Binding to thermolysin of phenolate-containing inhibitors necessitates a revised mechanism of catalysis

William L. MOCK* and Mohammed AKSAMAWATI

Department of Chemistry, University of Illinois at Chicago, Chicago, IL 60607-7061, U.S.A.

Competitive inhibition as a function of pH for the metallo-endoprotease thermolysin by derivatives of L- α -(2-hydroxyphenyl)benzenepropanoyl-L-tryptophanylglycylglycine exhibits a diagnostic bell shape. Binding is maximal between two pK_a values: on the acidic limb the apparent K_i value is regulated by an unchanging enzymic ionization (pK_a 5.3) which is also seen in the substrate-hydrolysis kinetics (k_{cat}/K_m), whereas the alkaline limb for inhibition varies and depends specifically on the pK_a of the phenolic group in the inhibitor. Although it should be the phenolate form of the inhibitor that co-ordinates more efficiently to the active-site Zn^{2+} , the apparent K_i shifts from pH-independent at pH values immediately below the inhibitor's pK_a to progressively weaker binding at higher pH. This is explained by an anomalous acidity for the exchangeable solvent molecule that is attached to enzymic Zn^{2+} in the absence of substrate or inhibitor. Since OH^- cannot be displaced from the enzyme as

readily as H_2O , a compensating pK_a of 5.3 possessed by Zn^{2+} -bound water rationalizes the binding characteristics, yielding the level pH profile exhibited at intermediate pH values. Recognition of the implicit heightened Lewis acidity of the metal ion in thermolysin leads to a revision of the mechanism of catalysis. The substrate amide bond becomes activated for hydrolysis by carbonyl-group co-ordination to the especially acidic Zn^{2+} ion (completely displacing the H_2O/OH^- species otherwise bound). The imidazole group of enzymic residue His-231, also discerned in the pH profile for k_{cat}/K_m from its pK_a of 8, provides general-base assistance for hydration of the activated scissile linkage in the first committed step of catalysis. Additional evidence from inhibition patterns shows how substrate-binding energy may be employed in this scheme to promote hydrolysis of peptides by thermolysin.

INTRODUCTION

A chemical mechanism for cleavage of peptide substrates by metalloproteases, exemplified by carboxypeptidase A and thermolysin, has been elusive. Although there is no shortage of speculative proposals, these have not always been comprehensive as regards the evidence concerning active-site functionality. In particular, the pH-dependence of the specificity constant k_{cat}/K_m is centrally important, as it constitutes the only indication of the protonation state of the enzyme residues involved in binding or activation of substrates. For the enzymes mentioned, there is evidently both a mechanistically essential proton donor and a similarly critical proton acceptor at the active site of the free enzyme, for the pH-dependence of catalysis is bell-shaped with a quenching of enzyme activity on the acidic as well as on the alkaline limbs of the pH profile. Any conjectural mechanism that misassigns one of these ionizations to the available active-site residues of an enzyme cannot be entirely correct.

Previously, a specifically designed probe was created for carboxypeptidase A, in order to identify the more acidic of the essential residues [1–3]. We now apply the same method to thermolysin, an unusually stable zinc endoprotease of bacterial origin for which extensive structural and kinetic information is available. The approach was to construct a competitive inhibitor that interacts in a uniquely interpretable way with a targeted enzyme residue. From the pH-dependence of K_i it is possible to

discern the pK_a of that residue. Because the evidence is thermodynamic in nature, it is intrinsically less ambiguous than is reliance on kinetic data involving substrate conversion. The results suggest that a previously unappreciated catalytic mechanism for thermolysin operates.

MATERIALS AND METHODS

Materials

Thermolysin (EC 3.4.24.4; recrystallized three times) was supplied by Sigma Chemical Co. or by Calbiochem. Enzyme concentrations in buffered stock solution (2.5 M sodium bromide, 0.1 M Hepes and 0.01 M $CaCl_2$) were estimated using $A_{1cm,280}^{1\%}$ 17.65 and a molecular mass of 34600 Da. Buffers employed in this work for kinetic analysis were (0.1 M each) as follows: 2-amino-2-methylpropane-1,3-diol, pH 8.75–10.0; Tris, pH 7.25–8.75; Mes, pH 5.3–7.25; pyridine-2,6-dimethanol, pH 4.2–5.3. All enzyme work was performed with solutions 0.01 M in $CaCl_2$. The assay substrate for routine kinetic work was *N*-[3-(2-furyl)acryloyl]glycyl-L-leucinamide (fua-Gly-Leu-NH₂, $K_m > 1$ mM at pH > 5 [4], $K_m \geq 30$ mM at pH 6.5 [5]) from Sigma, unless substrate-saturation phenomena were under investigation, in which case *N*-[3-(2-furyl)acryloyl]glycyl-L-phenylalanyl-L-alanine (fua-Gly-Phe-Ala-OH; $K_m = 0.15$ mM at pH 6.5) was employed. The latter was prepared by reaction between the Gly-Phe-Ala tripeptide (from Sigma) and 3-(2-

Abbreviations used: fua-Gly-Leu-NH₂, *N*-[3-(2-furyl)acryloyl]glycyl-L-leucinamide; fua-Gly-Phe-Ala-OH, *N*-[3-(2-furyl)acryloyl]glycyl-L-phenylalanyl-L-alanine; I(X), generic representation for phenol-ring-substituted derivatives of α -(2-hydroxyphenyl)benzenepropanoic acid: I(WGG), same for I(X) coupled with tripeptide as follows: I(WGG)a, L- α -(3-chloro-5-cyano-2-hydroxyphenyl)benzenepropanoyl-L-tryptophanylglycylglycine; I(WGG)b, L- α -(3,5-dichloro-2-hydroxyphenyl)benzenepropanoyl-L-tryptophanylglycylglycine; I(WGG)c, L- α -(5-chloro-2-hydroxyphenyl)benzenepropanoyl-L-tryptophanylglycylglycine; nor-I(WGG), benzenepropanoyl-L-tryptophanylglycylglycine; I(p-L), *N*-phospho-L-leucinamide.

* To whom correspondence should be addressed.

furyl)acrylic anhydride [6] in dimethylformamide in the presence of triethylamine. Descriptions of this material as well as of inhibitors I(WGG) and nor-I(WGG) are provided in the Appendix. The potassium salt of *N*-phospho-L-leucinamide [I(p-L)] was prepared as described by Kam et al. [7].

Methods

The pH-dependence of K_i for inhibitors with thermolysin was determined at $25.0 (\pm 0.1)^\circ\text{C}$ in buffers previously listed, with fua-Gly-Leu-NH₂ as assay substrate at a single initial concentration ($50\ \mu\text{M}$) of less than K_m , using spectrophotometric (322 nm, 2 cm path length) analysis and the method of initial rates. Enzyme concentration was maintained well below inhibitor concentration in K_i determinations (100-fold). Values of K_i were obtained at each pH by a non-linear least-squares fit of data from perturbation of $k_{\text{cat.}}/K_m$ to the appropriate inhibition equation

$$(k_{\text{cat.}}/K_m)_{\text{app.}} = (k_{\text{cat.}}/K_m)_{\text{lim.}} / (1 + [I]/K_i)$$

All pH values are calibrated pH-meter readings uncorrected for ionic-strength effects. Tolerances listed are standard errors from least-squares analysis.

RESULTS

Design of active-site probe

It has previously been shown that the phenol-containing substrate analogues I(X) are potent competitive inhibitors of carboxypeptidase A (measured K_i values in the micromolar range) [1,3]. The carboxylate and adjacent benzyl group are recognized by that enzyme (they are the chief specificity-conferring features in rapidly hydrolysed peptide substrates), and the phenolic oxygen in its anionic form co-ordinates with the active-site metal ion, as indicated by spectroscopic [1–3] and crystallographic investigations (G. Shoham and H. M. Greenblatt, personal communication). Thermolysin is similar to carboxypeptidase A in specificity and active-site configuration [8], but it does not readily bind this analogue of a C-terminal peptide fragment because the enzyme is an endoprotease. In order to mimic a more typical substrate for thermolysin, additional amino acid residues were introduced via the carboxy group of I(X). First, the active-site probe was coupled to the amino group of L-tryptophan (because such a residue in that position seems to contribute to the high affinity of the potent inhibitor phosphoramidon, and similarly to the complex of thermolysin with simple peptides such as Val-Trp [9]). As reactant I(X) was racemic whereas the tryptophan was chiral, diastereoisomers resulted which could be separated by crystallization into D,L and L,L forms (the first D/L descriptor referring to the stereogenic centre next to the phenol ring). In order to gain necessary additional enzyme affinity, the chain was further extended in both instances by two glycine residues, producing I(WGG), the class of thermolysin inhibitor subsequently characterized. One of the resulting diastereoisomers was consistently found to bind to the enzyme more firmly than the other; this allowed its configuration to be assigned as the L,L-isomer [with the N-terminus residue I(X) considered as an analogue of phenylalanine, a preferred amino acid in the S₁' position of substrates for thermolysin]. Inhibition data reported below are for the latter material (although it should be noted that the validity of our conclusions does not necessarily depend on the correctness of the stereoassignment). Truncated analogue nor-I(WGG), a counterpart to I(WGG) but lacking a phenol ring, was also prepared as a control (Figure 1).

Inhibition of thermolysin

Kinetic examination of I(WGG)b using various concentrations of the efficiently hydrolysed substrate fua-Gly-Phe-Ala-OH [5,10,10a] at pH 6.5 revealed strictly competitive inhibition (retardation of $k_{\text{cat.}}/K_m$ without perturbation of $k_{\text{cat.}}$ itself). Major importance in this work is attached to the pH-dependence of K_i . For three of the inhibitors examined, a pH profile is shown in log-log form in Figure 2 and considered in detail below. For comparison, the pH-dependence of the relevant second-order catalytic rate constant, $k_{\text{cat.}}/K_m$, is presented for the cleavage of the more convenient assay peptide fua-Gly-Leu-NH₂ under conditions of low substrate concentration (as used for the inhibition data in Figure 2). As previously established [4,11,12], there are enzyme residues with pK_a values of approx. 5.3 and 8.0 that regulate enzyme activity. The peptide-hydrolysis curve is bell-shaped, implying that the catalytically competent form of the enzyme (EH₁) has only one of these moieties protonated (i.e. both EH₂ and EH₀ are unproductive species). The major issue confronted in this paper is the identity and role of the residues responsible for this profile.

Figure 1 Structures of inhibitors

Figure 2 Plot showing pH-dependence of catalytic rate constant $k_{\text{cat.}}/K_m$ (top) and competitive inhibition constants K_i (bottom) for I(WGG) with thermolysin in buffered solution

The presentation is in a log-log form, i.e. $\log k$ and $-\log K_i$ (or pK_i) versus pH. For the kinetic data (upper portion, filled circles, obtained with assay substrate fua-Gly-Leu-NH₂) the curve is a least-squares fit to the expression $(k_{\text{cat.}}/K_m)_{\text{app.}} = (k_{\text{cat.}}/K_m)_{\text{lim.}} / \{ (1 + [H]/K_{a1}) (1 + K_{a2}/[H]) \}$, with $pK_{a1} = 5.3$ and $pK_{a2} = 8.0$, as denoted by breaks in the asymptotic dashed line (Dixon plot: slopes of +1, 0 and -1, formally indicating kinetic order in hydroxide for acidic, neutral and alkaline solution) and with $(k_{\text{cat.}}/K_m)_{\text{lim.}}$ as given by Kunugi et al. [4]. For the inhibition data (lower portion, same substrate with $[S] < K_m$) the symbols are: $\circ$, I(WGG)a; $\triangle$, I(WGG)b; $\square$, I(WGG)c. The curves for I(WGG)a and I(WGG)b are least-squares fits to a similar expression given in the text, with derived parameters listed there as well [asymptotic lines comparable with the upper plot are given for I(WGG)a, and on the alkaline limb only for I(WGG)b].

Existing data have already allowed assignment for the alkaline-limb ionization. The pK_a value of 8.0 must be attributed to 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 [13]. Moreover, the rate of chemical modification itself exhibits an expected sigmoidal pH-dependence, apparently characterized by the same pK_a value of ~ 8.0 [4]. His-231 is the only suitable active-site functionality; the imidazole side chain resides approx. 0.5 nm from the Zn^{2+} 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 Asp-226, according to crystallographic evidence [8,9,14].

We now turn to the inhibition data in Figure 2; binding of I(WGG)a and I(WGG)b to thermolysin exhibits a bell-shaped pH-dependence similar to that for catalysis ($k_{cat.}/K_m$). The acidic limb shows a decrease in pK_i (corresponding to diminished enzyme affinity) which is congruent with that seen in the kinetics. It is apparent that the same enzyme residue (pK_a value of 5.3) affects catalysis and inhibitor binding equivalently. On the alkaline limb, however, enzyme affinity for I(WGG) depends on the acidity of the phenol moiety incorporated in the inhibitor rather than on His-231. The downturn in the pK_i versus pH profiles correlates with pK_a values for the inhibitors. The latter have been independently measured in the same buffered solutions as used for kinetics, by determination of the phenol/phenolate u.v. absorbance as a function of pH. On fitting absorption intensity to the appropriate sigmoidal expression, the spectrophotometric measurements provide the following pK_a values: I(WGG)a, 6.1 (± 0.1); I(WGG)b, 7.7 (± 0.1); I(WGG)c, 9.5 (± 0.1). In fitting the kinetically obtained inhibition data of Figure 2 to a theoretical pH-dependence, the appropriate expression employed for I(WGG)a and I(WGG)b is:

$$(K_i)_{app.} = (K_i)_{lim.} (1 + [H]/K_{aE}) (1 + K_{aI}/[H])$$

where pK_{aE} in each case was assigned the value 5.3 (from the $k_{cat.}/K_m$ versus pH profile), and for which the parameters $(K_i)_{lim.}$ (the limiting value for K_i at intermediate pH) and pK_{aI} (the phenolic ionization causing the break on the alkaline limb) were then found by iterative least-squares adjustment. Values for the constants obtained by curve fitting to this expression are: I(WGG)a, $(K_i)_{lim.} = 15 (\pm 2) \mu M$, $pK_{aI} = 6.4 (\pm 0.1)$; I(WGG)b, $(K_i)_{lim.} = 26 (\pm 4) \mu M$, $pK_{aI} = 7.6 (\pm 0.2)$; I(WGG)c, $K_i = 100 (\pm 25) \mu M$. It may be seen that a satisfactory correspondence exists between the pK_{aI} values so obtained and the same constants independently acquired spectrophotometrically for I(WGG)a and I(WGG)b, confirming that the phenol group regulates binding. The least acidic inhibitor I(WGG)c appears not to have the same alkaline dependence for K_i . This is as it should be, because the pK_a value of this phenol lies outside the pH range accessible enzymically. This does leave open the question of whether I(WGG)c interacts with the enzyme (and its metal ion) in the same way as I(WGG)a and I(WGG)b. However, it may be noted that the curves for the other inhibitors cross over that for I(WGG)c, showing that it binds more tightly than the others on the alkaline limb. This suggests that I(WGG)c conforms to the pattern of I(WGG)a and I(WGG)b, although on the acidic limb it appears to behave more like nor-I(WGG).

Inhibitor nor-I(WGG), prepared as a control, generally binds more weakly (Figure 3), exhibiting K_i values of ~ 0.3 mM in the pH range 6–9. This confirms that the phenol group present in each of the L,L series of probe I(WGG) does make an additional contribution favouring complexation, which is absent in the case

Figure 3 Plot showing pH-dependence of pK_i for non-phenolic inhibitor nor-I(WGG)

The presentation is as for Figure 2. The equation for the fitted curve is $(K_i)_{app.} = (K_i)_{lim.} (1 + K_{a1}/[H]) (1 + K_{a2}/[H]) / (1 + K_{a3}/[H])^2$, with $(K_i)_{lim.} = 21 (\pm 1) \mu M$ (fitted value, acidic limb), and with inflections (denoted by breaks in the asymptotic dashed line) occurring at $pK_{a1} = 5.3$ and $pK_{a2} = 8.0$ (inserted as non-adjustable parameters, downwards deflections attributed to groups in free enzyme), and at $pK_{a3} = 7.1 (\pm 0.1)$, dual ionization (fitted value, upwards deflections attributed to same groups in E–I complex). The inset contains the same data in non-logarithmic form, illustrating the genuineness of K_i perturbation on the alkaline limb.

Figure 4 Plot showing variation in $k_{cat.}$ with pH

The presentation is as for Figure 2 (log k versus pH). The assay substrate was fua-Gly-Phe-Ala-OH; the curve is a least-squares fit to the expression $(k_{cat.})_{app.} = (k_{cat.})_{lim.} / (1 + [H]/K_a)$, with $(k_{cat.})_{lim.} = 190 (\pm 10) s^{-1}$ and $pK_a = 6.4 (\pm 0.1)$ as denoted by break in the asymptotic dashed line.

of nor-I(WGG). Unlike I(WGG), this truncated analogue binds most tightly at low pH; an interpretation of the rather different pH-dependence exhibited by nor-I(WGG) will be offered in the Discussion section. Members of the D,L series of I(WGG) do not bind any more efficiently than nor-I(WGG), and do not show the same pH-dependence as the L,L representatives of I(WGG), so that no inference of phenolic participation in the E–[D,L-I(WGG)] complexes can be drawn.

As shown by others [10,10a], the $k_{cat.}$ versus pH profile for thermolysin is simpler than for $k_{cat.}/K_m$, exhibiting a fall-off only on the acidic limb, with a pK_a value of 6.4 (Figure 4; assay substrate fua-Gly-Phe-Ala-OH). Although such apparent ionizations are sometimes only a ‘mirage’ caused by kinetic complexity, a plausible origin is suggested by the mechanism considered below.

Explanation for inhibitor pH profiles

As in the case of the binding of I(X) to carboxypeptidase A [1–3], the pattern of pH-dependence for K_i is counterintuitive for the phenol-containing inhibitors in Figure 2. Because enzyme affinity

for I(WGG) falls off abruptly at pH values above the pK_a of the inhibitor, it appears as if the phenol were binding and the phenolate were not. However, direct examination of E-I(X) complexes in the case of carboxypeptidase A has established that phenolate is the species present within the complex, a consequence of its co-ordination with the active-site Zn^{2+} ion [1,3]. The explanation for the pattern of pH-dependence, applied to the present case of thermolysin, is that binding depends absolutely on the protonation state of the enzyme residue with a pK_a of 5.3, giving the inflection seen in the pK_i versus pH profiles as well as on the acidic limb in the substrate kinetics. Were that group obliged to adopt its conjugate acid form in order that the phenolate moiety of the inhibitor could interact most firmly with the enzyme, then the pH profile would be rationalized, as exemplified below. In this instance where the pK_a value of the crucial group on the enzyme is lower than that on the entity bound, the phenomenon is categorized as reverse protonation [15]. It should be noted that ionization of the pK_a 8.0 residue (His-231) appears to perturb K_i only slightly at most [I(WGG)c] (Figure 2).

Although an identity for the kinetically manifested enzymic group with pK_a 5.3 has frequently been assigned to the carboxyl side chain of Glu-143 in the case of thermolysin (corresponding to Glu-270 for carboxypeptidase A), there is no direct experimental evidence to support this contention. On the contrary, a persuasive case can be made that the water molecule that binds to the active-site metal ion in the absence of substrate or inhibitor is responsible for the acidic-limb pK_a . A Zn^{2+} ion that has been rendered especially Lewis-acidic by the active-site environment (as is mechanistically credible) should as a consequence enhance the Brønsted acidity in a solvent water ligand [3]. Were that the case, the pK_i versus pH profiles would become immediately explicable [eqns. (1–4)]. The phenolate of the inhibitor (with dissociation constant designated as pK_{aI}) readily substitutes for H_2O on the active-site metal ion (pK_a 5.3); however, replacing HO^- entails much greater difficulty (and a non-ionized phenol displaces neither). At intermediate pH values, there is a mutual cancellation of the H^+ and HO^- dependences that appear on the wings of the pH profile, resulting in a level curve. Extensive documentation demonstrates this situation to be the case for carboxypeptidase A [1–3], and the conclusion carries over to thermolysin. The pertinent equilibria are summarized in eqns. (1–4), accounting for the overall bell shape of the pH profiles. In the equations, $(K_i)_{lim}$ is the limiting value of the competitive inhibition constant at intermediate pH as seen in Figure 2 (horizontal asymptote). The apparent dependence on pH derives, on the alkaline limb, from pK_{aE} [$(H_2O):Zn(Enz)$, value 5.3] and, on the acidic limb, from pK_{aI} (IArOH, value 6–9.5, depending on substituents), with cross-nullification at intermediate pH [reverse protonation, eqn. (3)].

Any attempted assignment of the pK_a of 5.3 to the carboxy group of Glu-143 is rendered untenable by the experimental observation that the acidic-limb ionization controls binding of I(WGG) absolutely. The pH profiles for K_i contain no detectable compensatory inflection corresponding to the same functional group within the enzyme-inhibitor complex [as is thermodynamically required, and as is described below for nor-I(WGG)]. Either the acidic group has been displaced from the enzyme (as we propose), or at least it has assumed a pK_a value greater than 9 in the complex (which is implausible for a carboxyl group). Furthermore, the preponderance of evidence indicating reverse protonation requires that the conjugate acid form of this enzymic functionality be the nominally productive species in the binding of metal-co-ordinating inhibitors, and this ought to hold true also for substrates. The latter observation would not be reconcilable with the widely assumed (but as yet unproven) general-base role for the Glu-143 side-chain carboxylate in the catalytic cycle, were this group the moiety with pK_a 5.3. We do not exclude the possibility that the ionization of $(H_2O):Zn(Enz)$ is influenced by the presence of Glu-143 (probably possessed of a typical carboxyl pK_a of ~ 4), but it is the second acid dissociation constant of the coupled system comprised of these residues that is detected kinetically [1,3].

Concordance with other metalloprotease inhibitors

In order to confirm this explanation for the pH-dependence of K_i for I(WGG), a comparable determination was carried out for *N*-phospho-L-leucinamide [I(p-L), $H_2O_3PNHCH(CONH_2)CH_2CH(CH_3)_2$]. This is a previously characterized relatively tightly binding inhibitor [7], for which a crystallographic structure unambiguously shows co-ordination of the phosphoramidate residue to the active-site Zn^{2+} ion with thermolysin [14]. The pH-dependence for K_i determined here (Figure 5) is similar to that previously reported for 2-benzyl-3-phosphonopropionic acid with carboxypeptidase A [16], and it should be interpreted in essentially the same way. The most pronounced break in the pK_i versus pH profile occurs at pH 8, corresponding to the second acid dissociation constant of the phosphoramidate moiety of the inhibitor (independently ascertained; Figure 5 inset). Once again, it appears that the superior metal ligand (RPO_3^{2-}) is rejected whereas its conjugate acid (RPO_3H^-) is preferentially bound. The explanation is the same as for I(WGG); the enzymic acid with pK_a 5.3 [$(H_2O):Zn(Enz)$] controls binding in a reverse protonation mode. As it is H_2O and not HO^- that is more readily displaced by the phosphoramidate dianion, the same cancellation of pH-dependences at intermediate pH as seen for I(WGG) is manifested in the pK_i profile for I(p-L). In this case the break at pH 5.3 cannot be clearly seen. This is because the monoanionic phosphoramidate group also binds (but more weakly), and is only moderately acidified in the E-I complex. Curve fitting suggests a pK_a of approx. 5.9 for $(RPO_3H^-)Zn(Enz)$. Such an ionization within E-I should cause an upward inflection in the pK_i versus pH profile, which evidently offsets the break concurrently observed at pH 5.3, as indicated by the asymptotes in Figure 5. In the case of the previously reported analogue 2-benzyl-3-phosphonopropionic acid with carboxypeptidase A, this deprotonation has actually been observed at a corresponding pH by ^{31}P -n.m.r. spectroscopy with the E-I complex [16]. It is noteworthy that the inflection seen on the extreme alkaline limb of the profile for I(p-L) is higher than first order; K_i diminishes with the square of the hydronium ion concentration at pH values above 8. This is attributable to concurrent deprotonation of His-231, which also has a pK_a of 8. In the E-I crystal structure the cationic side chain is seen to hydrogen-bond to the

Figure 5 Plot showing pH-dependence of pK_i for I(p-L)

The presentation is as for Figure 2. Equation for fitted curve: $(K_i)_{app} = (K_i)_{lim} \cdot (1 + K_{a1}/[H]) \cdot (1 + K_{a2}/[H])^2 / (1 + K_{a3}/[H])$, with $(K_i)_{lim} = 0.3 (\pm 0.1) \mu M$ (fitted value, acidic limb), and with inflections (denoted by breaks in the asymptotic dashed line) occurring at $pK_{a1} = 5.3$ and $pK_{a2} = 8.0$ (values inserted as non-adjustable parameters, the latter a dual ionization; all downwards deflections attributed to functional groups in free enzyme or to phosphoramidate group of inhibitor), and at $pK_{a3} = 5.9 (\pm 0.1)$ (fitted value, upwards deflection attributed to phosphoramidate group in E-I complex). Inset contains ^{31}P -n.m.r. chemical shift of I(p-L) as a function of pH in buffered water (5% 2H_2O), yielding a pK_a value of $7.9 (\pm 0.1)$ for the second acid ionization of the phosphoramidate group, as incorporated in the inhibition curve fit.

phosphoramidate dianion of the inhibitor via one of the pair of oxygens ligated to the metal ion, and this linkage apparently contributes significantly to the stability of the complex, unlike the cases of I(WGG) and nor-I(WGG). It is pertinent that inhibitor binding for this 'transition-state analogue' shows no trace of a signature, in the form of a levelling off in the value of pK_i for I(p-L) at high pH, arising from a perturbed ionization within the E-I complex of the pK_a 5.3 residue (because it has been displaced).

A cognate pattern of K_i dependence on pH has recently been reported for a related thermolysin inhibitor that contains a hydroxamic acid group as metal-ligating moiety [17]. The reverse-protonation interpretation described here was mentioned but was not adopted in that case, and a thermodynamically misconceived variation was advanced that does not adequately account for the acknowledged fact that hydroxamate anion [of basicity comparable with ArO^- in I(WGG)] is the bound species present within E-I, in the manner summarized by eqns. (1–4). Similar pH-dependence has been noted for binding to thermolysin of a chloromethyl ketone-hydroxamate affinity label [18]. Evidence connecting the acidic-limb pK_a in the substrate catalytic kinetics to $(H_2O:)Zn(Enz)$ is especially complete for carboxypeptidase A: in addition to previously cited data for phenolic inhibitors of structure I(X) and for 2-benzyl-3-phosphonopropionic acid [3,16], this interpretation provides the only convincing explanation for the pH-dependence in binding of benzylosuccinic acid [19], and for comparable sulfoximine- and sulphonylimine-containing inhibitors [20,21]. Members of the extensively employed family of metalloprotease regulators presenting a thiol group to the active-site Zn^{2+} probably also conform to this pattern [22,23].

DISCUSSION

Attribution of the acidic-limb pK_a value in the k_{cat}/K_m profile for thermolysin to the water molecule that co-ordinates to the active-site metal ion in the absence of substrate, rather than to the carboxyl side chain of Glu-143, calls for a re-evaluation of the enzyme mechanism. It is our contention that the 'metal-promoted water' reaction pathway advanced by crystallographers (and currently widely accepted) is not adequately substantiated by experimental evidence, and does not satisfactorily rationalize the extraordinary kinetic acceleration that is the hallmark of an enzymic process. We propose to evaluate critically the foundations for the latter mechanism, and then to offer an alternative that more appropriately explains the enzyme's efficacy.

The mechanism suggested by crystallography

The scheme advanced by Matthews [24] (and endorsed for carboxypeptidase by Christianson et al. [25]) features a nucleophilic water molecule initially co-ordinated to the active-site metal ion. It directly adds to the substrate carboxamide linkage, at the same time as the carbonyl oxygen enters the ligand sphere of the Zn^{2+} ion (Scheme 1). The hypothesized role of the carboxyl side chain of Glu-143 is to function first as a general base, deprotonating the metal-co-ordinated water nucleophile, and subsequently as an acid, protonating the scissile amide nitrogen during breakdown of the intervening tetrahedral adduct. In contradiction to our evidence, this interpretation assumes that Glu-143 contributes the pK_a of 5.3, and that the pK_a of $(H_2O:)Zn(Enz)$ must be greater than 9, since the latter does not appear in the kinetics. The role of the kinetically implicated imidazole (His-231) is somehow to provide essential stabilization of the tetrahedral adduct by continuously hydrogen-bonding (as an imidazolium ion, pK_a 8.0) to the oxygen atom that ends up co-ordinating to the metal ion. It is not explained why this function is so critical. Also proposed were additional hydrogen-bonding interactions of uncertain relevance, between other enzyme residues and reaction intermediates [24], but for conciseness these are not incorporated in the present version.

Before questioning the evidence adduced to support this mechanism, it is perhaps worth examining its chemical plausibility. The proposal is a concerted process, in which the Zn^{2+} ion activates both the substrate and the nucleophile water simultaneously. Although there is excellent documentation for a metal ion having the ability to execute each of these roles individually, no precedent exists in model systems for such a combined function involving a single Zn^{2+} ion [26–28], and particularly one that appears possessed of unexceptional electron deficiency, according to the ascribed properties [i.e. the imputed pK_a of ≥ 9 for $(H_2O:)Zn(Enz)$, which is similar to that for aquated Zn^{2+} in solution]. Both hypothetical capacities exploit the Lewis acidity of the metallic species, in one case straightforwardly to polarize the carbonyl group by co-ordination to its oxygen, and in the other to ease deprotonation of the water molecule by accepting electron density from the incipient hydroxy group (without comparably impairing its nucleophilicity, in model systems). However, it is not obvious that a single metal ion can concurrently execute both roles at the same time in anything other than a fungible manner. Logically, devotion of Lewis acidity to substrate activation diminishes that available for promoting the water ligand, and vice versa. There would appear to be no net benefit to be gained by centralizing both functions on one metal ion (which assertedly has only weak acidity). On the contrary, a long-accepted principle of enzyme catalysis is that the entropic barrier to polyfunctional concerted reactions can be

Scheme 1 Putative mechanism for thermolysin action advanced by Matthews [24]

Oxygen of the scissile carboxamide in the substrate enters the co-ordination sphere of the hydrated Zn^{2+} ion, as the pre-existing liganded water molecule concurrently migrates to the carbonyl carbon, assisted by the general-base Glu-143-CO_2^- . The conjugate acid $\text{Glu-143-CO}_2\text{H}$ thus generated subsequently aids the breakdown of the tetrahedral intermediate. His-231 continuously hydrogen-bonds to the oxygen atom initially present in the substrate carbonyl [24].

avoided by the gathering of a number of reactive groups in proper orientation at an active site, with each performing a separate task. It should first be ascertained that a better-supported mechanism employing separate groups for activation of substrate and nucleophile is unavailable, before embracing a completely novel hypothesis that explains nothing in terms of reactivity.

Putting aside the issue of chemical plausibility, one may ask what is the evidence that has led to the formulation of this particular mechanism? In part, it appears to be the tacit assumption that enzyme residue Glu-143 must be responsible for the $\text{p}K_a$ of 5.3, as manifested in the kinetics ($k_{\text{cat.}}/K_m$, acidic limb). There is in fact no direct experimental evidence that can be cited connecting this $\text{p}K_a$ with a carboxy group. Arguments purporting to associate the corresponding moiety in the case of carboxypeptidase A (Glu-270) with that enzyme's pH profile have been examined and found to be deficient [1–3]. There is no need to controvert the essentiality of Glu-143 for a functional enzyme [29]. An admission of indispensability does not connect it to the $\text{p}K_a$ 5.3 residue, and would not demonstrate a general-base capacity even if such a $\text{p}K_a$ assignment could be made. Furthermore, should the liganded aquo species on the Zn^{2+} ion actually exist in deprotonated form (as hydroxide) in the pH region of maximum enzyme activity as our evidence suggests, then the proposed proton-removing task for the carboxyl side chain of Glu-143 would appear to be quite unnecessary within the mechanism of Scheme 1. A role for this carboxylate group in a revised catalytic scheme will be considered below.

The primary basis for the previously proposed mechanism appears to be crystal structures of enzyme complexes with phosphonyl-containing 'transition-state analogue' inhibitors, of which I(p-L) may be regarded as a prototype. Although it cannot be disputed that peptide surrogates presenting a tetrahedral R_2PO_2^- anion to the active site have noteworthy affinity for metalloenzymes, this provides a doubtful premise for formulation of a detailed mechanism. The probable anionic tetrahedral reaction intermediate engaging with the metal ion $[\text{HO-C(R,NH)-O}^-]$ has negative charge localized on one oxygen

atom, in contrast with the phosphonyl moiety where it is equally shared by two $[\text{O}^{\delta-}\cdots\text{P(R)}_2\cdots\text{O}^{\delta-}]$. It should not be surprising that the latter functionality shows varying degrees of bidentate co-ordination with the active-site Zn^{2+} in enzyme-inhibitor complexes: in some E-I structures both oxygens of a phosphonyl group engage the metal ion, in others monodentate co-ordination involving one or other of the oxygens prevails [30]. Substrate-analogue 'carbonyl hydrate' $[\text{HO-C(R)}_2\text{-OH}]$ complexes have also been examined, for carboxypeptidase A, with crystallographic observation of similarly variable metal-ion co-ordination involving this moiety [25,31]. In the latter cases it seems likely from the pH-dependence of K_i [32] that the 'gem-diol' has its liganded oxygen deprotonated in the complex, i.e. the structures are simply hemiacetals formally derived from the addition of H-OZn(Enz) across the aldehyde or ketone group of the inhibitor. This would appear reasonable considering the extra acidification of zinc-co-ordinated water in the absence of substrate (and in view of the Zn-O distances reported), and as such is scarcely more remarkable than the existence of pyranose and furanose rings in carbohydrates. In point of fact, there is crystallographically no consistent stereochemical pattern of such metal ligation; for the phosphonyl as well as for the hemiacetal 'hydrate' complexes, the pro-*R* oxygen, the pro-*S* oxygen or some combination of the two apparently has a comparable chance of being found co-ordinated to Zn^{2+} . In an attempted accommodation [25,33,34], it has been suggested that such inconsistent behaviour is indicative of a facile reaction pathway, that the active-site metal ion may smoothly translocate from one oxygen to the other as in the mechanistic hypothesis being promulgated (Scheme 1). However, this would seem to be a positive interpretation of negative evidence; a healthy scientific skepticism suggests that no conclusion follows when the structures are as varied as reported.

In addition, the argument from kinetics creating a contention of transition-state analogy is itself questionable. Simple phospho inhibitors such as I(p-L) do not bind proportionately more tightly than comparable analogues presenting a non-tetrahedral carboxylate or thiolate to the active-site metal ion [35,36].

Moreover, the relationship advanced as the criterion for transition-state mimicry, namely, the existence within a series of paired substrates and phosphonyl inhibitors of a superior correlation between K_i and K_m/k_{cat} , compared with that between K_m and K_m/k_{cat} [37], has not been uniformly observed for metalloproteases [38], and has some important contingencies in any event [39]. As always, consistency with a hypothesis does not constitute proof of that hypothesis; although the absence of a K_i versus K_m/k_{cat} correlation might be taken to disprove transition-state homology, the obverse conclusion does not automatically follow when such a relationship is noted. At most, the latter correlation by itself indicates only that inhibitors are acting in a mode that is related to productive substrate binding. The extension to a conclusion of transition-state mimicry depends on concurrent observation of an inferior correlation between K_m and K_m/k_{cat} for substrates corresponding structurally to the inhibitors [37]. But it must be recognized that the substrate parameter K_m can manifest non-productive as well as productive binding; as a consequence there needs to be some assurance that the reference substrates employed in the correlation are also not binding to the enzyme in ways that fail to lead to catalysis. Examination of the data set employed [37] reveals that all substrate variation was confined to the S_2' position occurring two residues removed from the reaction centre, and was comprised of large and small side-chain residues (as well as a D-amino acid) placed in this normally hydrophobic enzyme locus [10,11,40,41]. It is not obvious how a poor fit to the enzyme at such a remote location could systematically perturb a transition-state structure within subsite S_1 . On the other hand, there might exist within E-S an unfavourable pre-equilibrium between productive (scissile carboxamide ligated to Zn^{2+}) and non-productive (substrate not yet within the co-ordination sphere of Zn^{2+} , for example) complexes. In that eventuality the incidence of a minor and variable percentage of productive binding might easily be influenced by a misfit within S_2' . This accords with the pattern of test substrates all appearing to have similar affinity (K_m 2–20 mM compared with K_i values spanning the micromolar to nanomolar range for analogous inhibitors). Predominant non-productive binding constitutes a chemically plausible rationalization for the nearly coequal K_m values observed with thermolysin: once the peptide linkage does become productively activated by Zn^{2+} ligation, to a different extent in each case, subsequent attainment of the transition states for hydrolysis (of identical configuration and $\Delta G^\ddagger$ with respect to subsite S_1) can then logically be indifferent to the binding contribution with regard to remote subsite S_2' . Should such mechanistically non-productive binding be the reason for a poor K_m versus K_m/k_{cat} correlation, then the superior K_i versus K_m/k_{cat} correlation comprises no probe of transition-state mimicry (the high chemical affinity between Zn^{2+} and $R_2PO_2^-$ being sufficient to ensure that all of the inhibitors employed adhere to the enzyme in a fashion related to productive substrate binding regardless of the S_2' residue). Although such correlations as have been provided in this approach may help to exclude a covalent acyl-enzyme intermediate in the course of peptide substrate hydrolysis, it seems questionable whether details of the catalytic process can be specified using such methods.

The kinetically indicated mechanism

We now advance a conceptually simpler and more adequately precedented chemical mechanism, possessing consistency with the pH-dependence of catalysis, and also rendering comprehensible the enzymic acceleration of peptide hydrolysis (Scheme 2). In essence, our proposal is that the metal ion functions solely

as a carbonyl-activating Lewis acid (albeit an especially potent one), and that His-231 takes care of the necessary proton transfers, initially as an imidazole general base deprotonating an incoming water molecule (which expressly is not also co-ordinated to Zn^{2+}) in the first committed steps of catalysis, and perhaps subsequently as an imidazolium ion acid to facilitate breakdown of the tetrahedral adduct. In actuality, the proton exchanges are handled by the side-chain diad consisting of His-231 backed up by Asp-226; we explicitly suggest an analogy to the serine proteases, where the same proton transfer, from attacking nucleophile to nitrogen atom in P_1' , is accomplished by such a combination of functionality.

The mechanism in Scheme 2 fits the pH-dependence for k_{cat}/K_m , and makes chemical sense of the enzyme activity in the process, as we shall now attempt to show. Although the bell-shaped pH profile for this specificity constant at first appearance suggests that the residue with the more acidic pK_a value is present as a base and that the imidazole functions as an acid, the reverse-protonated arrangement (pK_a 5.3 as acid and pK_a 8.0 as base) is in fact kinetically indistinguishable. The only information provided by kinetics is that an EH_1 species rather than EH_2 or EH_0 is the competent form of enzyme, but kinetics does not indicate at which site the single proton must reside for the system to work. We specify that activation of the substrate is provided by the Zn^{2+} ion, functioning as a Lewis acid, in the step designated as K_s . Biological evolution would be expected to have yielded an especially potent moiety for this role (by the established chemical means of diminishing the number of other donor ligands attached to the metal ion [42], for the most part). The key realization is that a strong Lewis acid (as required for effective catalysis) would necessarily induce abnormally high Brønsted acidity in the water molecule that occupies the ligation site on the enzyme in the absence of substrate. This is the explanation for the observed pK_a of 5.3, which must be attributed to $(H_2O):Zn(Enz)$. This hyperacidity represents the best evolutionary compromise between efficaciousness of Lewis acid as catalyst, and unavoidable inhibition by hydroxide competing with substrate for the enzymic Zn^{2+} ion in aqueous solution [3]. At pH values above 5, a proton must be taken up by the enzyme in order for substrate to be productively bound and activated (K_s). This is not seen in the kinetics, however, because the subsequent step has an inverse pH-dependence (k_2 , involving a general base). If the apparently essential imidazolium ion (pK_a 8.0) in His-231 functioned exclusively as its conjugate base in the first committed step of the catalytic cycle as depicted, a compensation would ensue within k_{cat}/K_m (numerically equal to k_2/K_s) [26], yielding the observed pH profile. The high value of the histidine pK_a (8.0; exceeding a more normal value of ~ 6.5) is rational; it may reflect the most potent base securable under the circumstances, just as the acidity of $(H_2O):Zn$ indexes the most favourable Lewis acidity attainable (but see a subsequent refinement of this generalization). Incidentally, because the enzymic Zn^{2+} ion should arrange to bring maximum electron deficiency to bear on the substrate, it should eschew as being counterproductive any interaction between the nucleophilic water molecule and this Lewis acid centre. Such interaction could only diminish the substrate activation while also reducing electron availability on the nucleophile, thereby retarding addition. Required 'activation' of the incoming water molecule is provided by the histidine residue in the form of general-base catalysis; attempted conceptual mixing of these catalytic functions on the metal ion as in Scheme 1 explains nothing mechanistically. There is no justification for supposing that the specific water molecule initially bound to the metal is that which ultimately cleaves the peptide (and no way experimentally to test such a hypothesis in any event).

Scheme 2 Proposed mechanism for thermolysin action

Substrate binding and activation (step K_s) involves complete displacement of water bound to the active-site Zn^{2+} ion (pK_a 5.3 in free enzyme). His-231 (pK_a 8.0 in free enzyme) induces peptide bond hydration of substrate thus activated in the first committed step of catalysis (k_2). Inhibition data and the mechanistic principle suggest that these pK_a values should be convergently perturbed in an initial E-S complex (see the Discussion section).

Whereas the anomalously low pK_a value of 5.3 for $(\text{H}_2\text{O})\text{Zn}(\text{Enz})$ provides one measure of electron deficiency for the active-site Zn^{2+} ion, more directly informative mechanistically is the energetic response of the enzyme to increasing oxyanion basicity, such as builds on the carboxamide oxygen in the course of nucleophilic addition. This parameter, dubbed the 'fluxionate' Lewis acidity, has been the subject of systematic investigation employing a series of phenolate inhibitors of structure I(X) with carboxypeptidase A [3]. It was found that the enzymic Zn^{2+} ion is indeed especially effective in this regard, i.e. it binds significantly more tightly to more basic oxyanions. Although the present limited evidence does not allow a comparable scaling with thermolysin, trends in the data are consistent and the conclusion ought to carry over.

Although the kinetic chemical advantage of an enzyme employing both an especially potent acid and a correspondingly strong base in concerted catalysis should be intuitively evident, full appreciation of the proposed scheme requires recognition of the role that E-S binding energy can play in promoting such a mechanism. There is a widespread misconception that a 'reverse-protonation' mechanism such as proposed here should be discounted, on the grounds that the free enzyme (before substrate complexation) would be grossly underpopulated as regards the productive species in solution. In the present instance only one of 500 enzyme molecules (at most) will be spontaneously 'set up for catalysis,' because the ratio of the two forms of EH_1 (i.e. that with the single H^+ on the Zn^{2+} ligand, relative to its counterpart with the proton on histidine) is governed solely by the acid dissociation constants of the two functional groups (for thermolysin: $\text{EH}_2 \rightarrow \text{EH}_1$, pK_a 5.3; $\text{EH}_1 \rightarrow \text{EH}_0$, pK_a 8.0). Such an arrangement with only a small fraction of the enzyme molecules 'active' seems prohibitively wasteful at first glance. However, the affinity of the substrate for the minor, more potently protonated, form of the enzyme may be much greater than for the more populous non-productive form (since that would be evolutionarily favoured). This is of cardinal significance; it is the protonation pattern within an initial E-S complex that matters catalytically, and the proton distribution can differ substantially from that for the unoccupied enzyme, in a direction facilitating

the nominally minor pathway. We may cite experimental evidence pertinent to this point.

The substrate-activation step that we designate as K_s in fact involves at least two stages: the formation of an encounter complex in which the specificity features of the substrate are recognized, followed by the displacement of water from the metal ion by the carboxamide group, which is the more pH-consequential process. Adequate evidence already exists showing that the first stage substantially perturbs the pK_a values for the kinetically manifested active-site residues. For example, a previously reported moderate sigmoidal pH-dependence for inhibition of thermolysin is exhibited in the case of some *N*-acetylated Phe-Ala dipeptides that are not hydrolysed appreciably by the enzyme [43]. The characteristic enzymic pK_a of 5.3 is unequivocally perturbed to a value of 6.5 or higher in the complexed enzyme [4,43]. It appears that the initial event of active-site occupancy (before displacement of the lyate species that is otherwise bound to the metal ion, a step that these catalytically inactive peptides are evidently unable to perform) elevates the pK_a of $(\text{H}_2\text{O})\text{Zn}(\text{Enz})$ by greater than one unit. (This type of enzymic ligation corresponds to the non-productive substrate-binding mode previously adumbrated in refutation of the transition-state-mimicry hypothesis for phosphonamide analogues; the peptide K_m and K_i values are comparable.)

Quite similar behaviour is noted in Figure 3 for inhibition of thermolysin by the non-phenolic analogue nor-I(WGG), which carries no substituent for displacing water from the Zn^{2+} ion. Paralleling the example of the inhibitory dipeptides just mentioned, the pK_i diminishes in value with increasing pH on the acidic limb of the profile, but in this case it then appears to level off and even to increase slightly on the alkaline limb. As indicated by the asymptotic lines, the fitted curve shows each of the pK_a values of the free enzyme (5.3 and 8.0) having been perturbed in a convergent fashion, resulting in dual compensatory inflections (right angle bend in the asymptote) at coincident pK_a values of 7.1 within the E-I complex. Actually, the perturbed enzymic pK_a values need not have converged to exactly the same value, but the inhibition data do not allow their analytical separation. Consequently, they are designated as being identical in curve fitting, so

as to restrict the number of adjustable parameters. The pK_a of 7.1 for E-I may be regarded as an averaged value.

If the behaviour of nor-I(WGG) is accepted as a model for what happens in the prefatory stage of productive substrate binding, then subsequent carboxamide activation by the metal ion becomes more easily comprehensible. With an initial E-S complex sustaining the same convergent pK_a perturbation, the imidazolium ion of His-231 may transfer its proton to $(HO^-)Zn$ without thermodynamic penalty (since the two groups now have nearly the same pK_a), allowing displacement from the metal ion of water rather than a difficult-to-dislodge HO^- anion. In this process the conjugate base form of His-231 is generated, as required in the ensuing step (k_2) of the new mechanism. Hence, within the E-S complex, the nominal statistical penalty associated with catalytically employing the 'minor' form of enzyme (as exists before complexation) may be much reduced or eliminated, as a consequence of differential substrate affinity favouring the productive form of the enzyme. Effectively, latent binding energy has been used to promote the reaction [44], by making intramolecular proton transfer [$His:H^+$ to $(HO^-)Zn$] feasible so as to create an adequate percentage of the more suitable acid-base combination, but only subsequent to formation of an initial E-S complex. In this connection, it ought to be remembered that breaks in the curve for $\log k_{cat}/K_m$ always yield exclusively the pK_a values of the uncomplexed enzyme (in the situation of equilibrium binding of substrate); shifted pK_a values, caused by substrate ligation, might show up in the k_{cat} versus pH profile, but can easily be further perturbed kinetically there [26].

Critical to the legitimacy of this mechanistic possibility is the fact that intramolecular proton shifts can be several orders of magnitude more rapid than the diffusion limit for aggregates of heavier atoms, particularly when the transfer can be along a hydrogen bond, as would seem to be realizable according to the crystal structure giving rise to Scheme 1. The larger significance is that the usual kinetic competency argument commonly employed to discount reverse-protonation mechanisms for very rapid enzymes can be evaded. This problem has been surmounted by having the substrate initially bind to the predominant form of uncomplexed enzyme in solution, which then assumes the correctly protonated configuration for thermodynamic reasons before the first committed step of catalysis. There is only one price to be paid in following this course of events: convergent perturbation of the pK_a values of the controlling functionalities (somewhat diminishing the chemical potential inherent in the reverse-protonated state) is an inescapable consequence of exploitation of binding energy to populate the productive enzyme species [45]. But this alone constitutes necessary and sufficient conditions for removing any statistical barrier to the proposed mechanism, since it may be rigorously shown thermodynamically that $(\Delta pK_a)_{ES} = (\Delta pK_a)_E - (\Delta pK_a)_S$, where the ΔpK_a terms refer to the differential in acidity of the two ionizable functionalities in the E-S complex and between the same groups in the free enzyme, and ΔpK_a corresponds to the differential in affinities of substrate towards normally protonated and reverse-protonated forms of enzyme [45]. The preceding considerations provide a crucial mechanistic insight; consumption of latent binding energy to enforce utilization of the more potent acid-base combination in the catalytic step explains why the enzyme works, as well as how.

It might be noted that the pH-dependence of k_{cat} , presented in Figure 4, is consistent with the new scheme. Although mechanistic interpretation of such profiles ought to be approached with caution, we suggest that a fall-off in activity occurring only on the acidic limb can be attributed to His-231 functioning as a general base in step k_2 . This pH profile contains only one

inflection, since the metal-bound water molecule that provides the other ionization seen in k_{cat}/K_m has been displaced by substrate in a previous step. The fitted pK_a value of 6.4 is comparable with the perturbed pK_a for the histidine side chain within the E-I complex involving nor-I(WGG) (although nothing requires the same value for both E-I and E-S). While the pK_a value of 8 seen for His-231 in the free enzyme may have indicated a more potent base, the group must exist unprotonated to act in that capacity, and a histidine of normal basicity [46], as here suggested, represents the most efficacious proton acceptor that can be available in neutral solution [26]. The pK_a value of 8 probably reflects hydrogen-bond bridging of the imidazolium ion to $(HO^-)Zn$, which of course is relinquished when substrate replaces the lyate species on the metal ion. We note that no comparably straightforward rationalization of the k_{cat} profile is offered by the mechanism of Scheme 1, particularly as regards His-231 on the alkaline limb.

Conspicuously absent until now in the newly advanced mechanism is a role for the carboxylate of enzymic residue Glu-143. Our scheme does not require it to have an abnormal pK_a value, nor is it envisaged to function as a general base. Glu-143 is not particularly well equipped for the latter task. For the side chain of this residue, nominally free rotations about no less than three carbon-carbon single bonds would have to be frozen ($>CH-CH_2-CH_2-CO_2^-$), were the carboxylate to be maintained in a fixed position to serve as a proton acceptor, as designated in the proscribed mechanism of Scheme 1. There are no obvious conformational constraints on the side chain such as would be needed to orient it rigidly in a manner that seems to be a prerequisite for effective intramolecular general-base catalysis [47]. In contrast, the more suitably basic imidazole of His-231 is tethered with a shorter chain, and is held in proper orientation by backside hydrogen-bonding to Asp-226, as in the case of the serine proteases. However, were the Glu-143 carboxylate to have only a charge-neutralization role in the catalytic mechanism, electrostatically satisfying electron deficiency alternately in the metal ion or in the substrate carbonyl group at various stages of the catalytic cycle, its conformational flexibility could be of benefit in allowing optimal interaction guided by electronic attraction. This ostensibly passive role could be just as critical to the mechanism as would be the hypothetical acid-base capacity commonly assigned to this residue. As ionic charge by itself is known to be of major significance in active-site integrity, it ought not to be surprising if mutationally induced loss of the carboxylate should disable the enzyme, as is the case [29].

We suggest that the emphasis previously given to this enzyme residue arises partly from a contingency of the structure of carboxypeptidase A having been described first. Whereas most of the known metalloendoproteases such as thermolysin appear to contain the His-231-Asp-226 diad [48-50], the characterized metalloexoproteases exemplified by carboxypeptidase A do not. This has led to an exclusionary focusing on the active-site glutamate residue that is common to both types, and to an interpretational over-reliance upon that moiety. However, we have previously indicated that fundamentally the same mechanism as suggested for thermolysin can apply to the carboxypeptidases. Although these lack the catalytic histidine, the specificity-conferring substrate carboxylate group is appositely situated within enzyme-peptide complexes for carrying out the necessary proton transfers in the case of the zinc carboxypeptidases [21]. According to enzyme crystal structures, this functional group is generally placed over the same face of the scissile linkage of substrate as is His-231 in the case of thermolysin. As a potential proton acceptor during catalysis, the substrate carboxylate may seem less adequate than an imidazole,

but one cannot logically argue that it is inferior to Glu-270 (the carboxypeptidase counterpart to thermolysin's Glu-143) for that purpose. The substrate- CO_2^- within an E-S complex of carboxypeptidase A would also appear to be more rigidly oriented than the enzyme glutamic carboxylate with respect to the reaction centre, because of E-S binding interactions that freeze internal bond rotations within the substrate. We suggest that utilization of substrate- CO_2^- for catalysis as well as for recognition in the carboxypeptidases has an evolutionary root: once adopted for the role of proton transfer within these enzymes, the carboxylate's presence within the substrate never allowed mutational replacement by a histidine. In the final analysis, there is no experimental evidence indicating that the role of thermolysin residue Glu-143 (or 270 in carboxypeptidase A) in the catalytic mechanism of the metalloproteases embodies anything more than the electrostatic contribution that we have outlined.

Conclusion

The new mechanism we have provided (Scheme 2) is but a theory, needing additional confirmation. However, we aver that it constitutes the most parsimonious explanation as to how the enzyme works. It explicitly accounts for the pH-dependence of catalysis (using substantiated pK_a assignments), and it economically invokes active-site functionality of enhanced reactivity, coupled in well-precedented and chemically rational roles. It is fully compatible with the extensive crystallographic evidence for thermolysin. We believe that this most plausible chemical process has been overlooked until now for four reasons: (i) an erroneous attribution of the acidic limb pK_a in the kinetics to Glu-143; (ii) an unsustainable rationale as regards phosphoramidate inhibition; (iii) the historical contingency that the structure of carboxypeptidase A was described first, leading to a relative neglect of His-231; (iv) insufficient recognition of the kinetic indistinguishability of reverse-protonation in a catalytic mechanism, and of the inherent opportunity for exploitation of binding energy for promotion of reaction that it offers. The same fundamental mechanism ought to hold relevance for other metalloendoproteases that contain a counterpart to His-231 at the active site [48–51], although it would be rash to presume that all metalloproteases employ exactly the same chemical steps. But until disproved, the process depicted in Scheme 2 ought at least to be designated as the kinetically indicated mechanism for thermolysin.

This work was supported by the U.S. National Institutes of Health.

REFERENCES

- Mock, W. L. and Tsay, J.-T. (1986) *Biochemistry* **25**, 2920–2927
- Mock, M. L. and Tsay, J.-T. (1988) *J. Biol. Chem.* **263**, 8635–8641
- Mock, W. L., Freeman, D. J. and Aksamawati, M. (1993) *Biochem. J.* **289**, 185–193
- Kunugi, S., Hirohara, H. and Ise, N. (1982) *Eur. J. Biochem.* **124**, 157–163
- Izquierdo, M. C. and Stein, R. L. (1990) *J. Am. Chem. Soc.* **112**, 6054–6062
- Pozdnev, V. F. (1986) *J. Gen. Chem. USSR* **56**, 613–617
- Kam, C.-M., Nishino, N. and Powers, J. C. (1979) *Biochemistry* **18**, 3032–3038
- Kester, W. R. and Matthews, B. W. (1977) *J. Biol. Chem.* **252**, 7704–7710
- Holden, H. M. and Matthews, B. W. (1988) *J. Biol. Chem.* **263**, 3256–3260
- Moriwaka, K. and Tsuzuki, H. (1970) *Eur. J. Biochem.* **15**, 374–380
- 10a Fukuda, M. and Kunugi, S. (1984) *Bull. Chem. Soc. Jpn.* **57**, 2965–2970
- Feder, J. and Schuck, J. M. (1970) *Biochemistry* **9**, 2784–2791
- Pangburn, M. K. and Walsh, K. A. (1975) *Biochemistry* **14**, 4050–4054
- Burstein, Y., Walsh, K. A. and Neurath, H. (1974) *Biochemistry* **13**, 205–210
- Tronrud, D. E., Monzingo, A. F. and Matthews, B. W. (1986) *Eur. J. Biochem.* **157**, 261–268
- Cleland, W. W. (1982) *Methods Enzymol.* **87**, 390–405
- Goli, U. B., Grobelny, D. and Galaray, R. E. (1988) *Biochem. J.* **254**, 847–853
- Izquierdo-Martin, M. and Stein, R. L. (1992) *J. Am. Chem. Soc.* **114**, 325–331
- Rasnick, D. and Powers, J. C. (1978) *Biochemistry* **17**, 4363–4369
- Byers, L. D. and Wolfenden, R. (1973) *Biochemistry* **12**, 2070–2078
- Mock, W. L. and Tsay, J.-T. (1989) *J. Am. Chem. Soc.* **111**, 4467–4472
- Mock, W. L. and Zhang, J. Z. (1991) *J. Biol. Chem.* **266**, 6393–6400
- Blumberg, S. and Tauber, Z. (1983) *Eur. J. Biochem.* **136**, 151–154
- Shapiro, R. and Riordan, J. F. (1984) *Biochemistry* **23**, 5225–5233
- Matthews, B. W. (1988) *Acc. Chem. Res.* **21**, 333–340
- Christianson, D. W., David, P. R. and Lipscomb, W. N. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1512–1515
- Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd edn., chapters 2 and 3, W. H. Freeman, New York
- Fife, T. H. (1991) *Perspect. Bioinorg. Chem.* **1**, 43–93
- Gellman, S. H., Petter, R. and Breslow, R. (1986) *J. Am. Chem. Soc.* **108**, 2388–2394
- Toma, S., Campagnoli, S., Gregoriis, E. De, Gianna, R., Margarit, I., Zamai, M. and Grandi, G. (1989) *Protein Eng.* **2**, 359–364
- Christianson, D. W. and Lipscomb, W. N. (1988) *J. Am. Chem. Soc.* **110**, 5560–5565
- Shoham, G., Christianson, D. W. and Oren, D. A. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 684–688
- Mookhtiar, K. A., Grobelny, D., Galaray, R. E. and Van Wart, H. E. (1988) *Biochemistry* **27**, 4299–4304
- Christianson, D. W. and Lipscomb, W. N. (1986) *J. Am. Chem. Soc.* **108**, 4998–5003
- Christianson, D. W. and Lipscomb, W. N. (1989) *Acc. Chem. Res.* **22**, 62–69
- Nishino, N. and Powers, J. C. (1979) *Biochemistry* **18**, 4340–4347
- Holmquist, B. and Vallee, B. L. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6216–6220
- Bartlett, P. A. and Marlowe, C. K. (1983) *Biochemistry* **22**, 4618–4624
- Grobelny, D., Goli, U. B. and Galaray, R. E. (1985) *Biochem. J.* **232**, 15–19
- Hanson, J. E., Kaplan, A. P. and Bartlett, P. A. (1989) *Biochemistry* **28**, 6294–6305
- Pank, M., Kirret, O., Paberit, N. and Aaviksaar, A. (1982) *FEBS Lett.* **142**, 297–300
- Hangauer, D. G., Monzingo, A. F. and Matthews, B. W. (1984) *Biochemistry* **23**, 5730–5741
- Woolley, P. (1975) *Nature (London)* **258**, 677–682
- Bateman, Jr., R. C., Rodriguez, G., Vijayaraghavan, J. and Hersh, L. B. (1990) *Arch. Biochem. Biophys.* **279**, 355–362
- Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol.* **43**, 219–409
- Mock, W. L. (1992) *Bioorg. Chem.* **20**, 377–381
- Tanford, C. (1962) *Adv. Protein Chem.* **17**, 69–165
- Kirby, A. J. (1980) *Adv. Phys. Org. Chem.* **17**, 183–278
- Stoeva, S. (1991) *Int. J. Peptide Protein Res.* **37**, 325–330
- Stark, W., Paupit, R. A., Wilson, K. S. and Jansonius, J. N. (1992) *Eur. J. Biochem.* **207**, 781–791
- Thayer, M. M., Flaherty, K. M. and McKay, D. B. (1991) *J. Biol. Chem.* **266**, 2864–2871
- Bode, W., Gomis-Ruth, F. X., Huber, R., Zwilling, R. and Stöcker, W. (1992) *Nature (London)* **358**, 164–167

APPENDIX

Preparation of inhibitors I(WGG)

The synthesis of I(WGG)b illustrates the procedure. To a slurry of 0.62 g (3.0 mmol) of L-tryptophan and 600 mg of triethylamine in 2 ml of dimethylformamide was added 0.89 g (3.0 mmol) of 5,7-dichloro-3-(phenylmethyl)-2^[3H]-benzofuranone [obtained by lactonization of the corresponding I(X) with acetic anhydride]

at 85 °C. After 15 min, the resulting solution was diluted with 60 ml of 1 M HCl and extracted ($\times 2$) with ethyl acetate. The extracts were washed ($\times 4$) with water and with saturated NaCl and then combined and dried with anhydrous CaCl_2 . Evaporation gave an oil which was taken up in 30 ml of methanol, to

which was added 0.6 g of dicyclohexylamine. The resulting solution was slowly rotary evaporated to a volume of ~4 ml, during which a precipitate separated. After standing at 0 °C, 0.80 g of a single diastereoisomer salt was collected (m.p. 237–238 °C). A second crop of 0.1 g was obtained on further concentration and dilution with propan-2-ol (yield 88%). This salt (0.85 g) was converted into the acid by dissolution in 25 ml of 1 M NaOH and extraction ($\times 2$) of the liberated dicyclohexylamine with toluene. The resulting aqueous solution was acidified with HCl, resulting in a white precipitate which was collected and washed with water, yielding 0.64 g (97%) of D- α -(3,5-dichloro-2-hydroxyphenyl)benzenepropanoyl-L-tryptophan. It was recrystallized by dissolution in a few millilitres of ethyl acetate, filtering, and dilution with several volumes of hexane (m.p. 105 °C; spectral properties similar to the L,L-isomer following).

The supernatant from the original crystallization gave, on evaporation, a second dicyclohexylamine salt as an oil which was converted into an impure acid in the same way. The acid was taken up in 3 ml of chloroform, and 0.24 g of 4-methylbenzylamine was added. The resulting solution was then diluted to the cloud point with cyclohexane. On standing, 0.72 g (78%) of a fine white precipitate of the 4-methylbenzylamine salt of L- α -(3,5-dichloro-2-hydroxyphenyl)benzenepropanoyl-L-tryptophan was obtained (m.p. 224 °C) [free of diastereoisomer by t.l.c.; the two tryptophan derivatives were separable on analytical silica t.l.c.; ethyl acetate/ethanol (10:1, v/v); second component slower moving]. The acid itself (obtained as a froth by chloroform extraction of a dilute HCl solution of the methylbenzylamine salt followed by evaporation and intensive evacuation) could not be induced to crystallize; 400 MHz ^1H n.m.r. δ (p.p.m.) [(^2H)acetone] 3.16 (1 H, dd, J 8.4, 13.5 Hz), 3.23 (1 H, dd, J 5.1, 14.8 Hz), 3.34 (1 H, dd, J 5.3, 14.8 Hz), 3.38 (1 H, dd, J 7.6, 13.5 Hz), 4.17 (1 H, t, J 8 Hz), 4.81 (1 H, ddd, J 5.3, 6.1, 7.4 Hz), 6.82 (1 H, s), 6.82 (1 H, t, J 8 Hz), 6.97 (1 H, d, J 2.3 Hz), 7.02 (1 H, t, J 8 Hz), 7.07–7.2 (5 H, m), 7.29 (1 H, d, J 2.3 Hz), 7.30 (1 H, d, J 8 Hz), 7.32 (1 H, d, J 8 Hz), 8.05 (1 H, d, J 7.4 Hz), 9.97 (1 H, s).

To a solution of 613 mg (1.23 mmol) of the L,L-acid, 580 mg (2.95 mmol) of ethyl glycylglycinate hydrochloride, 300 mg (2.24 mmol) of 1-hydroxybenzotriazole and 290 mg (2.9 mmol) of triethylamine in 16 ml of methylene chloride was added 740 mg (1.74 mmol) of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodi-imide metho-*p*-toluenesulphonate, and the mixture was allowed to stand overnight at 25 °C. The resulting solution was diluted with chloroform and extracted ($\times 2$) with 1 M HCl and then with saturated NaCl. On concentration the major product separated as a voluminous solvate which was collected in several crops and dried to a powder [single component by t.l.c. (benzene/acetone, 3:1, v/v)]. It was recrystallized from propan-2-ol by dilution with hexane to give 383 mg (48%) of L- α -(3,5-dichloro-2-hydroxyphenyl)benzenepropanoyl-L-tryptophanyl-glycylglycine ethyl ester {m.p. 191–192 °C; i.r. (KBr) 1740 cm^{-1} , 1670, 1635; 400 MHz ^1H n.m.r. δ (p.p.m.) [(^2H)methanol] 1.24 (3 H, t, J 7.1 Hz), 4.12 (2 H, q, J 7.1 Hz), plus other signals characteristic of I(WGG)b (below)}.

The ester (350 mg) was taken up in ~1 ml of methanol, diluted with 3 ml of 1 M NaOH and the resulting solution was rotary evaporated at 25 °C for 15 min in order to remove the methanol. On acidification, a precipitate formed and was collected. It was dissolved in propan-2-ol and treated with charcoal, and the residue obtained from the filtrate was redissolved in 1 M NaOH with subsequent reprecipitation with acid to give 302 mg (91%) of L- α -(3,5-dichloro-2-hydroxyphenyl)benzenepropanoyl-L-tryptophanyl-glycylglycine [I(WGG)b] as a fine tan powder {m.p.

(sinter) 115–120 °C; i.r. (KBr) 1720 cm^{-1} , 1650; 400 MHz ^1H n.m.r. δ (p.p.m.) [(^2H)methanol] 2.98 (1 H, dd, J 8, 13.6 Hz), 3.06 (1 H, dd, J 7, 14.5 Hz), 3.23 (1 H, dd, J 7, 14.5 Hz), 3.24 (1 H, dd, J 8, 13.6 Hz), 3.71 (1 H, d, J 16.9 Hz), 3.82 (1 H, d, J 16.9 Hz), 3.84 (2 H, s), 4.10 (1 H, t, J 8 Hz), 4.61 (1 H, t, J 7 Hz), 6.90 (1 H, s), 6.91 (1 H, t, J ~8 Hz), 6.92 (1 H, d, J 2.3 Hz), 7.02 (1 H, t, J ~8 Hz), 7.05–7.17 (5 H, m), 7.19 (1 H, d, J 2.3 Hz), 7.26 (1 H, d, J ~8 Hz), 7.41 (1 H, d, J ~8 Hz); 100 MHz ^{13}C n.m.r. δ (p.p.m.) [(^2H)methanol] 28.3, 38.2, 41.8, 43.4, 51.1, 56.1, 110.3, 112.3, 119.1, 119.8, 122.4, 123.4, 124.5, 125.4, 127.4, 128.5, 129.1, 129.3, 129.9, 130.0, 137.8, 140.1, 151.2, 171.7, 172.9, 174.0, 175.7; mass spectrum (f.a.b., MH^+) m/e 611.1492 $\pm$ 0.0011 (5); $\text{C}_{30}\text{H}_{29}\text{N}_4\text{O}_6\text{Cl}_2$ requires 611.1464 (Found: C, 57.6; H, 4.7; N, 8.8; $\text{C}_{30}\text{H}_{28}\text{N}_4\text{O}_6\text{Cl}_2\cdot\text{H}_2\text{O}$ requires C, 57.2; H, 4.8; N, 8.9%).

I(WGG)a and I(WGG)c (plus the enzymically less active D,L-diastereoisomers, not described) were similarly prepared. L- α -(3-chloro-5-cyano-2-hydroxyphenyl)benzenepropanoyl-L-tryptophanyl-glycylglycine [I(WGG)a]: m.p. 159–160 °C; i.r. (KBr) 2210 cm^{-1} , 1730, 1660–1640; 400 MHz ^1H n.m.r. δ (p.p.m.) [(^2H)methanol] 2.97 (1 H, dd, J 8.0, 13.5 Hz), 3.03 (1 H, dd, J 8.2, 14.6 Hz), 3.23 (1 H, dd, J 8.0, 13.5 Hz), 3.25 (1 H, dd, J 6.2, 14.6 Hz), 3.74 (1 H, d, J 16.9 Hz), 3.85 (1 H, d, J 16.9 Hz), 3.87 (2 H, s), 4.09 (1 H, t, J 8.0 Hz), 4.66 (1 H, dd, J 6.2, 8.2 Hz), 6.88 (1 H, t, J ~8 Hz), 6.91 (1 H, s), 7.00 (1 H, t, J ~8 Hz), 7.03–7.2 (6 H, m), 7.23 (1 H, d, J ~8 Hz), 7.41 (1 H, d, J ~8 Hz), 7.53 (1 H, d, J 1.9 Hz); 100 MHz ^{13}C n.m.r. δ (p.p.m.) [(^2H)methanol] 28.3, 38.2, 41.7, 43.4, 51.3, 56.1, 110.4, 112.3, 119.0, 119.8, 122.4, 123.5, 124.5, 127.6, 128.6, 129.4, 130.0, 133.4, 133.6, 137.8, 139.8, 156.9, 171.7, 172.8, 173.9, 175.4; mass spectrum (f.a.b., MH^+) m/e 602.1820 $\pm$ 0.0007 (5); $\text{C}_{31}\text{H}_{29}\text{N}_5\text{O}_6\text{Cl}$ requires 602.1806 (Found: C, 60.2; H, 4.8; N, 11.3; $\text{C}_{31}\text{H}_{28}\text{N}_5\text{O}_6\text{Cl}\cdot\text{H}_2\text{O}$ requires C, 60.1; H, 4.9; N, 11.3%). L- α -(5-chloro-2-hydroxyphenyl)benzenepropanoyl-L-tryptophanyl-glycylglycine [I(WGG)c]: m.p. (sinter) ~125 °C; i.r. (KBr) 1720 cm^{-1} , 1650; 400 MHz ^1H n.m.r. δ (p.p.m.) [(^2H)methanol] 2.94 (1 H, dd, J 7.3, 13.7 Hz), 3.03 (1 H, dd, J 7.3, 14.6 Hz), 3.15 (1 H, dd, J 6.3, 14.6 Hz), 3.22 (1 H, dd, J 8.3, 13.7 Hz), 3.65 (1 H, d, J 16.9 Hz), 3.75 (1 H, d, J 16.9 Hz), 3.78 (2 H, s), 4.11 (1 H, dd, J 7.3, 8.3 Hz), 4.53 (1 H, dd, J 6.3, 7.3 Hz), 6.69 (1 H, d, J 8.6 Hz), 6.81 (1 H, s), 6.89 (1 H, t, J 7.4 Hz), 6.95–7.12 (8 H, m), 7.24 (1 H, d, J 8 Hz), 7.39 (1 H, d, J 8 Hz); 100 MHz ^{13}C n.m.r. δ (p.p.m.) [(^2H)methanol] 28.2, 37.9, 41.7, 43.5, 49.4, 55.9, 110.3, 112.3, 118.0, 119.1, 122.4, 124.5, 125.3, 127.2, 128.3, 128.5, 129.0, 129.2, 129.8, 129.9, 137.8, 140.5, 154.9, 171.7, 172.8, 174.2, 175.9; mass spectrum (f.a.b., MH^+) m/e 577.1846 $\pm$ 0.0013 (5); $\text{C}_{30}\text{H}_{30}\text{N}_4\text{O}_6\text{Cl}$ requires 577.1854 (Found: C, 61.1; H, 5.3; N, 9.7; $\text{C}_{30}\text{H}_{29}\text{N}_4\text{O}_6\text{Cl}\cdot 0.67\text{H}_2\text{O}$ requires C, 61.2; H, 5.2; N, 9.5%).

Preparation of nor-I(WGG)

A suspension of 500 mg (1.57 mmol) of L-tryptophanyl-glycylglycine (Sigma) and 180 mg (1.75 mmol) of triethylamine in 10 ml of dimethylformamide was warmed briefly with stirring to form the insoluble amine salt, and then allowed to cool to 25 °C. To a solution of 260 mg (1.73 mmol) of 3-phenylpropanoic acid and 180 mg (1.75 mmol) of triethylamine in 5 ml of methylene chloride at 0 °C was added 236 mg (1.73 mmol) of isobutyl chloroformate, and the mixture was allowed to stand for a few minutes until triethylamine hydrochloride had separated. The methylene chloride solution was slowly added to the dimethylformamide suspension at 25 °C by dropper with vigorous mixing. All solids gradually went into solution. The methylene chloride was removed by rotary evaporation and the residue was diluted into 1 M HCl. The resulting

solution was extracted with ethyl acetate and the extracts were thoroughly washed with water to remove dimethylformamide. On drying with CaCl_2 , a gum separated from the ethyl acetate solution and was discarded. The solution was concentrated, filtered and diluted with cyclohexane to give a white amorphous precipitate. This was taken up in 1 M NaOH, filtered and carefully acidified with HCl. After standing at 0 °C, 140 mg (20%) of *benzenepropanoyl-L-tryptophanylglycylglycine* [nor-I(WGG)] was collected (single component by silica t.l.c. (ethanol), m.p. 90 °C, i.r. (KBr) 1730 cm^{-1} , 1660sh, 1630; 400 MHz ^1H n.m.r. δ (p.p.m.) [(^2H)methanol] 2.47 (2 H, t, J 7.4 Hz), 2.77 (2 H, t, J 7.4 Hz), 3.09 (1 H, dd, J 7.8, 14.6 Hz), 3.25 (1 H, dd, J 6.6, 14.6 Hz), 3.71 (1 H, d, J 17.0 Hz), 3.83 (2 H, s), 3.85 (1 H, d, J 17.0 Hz), 4.57 (1 H, dd, J 6.6, 7.8 Hz), 7.01 (1 H, dd, J 7.2, 8.0 Hz), 7.04 (1 H, s), 7.05–7.2 (6 H, m), 7.33 (1 H, d, J 8.0 Hz), 7.55 (1 H, d, J 8.0 Hz); 100 MHz ^{13}C n.m.r. δ (p.p.m.) [(^2H)methanol] 28.4, 32.5, 38.5, 41.6, 43.5, 56.2, 110.8, 112.4, 119.3, 119.9, 122.5, 124.6, 127.1, 128.7, 129.3, 129.4, 138.0, 142.1, 171.9, 172.8, 174.8, 175.6; mass spectrum (f.a.b., MH^+) m/e

451.1981 ± 0.0007 (5); $\text{C}_{24}\text{H}_{27}\text{N}_4\text{O}_5$ requires 451.1981 (Found: C, 62.3; H, 5.9; N, 11.9; $\text{C}_{24}\text{H}_{26}\text{N}_4\text{O}_5$, 0.75 H_2O requires C, 62.1; H, 6.0; N, 12.1%).

New substrate

Characterization is provided for *N*-(3-[2-furyl]acryloyl)glycyl-L-phenylalanyl-L-alanine (fua-Gly-Phe-Ala-OH) {m.p. 209–210 °C (from chloroform/petroleum ether); 400 MHz ^1H n.m.r. δ (p.p.m.) [(^2H)dimethyl sulphoxide] 1.30 (3 H, d, J 7.3 Hz), 2.74 (1 H, dd, J 9.9, 13.8 Hz), 3.03 (1 H, dd, J 3.9, 13.8 Hz), 3.66 (1 H, d, J 16.6 Hz), 3.82 (1 H, d, J 16.6 Hz), 4.20 (1 H, m), 4.55 (1 H, m), 6.46 (1 H, d, J 15.6 Hz), 6.58 (1 H, dd, J 1.8, 3.3 Hz), 6.77 (1 H, d, J 3.3 Hz), ~7.2 (7 H, m), 7.76 (1 H, s), 8.09 (1 H, d, J 8.5 Hz), 8.36 (1 H, m); i.r. (KBr) 1710 cm^{-1} ($-\text{CO}_2\text{H}$), 1670, 1660 ($-\text{CONH}-$), 1615 ($\text{C}=\text{C}$); mass spectrum (f.a.b., MH^+) m/e 414.1664 ± 0.0013 (5); $\text{C}_{21}\text{H}_{24}\text{N}_3\text{O}_6$ requires 414.1665 (Found: C, 60.6; H, 5.5; N, 9.9; $\text{C}_{21}\text{H}_{23}\text{N}_3\text{O}_6$ requires C, 61.0; H, 5.6; N, 10.2%).