at intermediate pH is also given in the legend. The influence of side-chain size and especially configuration in the P_2' position is readily apparent. For example, presence of a D-Ala residue there yields a 4700-fold rate reduction in comparison to L-Ala. An explanation of relative rates, and the identity and role of enzymic moieties responsible for the observed pH dependence, is offered subsequently.

Michaelis−Menten Parameters. Although the specificity profile for peptide cleavage as displayed in Figure 2 is conventionally bell-shaped, the component parameters k_{cat} and K_m have a markedly different pH dependence. The complexity of the situation is shown for the most active substrate Aaf-Leu-Leu-OH in Figure 3, again employing a log scale. The experimental K_m only attains its minimum value, 0.78 μM, on the acidic-limb of the pH range, and it appears to increase by >4 orders of magnitude on passing to a pH of 9. Conversely, the value of k_{cat} is maximal at high pH (limiting value of 0.63 s^{-1}), and it suffers a monotonic decrease with diminishing pH, again by nearly 4

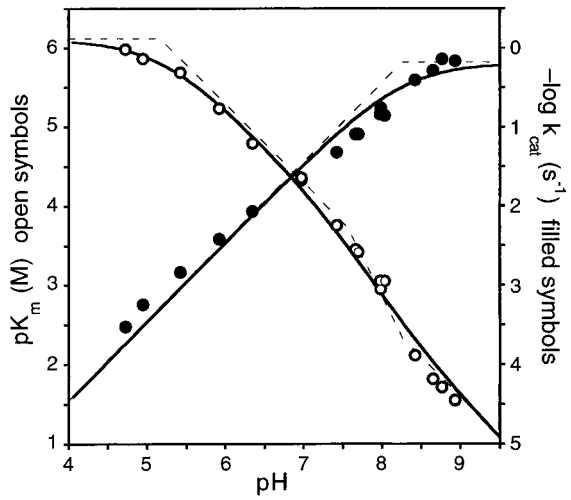


FIGURE 3: Dixon plots for substrate Aaf·Leu-Leu-OH, of pK_m (−log K_m, open symbols, left-hand axis) and of log k_{cat} (filled symbols, right-hand axis) *versus* pH for catalytic hydrolysis with thermolysin. Curves shown are nonlinear least-squares (log) fits to equations $(k_{cat})_{apparent} = (k_{cat})_{lim}/(1 + [H]/K_a)$ and $(K_m)_{apparent} = (K_m)_{lim}\ (1 + 10^{-8.26}/[H])/\{(1 + K_a/[H])(1 + 10^{-7.56}/[H])\}$. Fitted parameters are as follows: $(k_{cat})_{lim}$ = 0.63 (±0.18) s^{-1}, pK_a = 8.26 (±0.16), and $(K_m)_{lim}$ = 0.78 (±0.24) μM, pK_a = 5.16 (±0.15). Minor double inflection at high pH in the pK_m profile is required by the differential between alkaline-limb pK_a values for primary rate parameters k_{cat} and k_{cat}/K_m (8.26 and 7.56, respectively). Dashed line asymptotes with breaks at pK_a values are also shown.

log units. A pK_a of 5.1 characterizes the major inflection in the pK_m profile, and a pK_a of 8.3 marks the downturn in log k_{cat}. Because of this kinetic expression within K_m and k_{cat} of a mutually compensatory $[HO^-]$ and $[H^+]$ dependence, an essentially level profile results for log k_{cat}/K_m at intermediate pH values (Figure 2). It is noteworthy that detection of such an extreme pattern of pH variation for the individual Michaelis−Menten parameters was made feasible only by the exceptional spectrokinetic sensitivity of the new substrates. Because of the large absorptivity change accompanying hydrolysis, it is practical to follow kinetics over a $>10^5$-fold range of substrate concentrations (by adjusting cell path length and assay wavelength), as was necessary to establish the experimental K_m dependence. Comparable observations indicate that the other arazoformyl−dipeptide substrates follow the same pattern, although unfavorable K_m values and substrate solubility limitations prevent full discernment for several of the less active members of the series. For a pH of 7, the following experimental K_m and k_{cat} values were obtained: Aaf·Leu-Leu-OH, 0.044 (±0.002) mM, 0.021 (±0.001) s^{-1}; Aaf·Leu-Ala-OH, 1.4 (±0.1) mM, 0.37 (±0.02) s^{-1}; Aaf·Leu-Phe-OH, 0.36 (±0.06) mM, 0.027 (±0.002) s^{-1}; Aaf·Leu-Gly-OH, 4.5 (±0.2) mM, 0.079 (±0.002) s^{-1}; Aaf·Leu-NH_2, ≥15 mM (extrapolated K_m value, k_{cat} undiscernable); Aaf·Leu-D-Ala-OH, K_m, k_{cat} inaccessible.

Kinetic Resemblance to More Typical Substrates. In comparison with normal oligopeptides or with *N*-furanacryloyl derivatives, the arazoformyl dipeptides seem to be somewhat less active enzymically. The specificity constants (k_{cat}/K_m values) for the new substrates appear to be reduced by several orders of magnitude relative to authentic peptide species carrying the same $P_1'P_2'$ residues, as will subsequently be considered. As a practical matter this is compensated, in large measure, by the great spectral sensitivity of kinetic assay in the case of the arazoformyl substrates, and by

generally lower K_m values, easing solubility complications. A previously observed and curious feature of thermolysin catalysis is "superactivation" by chemical modification. Brief treatment of solutions of the enzyme with *N*-acetyl-L-phenylalanyl-*N*-hydroxysuccinimide has the kinetic consequence of enhancing catalytic activity by more than an order of magnitude with some of the normal substrates ($\geq$70-fold increase in exceptional cases) (Blumberg & Vallee, 1975; Holmquist et al., 1976). The phenomenon has been traced to O-acylation of the phenol side chain of enzymic residue Tyr 110 by acetyl-Phe (Blumberg, 1979). We find no such kinetic effect in the case of the arazoformyl dipeptides. Thermolysin that has been activated successfully with respect to cleavage of a furanacryloyl-type substrate shows only a slight *deactivation* in the new assay (k_{cat}/K_m), with observed relative rates for enzymic hydrolysis $\sim$0.8 of that with the native (unmodified) enzyme, for all of the arazoformyl derivatives. The latter finding carries mechanistic significance, as emerges from the following analysis.

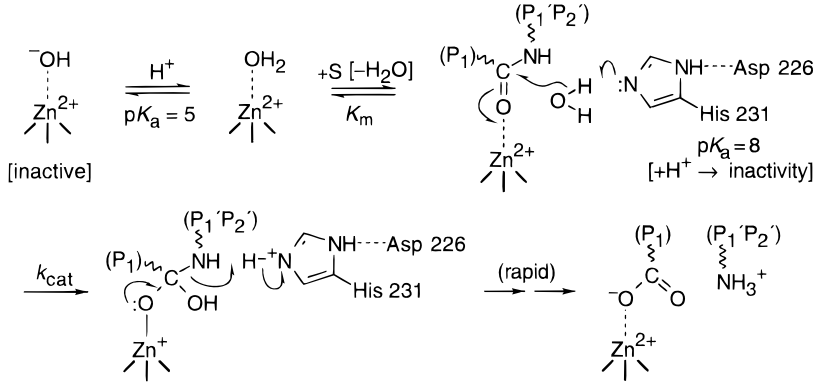
DISCUSSION

Effect of pH on Catalysis. The steady-state kinetic results obtained with this new type of substrate clarify the mechanism of thermolysin. Since catalysis rates are several hundred-fold slower than for the best normal oligopeptide substrates, it is highly likely that equilibrium binding of the arazoformyl dipeptides to the enzyme is occurring. As a function of pH, the specificity constant k_{cat}/K_m has the bell-shaped profile commonly observed with thermolysin. This signifies that ionizations perturbing the kinetics occur on the substrate or on the enzyme. Because pertinent deprotonation of substrate (e.g., Aaf•Leu-NH_2) does not take place within the pH range examined, the presence of two ionizable and mechanistically significant functional groups at the enzyme active site is indicated, with pK_a values of 5 and 7.7 on the acidic- and alkaline-limbs, respectively. From Figures 2 and 3 it is obvious that the relative insensitivity to pH for k_{cat}/K_m near neutrality is the product of opposing strong pH dependences for the individual factors within that quotient. This aspect of thermolysin kinetics has been given only cursory examination previously (Morihara & Tsuzuki, 1970; Fukuda & Kunugi, 1984; Mock & Aksamawati, 1994). The pK_a value of 5 is seen to be associated with the Michaelis−Menten constant K_m; apparent enzyme−substrate affinity is greatest at low pH and diminishes linearly with increasing alkalinity. On the other hand k_{cat} is maximized near the pK_a of $\sim$8, and below that pH reaction velocity decreases in response to increased acidity. In each case a first-order [H^+] or [HO^-] dependence is seen for the kinetic parameter. Quite evidently, productive substrate binding takes place only in the presence of the conjugate acid form of the functional group having pK_a of 5, and actual chemical conversion depends upon the conjugate base form of the group having pK_a of $\sim$8. This pattern meets the definition of a "reverse protonation" mechanism of catalytic action for thermolysin. The nominal enzyme species yielding catalysis when [S] $\ll$ K_m is an apparently minor and thermodynamically disfavored constituent within the prevailing distribution of enzymic protonation states, namely, that form with but a single proton present at the active site and attached to the *less basic member* of the implicated duo of catalytic residues. In the active enzyme *protonation is reversed* from that normally expected (Mock, 1992; Mock & Aksamawati, 1994). A valid attribution for both the pH 5 and 8 enzymic ionizations to appropriate, crystallographically-observed residues present at the active site of thermolysin is obviously essential for a correct understanding of the catalytic process. This is a matter that has been subject to misconceptions in the past, as regards metalloproteases generally.

The alkaline-limb pK_a of $\sim$8 for thermolysin can be assigned with a good deal of assurance to the side chain of essential active site residue His 231. The enzyme can be inactivated by the imidazole-specific reagent diethyl pyrocarbonate in a time-dependent manner, with protection conferred by a competitive inhibitor and with inactivation reversible by hydroxylamine (Burstein et al., 1974). Moreover, the rate of chemical modification itself exhibits an expected sigmoidal pH dependence, apparently characterized by the same pK_a value of $\sim$8 (Kunugi et al., 1982). His 231 is the only suitable active site functionality explaining this behavior; its side-chain imidazole resides within the active site cleft approximately 5 Å from the zinc ion, and it is linked through a backside hydrogen bond, having an orientation distal to the metal ion (via N_δ), to the side-chain carboxylate of residue Asp 226, according to crystallographic evidence (Matthews, 1988). Present kinetic results (Figure 3) indicate that the cationic imidazolium form of His 231 is an inactive species catalytically and that in all probability the neutral imidazole ring functions instead as a base in the course of amide bond scission, thereby showing up in k_{cat} (Mock & Aksamawati, 1994). Our evidence suggests that binding of substrate causes a half-unit increase in the pK_a value of this moiety, from 7.7 in the free enzyme (k_{cat}/K_m) to $\geq$8.2 in E•S (k_{cat}). Such a perturbation is unexceptional and produces only the expected 0.5 log unit double inflection in the K_m profile (Figure 3); i.e., prototopic change involving His 231 influences only slightly the binding of susceptible substrates. By way of confirmation, a site-directed mutagenesis study with the homologous extracellular protease from *Bacillus stearothermophillus* showed that excision of the side chain imidazole moiety (point mutation H231A) results in a reduction of $\sim$500-fold in the specificity constant k_{cat}/K_m, with an accompanying deletion of the alkaline-limb pK_a value otherwise appearing in that kinetic parameter [Beaumont et al., 1995; see also Toma et al. (1989)]. The catalytic variation resided almost entirely in k_{cat}, indicating that binding of substrate was unaffected.

Previously, the acidic-limb pK_a of 5 that is noted in K_m has been widely (but erroneously) attributed to enzymic residue Glu 143, the side chain of which is located at the bottom of the active site cleft of thermolysin and in the vicinity of the zinc ion. This glutamate residue has an omnipresent counterpart in other metalloproteases. However, such prior assignments to that carboxylic acid functional group, for the kinetic perturbation occurring in acidic solution, have never had any chemical substantiation (Mock & Tsay, 1988). Instead, the pK_a of 5 ionization for thermolysin is now known from inhibitor binding studies to be due to the *water molecule* that binds to the active-site zinc ion in the absence of substrate (Mock & Aksamawati, 1994). While such an enhanced $(Zn^{2+})OH_2$ acidity might seem surprising, it has been demonstrated in several other metallopeptidases (Mock & Tsay, 1986, 1988; Mock et al., 1993; Mock & Liu, 1995). An explanation for the observed pH dependence of K_m is that the aquo unit present in enzymic $(Zn^{2+})OH_2$ has to be *displaced* from the metal ion in the

Scheme 1



course of productive substrate binding (Mock et al., 1981). However, a relatively nonbasic amide carbonyl such as encountered in substrates cannot compete well with hydroxide in ligation to that Lewis acid center. Consequently, the lowest values of K_m are only achieved below a pH of 5, and binding becomes progressively weaker (apparent pK_m diminishes) as alkalinity of the medium increases and enzymic $(Zn^{2+})OH^-$ comes to predominate. This relatively low value of pK_a for metal-bound water has to be understood in terms of heightened *Lewis* acidity on the part of the zinc ion, which has been evolutionarily optimized for most effective chemical activation of substrate carbonyl (Mock et al., 1993). Electrodative restrictions in the immediate ligand environment of that Zn^{2+} (four-coordination) intensifies the cationic electron deficiency, rendering it especially potent for the purpose of stabilization of a tetrahedral adduct oxyanion in the course of catalysis but also unavoidably inducing deprotonation of ligated H_2O in the absence of substrate. The pK_a of 5 represents a fittest compromise between catalytically beneficial Lewis acidity of enzymic Zn^{2+}, and detrimental Brønsted acidity of the corresponding (Zn^{2+})-OH_2.

The chemical mechanism according to this explanation is summarized in Scheme 1 for normal peptides, with an analogous process envisioned for the arazoformyl analogues. In the catalytic cycle, H_2O/HO^- first becomes displaced from Zn^{2+} by substrate (productive binding), and then the imidazole side chain of His 231, in its unprotonated form, functions as a general base to assist hydration of the metal-activated substrate in the k_{cat} step. Subsequent conversion to products, through a Zn^{2+}-ligated tetrahedral adduct derived from the scissile linkage, is probably facilitated through imidazolium general acid catalysis as shown also.[2]

As previously noted, genetic deletion of the His 231 side chain from the active site causes a 500-fold reduction in k_{cat}, with accompanying disappearance of the alkaline-limb pK_a in the k_{cat}/K_m profile (Beaumont et al., 1995). According to the reported pH dependence for catalysis, the H231A mutant enzyme evidently achieves its residual hydrolytic activity through the default pathway of a specific base mechanism (nucleophile = lyate HO^- operating upon the zinc-activated substrate), which process must be nearly diffusion-limited in view of the rate constants reported. The latter mechanism apparently is only 2−3 orders of magnitude less favorable than the general base pathway of the intact enzyme (nucleophile = H_2O, undergoing deprotonation by His 231 in the transition state for addition). Such behavior has precedent in ordinary acyl-group transfers; specific and general base mechanisms commonly are competitive with one another. Alternatively, the less acidic side chain of active site residue Tyr 157, which we suggest normally serves to orient the nucleophilic water molecule by hydrogen-bond donation (*vide infra*), could ionize and step in as a reverse-protonation catalytic general base (phenolate) in the case of the H231A mutant [precedent in Mock and Xu, 1994)].

We note that an alternative attribution of the pK_a of 5 to enzymic residue Glu 143 cannot be sustained in the face of the present evidence. It is improbable that deprotonation of that side-chain carboxyl group should completely suppress productive substrate binding as we find kinetically. And in any event, should that be so then the catalytically active form would be Glu 143-$CH_2CH_2CO_2H$, and the carboxylate *anion* would then be unavailable within an E·S complex for the mechanistic general base function previously assigned to it. The pH dependence of K_i for competitive inhibitors that ligate to the active site metal ion is unequivocal in identifying the pK_a of 5 with $(Zn^{2+})OH_2$ (Mock & Aksamawati, 1994). In all probability Glu 143 has a normal pK_a (value <5), and its essentiality (Toma et al., 1989) arises from the anionic side-chain carboxylate group serving as a negatively charged counter-ion providing electrostatic stabilization for electron-deficient reaction intermediates that are generated in proximity during the catalytic cycle.[3]

Productive versus Nonproductive Binding. It remains to be shown how this mechanism clarifies formerly confusing features of thermolysin catalysis. Table 1 contains a compilation of items of relevant kinetic behavior for the enzyme, either from this investigation or previously reported. Of special significance is the *superactivation* of thermolysin by chemical modification. Selective acylation of the phenolic side chain of enzymic residue Tyr 110 with acetylphenylalanine reportedly enhances catalytic activity toward various peptide-type substrates by a factor of 2−70-fold (Blumberg & Vallee, 1975; Holmquist et al., 1976; Blumberg, 1979).

[2] A reservation that might be expressed about the new mechanism is that the largely analogous zinc enzyme carboxypeptidase A does not have an equivalent active site histidine residue. As recently shown, however, carboxypeptidase A probably uses the catalytically-obligatory carboxylate present in its substrates for intramolecular general base catalysis in lieu of His 231 (Mock & Zhang, 1991; Mock & Xu, 1994).

[3] Because Glu 143-$CH_2CH_2CO_2H$ and $(Zn^{2+})OH_2$ reside within H-bonding distance of one another, we do not exclude that their ionizations should mutually influence each other. However, the measured pK_a of 5 would be the *second* ionization of such a coupled system.

Table 1: Kinetic Parameters for Substrates/Inhibitors P_1·Leu-P_2' (· = Cleavage Site)

	P_2' residue					
P_1 residue	Leu-OH	Ala-OH	Phe-OH	Gly-OH	NH_2	D-Ala-OH
			k_{cat}/K_m (s^{-1} M^{-1})			
1. ArN═NCO[a]	580	470	100	20	5.4	0.1
2. Cbz-Gly[b]	144 000	78 000	50 000	6 100	5 100	300
3. Fua-Gly[c]		870 000	300 000	83 000	22 000	
			K_m (mM, pH = 7)			
4. Cbz-Gly[b]	2.6	10.6	2.4	10.8	20.6	16.6
5. ArN═NCO	0.044	1.4	0.36	4.5	≥15	
			K_i (competitive inhibition by substrate analogues)			
6. Cbz-Gly[P] (nM)[d]	9.1	16.5	78	270	760	1700
7. P_1 absent (mM)[e]	1.0	6.3	1.1	18		
			"superactivation" factor (× rate enhancement upon acylation of Tyr 110)			
8. Fua-Gly[c]		3.5	15	20	20	
9. ArN═NCO	0.9	0.8	0.8	0.7	0.8	

[a] Limiting values from Figure 2. [b] Data for pH = 7 from Morihara and Tsuzuki (1970). [c] Data from Blumberg and Vallee (1975). [d] Phosphonamidates: P_1 = Cbz-$NHCH_2PO_2^-$. Data from Bartlett and Marlowe (1983). [e] $P_1'P_2'$-fragment analogs: P_1' = $C_6H_5CH_2CH_2C(=O)$ residue instead of Leu. Data from Murphy et al. (1980).

Table 1, row 8, gives literature data for Fua-Gly·Leu-Xaa-OH substrates, for which specificity constants are given in row 3. The effect is greater for less readily cleaved substrates (Holmquist et al., 1976; Blumberg, 1979). This phenomenon has been unexplained, and it is a puzzle that an enzyme with a presumed evolutionary history should harbor such latent but easily accessible extra activity. According to crystallography the enzymic tyrosine residue that becomes modified lies on the rim of the active site cleft, in the vicinity of the metal ion, but apparently it is too remote from the reaction center for direct chemical-catalytic participation. The germane observation is that while the activation phenomenon appears to be general for peptidic substrates having K_m values in the millimolar range, it is absent for our Aaf·Leu-Xaa-OH substrates with rather lower K_m values (rows 1, 5, and 9 of Table 1). This circumstance will subsequently be rationalized.

A second important clue to the catalytic mechanism lies in the Morihara−Tsuzuki specificity order. Normal peptide cleavage with thermolysin is very sensitive to the nature of the P_2' residue within the substrate. As recorded in Table 1, for a series of substrates having structure Cbz-Gly·Leu-Xaa-OH (row 2), the value for k_{cat}/K_m varies by ∼500-fold according to the size and stereochemistry of the substrate side chain in that position (generic residue Xaa = P_2', cleavage site = ·). By way of rationalization, a hydrophobic pocket registering with the P_2' portion of substrate is evident in crystal structures of thermolysin (enzymic subsite S_2'). However, the value of the kinetic parameter K_m is scarcely dependent upon chemical structure for the physiological-type substrates. As also given in Table 1 (row 4), K_m values for the normal peptides cluster around a value of 10 mM, with the lower values (for Xaa = Leu or Phe) primarily attributable to desolvation of the P_2' side chain in the course of association with the enzyme and not necessarily to any specific E−S interaction involving the S_2' subsite. The latter interpretation is supported by the relative magnitudes of K_i values for competitive inhibitors having structure $C_6H_5CH_2$-CH_2CO-Xaa-OH (e.g., a truncated substrate fragment nominally corresponding to $P_1'P_2'$), also recorded in Table 1 (row 7). Here the same hydrophobicity pattern is reflected, for a set of non-reacting analogues that merely associate reversibly with the enzyme. Moreover, one member of this inhibitor family has been shown crystallographically to bind across the enzymic S_1S_1' subsites, leaving S_2' empty (Kester & Matthews, 1977). In summary, what is peculiar about the Morihara−Tsuzuki kinetic data is that fit of substrate to the enzyme, at a position which is relatively remote from the reaction center, should be more important for a catalytic step (k_{cat}/K_m) than for apparent substrate binding (K_m).

The thermolysin reactivity shown by the new arazoformyl dipeptide substrates conforms only as regards the relative magnitudes of k_{cat}/K_m. As may be seen in Table 1, overall catalytic susceptibility is reduced by several orders of magnitude in comparison to normal peptide substrates, but specificity still changes across the P_2'-varied series in the same fashion (compare rows 1−3). Unlike the normal peptides, however, the reactivity trend is also tracked in K_m values (row 5). Although the hydrophobicity effect of the larger side chains still shows, the range of K_m values (>300-fold) matches (inversely) that of k_{cat}/K_m. Furthermore, as previously noted the new substrates do not benefit catalytically from enzymic chemical modification. Acylation of Tyr 110, which reportedly produces a 20-fold enhancement in the rate of hydrolysis of the poorer *N*-furanacryloyl substrates, has a uniform and slightly adverse effect upon cleavage of arazoformyl dipeptides (row 9 of Table 1).

For the preceding complex pattern of kinetic behavior we can supply a coherent and quite simple explanation, having major ramifications for the catalytic mechanism. We are forced to the conclusion that commonly studied small peptide substrates for thermolysin, other than our arazoformyl derivatives, bind to the enzyme *nonproductively* for the most part (Fukuda & Kunugi, 1984; Kunugi et al., 1995). Specifically, the S_2' receptacle on the enzyme may not be filled to any extent in equilibrium complexes with such substrates. Of course a competing but less accessible productive mode necessarily does exist, probably involving activation of the substrate by metal-ion coordination as depicted in Scheme 1 (and having P_2' engaged with S_2'), but that complex is not a thermodynamically favored manner of E·S binding for many normal peptides. We further suggest that all substrates of a given type, once they do achieve productive binding, experience a similar rate of chemical conversion. According to this postulate, the Morihara−Tsuzuki reactivity scale for normal peptides is

solely a consequence of differing amounts of *productive* enzyme−substrate association, attributable to a $P_2'-S_2'$ substituent interaction occurring within the *minor* amount of E·S that is so configured at equilibrium. This explains why K_m values are nearly the same for all such substrates (i.e., the P_2' residue is disengaged from its receptacle in the thermodynamically-favored nonproductive mode, which accounts for most of E·S). The explanation for "superactivation" by chemical modification is that it tilts the balance between productive and nonproductive peptide binding. There need be no effect upon the actual chemical conversion step, so long as the modified enzyme yields a greater fraction of productively bound substrate. The experimental observation that poorer peptide substrates typically experience a greater degree of activation indicates that this is so. The rationalization is that they had a greater proportion of nonproductive binding initially, i.e., the better substrates are superior only because they have less of an energetic hill to climb in the course of converting from a nonproductive to the productive mode within E·S.

The kinetic behavior of our arazoformyl dipeptide substrates neatly completes this picture. The evidence indicates that, unlike physiological-type peptides, they do bind productively for the most part. The Morihara−Tsuzuki specificity with these substrates is reflected in K_m (corresponding to fit of the P_2' side chain) and with one exception k_{cat} does not vary greatly with the nature of the side chain (once substrate has been bound and activated, remote subsite S_2' evidently cannot influence the activation barrier for cleavage within subsite S_1).[4] These substrates are not susceptible to "superactivation" for that reason. For this to be plausible, the arazoformyl substrates ought to bind intrinsically more tightly to the enzyme. Such is the case; at a pH of 7 the K_m value for Cbz-Gly·Leu-Leu-OH indicates a 40-fold weaker association than for Aaf·Leu-Leu-OH, despite the latter containing one less amino acid residue (Table 1). Due to some cause that presently can only be surmised, the arazoformyl moiety strongly enhances productive binding over the nonproductive mode that is favored with normal peptides.

Enzyme Efficacy. This greater enzyme affinity in productive association is probably connected with the reduced enzymic susceptibility of the arazoformyl derivatives relative to normal peptides (Table 1, rows 1−3), for according to current theory, excessive binding energy squanders catalytic potential. In the case of the more extended proteinacious chains that this *endopeptidease* usually cleaves in nature, the enzyme may be poised so that productive and nonproductive binding modes of the scissile carboxamide linkage are more closely in balance than for small peptides. Potent Lewis acidic Zn^{2+} coordination to the amide carbonyl such as we have postulated in the course of productive binding can be regarded as a ground-state *destabilizing* interaction, an optimized form of "electronic strain induction" within the peptide linkage, which is ordinarily promoted by compensatory noncovalent E·S interactions adjacent to the active site. Were that the case, thermodynamic accessibility of an alternate but unproductive pattern of substrate binding might be expected, one that avoids that implicit instability at the actual reaction center. Evolutionary refinement of the enzyme by natural selection can only reduce incidence of such an unstrained but abortive mode until it becomes incipiently competitive with that leading to catalysis, for *targeted* substrates. The less specific assay peptides commonly used with thermolysin would then not show benefit from strain induction, on account of favored nonproductive binding. By way of confirmation, Fua-Phe·Leu-Gly-OH is an example of a high-specificity normal peptide substrate ($k_{cat}/K_m = 2.3 \times 10^6$ s^{-1} M^{-1}); it, too, shows diminished rather than enhanced catalytic activity as a result of enzymic chemical modification (Holmquist et al., 1976).

The pronounced pH dependence of K_m does not mean that functional capability for thermolysin is necessarily wasted on account of a predominant amount of catalytically inactive enzyme, $(Zn^{2+})OH^-$, in less acidic medium. Adequate evidence already exists showing that the first stage of E−S interaction substantially perturbs the pK_a value for the kinetically-manifested active site residue, $(Zn^{2+})OH_2$. For example, a previously reported sigmoidal pH dependence for inhibition of thermolysin is exhibited in the case of some *N*-acetylated Phe-Ala dipeptides that are not hydrolyzed appreciably by the enzyme (Bateman et al., 1990). The characteristic enzymic pK_a of 5 is unequivocally perturbed upward to a value of ≥ 6.5 in the E·I complex (from plotting of pK_i *versus* pH profiles). In these cases it appears that active site occupancy by peptide elevates the pK_a of (Zn^{2+})-OH_2 by 1.5 units, without concurrent displacement of the aquo unit, at least for higher pH values where the water-ligand appears to deprotonate within the E·I complex. Occurrence of such a pK_a perturbation suggests that in neutral solution, contingent formation of a Zn^{2+}-activated peptide linkage within E·S complexes of *vulnerable* substrates should be accompanied by a negligible "population penalty" as regards enzymic protonation. The pK_a of metal-bound water becomes raised in response to the initial act of active site occupancy, facilitating conversion to productive binding on the part of susceptible substrates, which will have combined initially with the enzyme in the nonproductive manner (Kunugi et al., 1995). That shifted pK_a can actually be seen in the vicinity of pH 6.5−7 in the k_{cat} *versus* pH profile for cleavage of normal peptides, in the form of a leveling off in the otherwise strong $[HO^-]$ dependence noted in Figure 3 (Morihara & Tsuzuki, 1970; Fukuda & Kunugi, 1984; Mock & Aksamawati, 1994). Occurrence of this energy-consumptive pK_a perturbation constitutes another manifestation of E·S binding interactions being used to promote reaction (Mock, 1992). The foregoing evidence that the kinetic behavior of arazoformyl dipeptides does reflect productive binding, taken with the substantial pH dependence for k_{cat} and K_m that is exhibited, validates Scheme 1 as the true "reverse-protonation" mechanism for thermolysin.

Reconsideration of Phosphonamidate Transition-State Mimicry. The finding that normal peptide substrates bind nonproductively for the most part to thermolysin has significance for the "transition-state mimicry" by phosphonamidate inhibitors as espoused by Bartlett and others. That

[4] Aaf·Leu-Ala-OH appears to be an anomaly; its k_{cat} is an order of magnitude greater than for its congeners. It might be noted that subtracting the data for Cbz-Gly·Leu-D-Ala-OH from the set of physiological substrates reduces the range of k_{cat} values for normal peptides to a span of 6-fold. The transition-state mimicry hypothesis subsequently reconsidered (Bartlett & Marlowe, 1983) requires a range closer to that of k_{cat}/K_m. Nonproductive substrate binding offers explanation for several other kinetic irregularities with thermolysin: Virtually any mutation of residue 119 yields kinetic activation (Kidokoro et al., 1995), and "synergistic" inhibitor binding effects may be related (Pfuetzner & Chan, 1993).

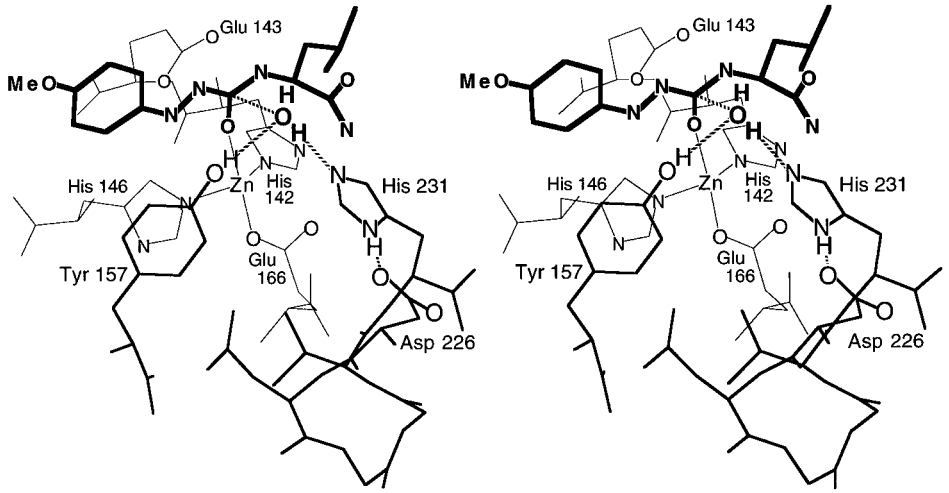


FIGURE 4: Stereodepiction of active site of thermolysin, showing productive binding of Aaf·Leu-NH_2 (bold) in relation to catalytically participating enzymic residues. Model has been derived from crystallographic coordinates. Nucleophilic H_2O (also bold) has been placed on the Bürgi−Dunitz reaction pathway (a vector approaching the Zn^{2+}-activated substrate at an approximate angle of 105° to the C═O bond), and at the appropriate interatomic distance from the C-atom whence geometric perturbation of the carbonyl commences (2.7 Å) according to model systems (Bürgi et al., 1973). This position is at optimal H-bonding distance (also 2.7 Å) relative to N_ϵ of His 231, the catalytic general base. The latter imidazole group is also aligned with Asp 226 (part of protein loop in foreground). Positioning of the reactive H_2O may be aided by Tyr 157. The anionic side chain of residue Glu 143 (backside) provides essential electrostatic stabilization of electron-deficient intermediates but does not otherwise participate in catalysis. The latter carboxylate group is spatially located at the bottom of the active site cleft and becomes sequestered from solvent H_2O by substrate, disallowing its functioning as a base.

argument (Bartlett & Marlowe, 1983) hinges on two linear free energy relationships: (i) When they are plotted against one another, values of $\log(k_{cat}/K_m)$ for Cbz-Gly·Leu-Xaa-OH substrates correlate *unitarily*, linear slope of 1.04 ± 0.18, with pK_i values for a set of corresponding phosphonamidate inhibitors Cbz-Gly^P-Leu-Xaa-OH (Gly^P = aminomethylphosphonyl anion residue; kinetic data given in rows 2 and 6 of Table 1). (ii) A similar comparison of substrate pK_m with inhibitor pK_i values for these species (rows 4 and 6 of Table 1) gives a non-integral correlation, slope of 0.31 ± 0.16, with the latter due to the similarity of K_m values previously commented upon. The claim is that because free energies are being measured ($\log k \approx \Delta G^{\ddagger}$ and $\log K \approx \Delta G°$), the phosphonamidates must consequently bind to thermolysin in a fashion physically resembling the transition state for peptide cleavage and explicitly not in a manner related to the antecedent ground state, thereby allowing structural inferences to be drawn about the metastable catalytic species (tetrahedral adduct) in transit. However, that contention breaks down should the substrates employed in the correlation actually prefer to bind unproductively, as the superactivation phenomenon suggests. Comparable plotting of the new kinetic data clarifies the situation: For cleavage of productively-binding substrates Aaf-Leu-Xaa-OH, a linear and unitary correlation between $\log(k_{cat}/K_m)$ and the same phosphonamidate pK_i values persists, slope of 1.08 ± 0.07 (comparing rows 1 and 6 of Table 1). However, we also observe a virtually identical *unitary* relationship between pK_m and phosphonamidate pK_i, slope of 1.04 ± 0.37 (comparing rows 5 and 6 of Table 1, with the relatively large standard error reflecting an independent hydrophobic effect previously considered, as exemplified in rows 4 and 7). The simpler interpretation is that, because of an intrinsically strong $Zn^{2+}\cdots{}^{-}O(P{=}O)$ interaction, *phosphonamidates merely mimic productive binding of substrates*, which is the mode detected in K_m for arazoformyl dipeptides but not for normal substrates such as used in the previous correlation. Should that be so, then structure−activity comparisons and crystallographic investigations involving phosphonamidates cannot justifiably be said to constitute a probe of transition-state morphology, which the phosphonamidates do not resemble in terms of electronic charge distribution in any event.

Placement of the Nucleophilic Water Molecule. Previously-advanced mechanisms for metalloproteases have featured a "promoted water molecule" as the amide-cleaving species. In a widely circulated mechanistic proposal for thermolysin (Weaver et al., 1977; Tronrud et al., 1986; Matthews, 1988) it has been postulated that an H_2O that is initially coordinated to the enzymic zinc ion becomes deprotonated (by Glu 143) as it migrates to the scissile carboxamide linkage to yield a tetrahedral adduct. Since it is now clear that diametrically opposed (with respect to the active-site cleft) residue His 231 is the catalytic base, one might be tempted to turn this scheme around and to advance the same concerted process from a contrary direction. However, we find no merit in that interpretation. For one thing, the water molecule in question is already acidified to such an extent, pK_a of ∼5, that proton abstraction by His 231 (or by Glu 143) would be unneeded in this scheme. Secondly, the hypothesis of concertedness is untestable, since water is the lyate species, freely exchanging onto the enzyme. We know of no experiment that could tell whether it is the initially metal-bound H_2O or an external solvent H_2O that becomes incorporated into cleavage-product carboxylate. The posed conflation of the two is unconfirmable. Additionally, the claimed transition-state mimicry by phosphonamidate inhibitors, which allegedly models the catalytic process, is unfounded according to the preceding paragraph. The supposition of a synchronous reaction is baseless and so cannot contribute to mechanistic understanding. Moreover, the latter catalytic hypothesis appears counterproductive on its face. The amide-cleaving species (incipient hydroxide) should have its nucleophilicity maximally directed to the substrate carbonyl; any diversion of electron availability in HO^- onto the enzymic metal ion within the transition state *defeats* catalysis (and should also diminish the Lewis acidity

of the zinc in its role of activating substrate). The mechanism as we formulate it in Scheme 1 specifies that a water is displaced from zinc in the productive binding step (activation of scissile carboxamide) and that a water (held only by hydrogen bonds) subsequently enters the substrate with the aid of general base His 231. Opposing pH dependence within K_m and k_{cat} affirms that these must be separate processes. There is no necessity to suppose that it is the same H_2O molecule in each step, and since that issue is untestable, we do not further consider it. A model for a productively bound substrate within the crystallographically-established active site of thermolysin, according to the pathway of Scheme 1 and depicting the location of the nucleophilic H_2O, is presented in Figure 4.

Conclusion. The arazoformyl indicator-residue introduced in this article provides a significant improvement in kinetic assay for metallopeptidases, and perhaps for other types of proteases as well. Notable mechanistic insight is provided by our investigations; the question of *why* thermolysin works is explained in addition to *how*. During catalysis the substrate carbonyl becomes activated toward addition of H_2O by an especially potent Lewis acid. The observed pK_a of 5 for an enzymic metal-bound water in the absence of a substrate correlates with the greatest degree of reaction-promoting cationic character attainable for active site Zn^{2+} in aqueous solution, in the face of unavoidable hydroxide inhibition. Conversion of that electrophilic chemical potential into transition state stabilization during catalysis has been quantitatively appraised previously, in the concept of a "fluxionate Lewis acidity" coefficient α (a Brønsted-type parameter expressing the energetic response of the enzymic zinc ion to changing basicity in an oxyanion being created in coordination to it). The latter aspect of catalysis has received circumstantial evaluation from competitive inhibition data with carboxypeptidase A [Mock et al., 1993; see also Mock and Liu (1995)]. Similarly, the pK_a of 8 for His 231 represents the most practicable general base that may be employed by the enzyme. A weaker H^+-acceptor (such as a carboxylate group) might not suffer from competitive protonation in neutral solution as does the imidazole group, but would also be less potent for the purpose of activating the nucleophilic water molecule. It should be noted that the latter imidazole base in thermolysin is structurally "backed up" by Asp 226 in the manner of the serine proteases, probably reflecting its similar mechanistic role. However, it does not appear that the active site of a serine protease can provide anything like the degree of substrate activation yielded by a *hyper*-Lewis acidic zinc ion (Mock & Chua, 1995). In this regard it can now be contended that zinc peptidase catalysis is actually better understood than is the chemical mechanism for any of the other categories of proteolytic enzymes. Moreover, the explanatory reactivity principles that we have described ought to be extendible to other types of metalloenzymes, providing comparable interpretive insight.

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