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Importance of Tetrahedral Intermediate Formation in the Catalytic Mechanism of the Serine Proteases Chymotrypsin and Subtilisin

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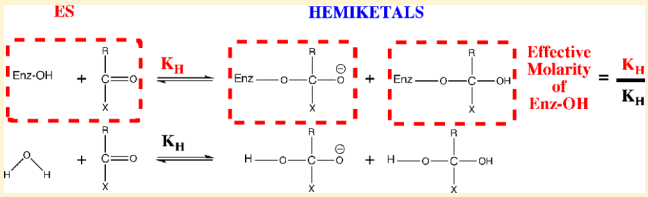
ABSTRACT: Two new inhibitors in which the terminal α-carboxyl groups of Z-Ala-Ala-Phe-COOH and Z-Ala-Pro-Phe-COOH have been replaced with a proton to give Z-Ala-Ala-Phe-H and Z-Ala-Pro-Phe-H, respectively, have been synthesized. Using these inhibitors, we estimate that for α-chymotrypsin and subtilisin Carlsberg the terminal carboxylate group decreases the level of inhibitor binding 3−4-fold while a glyoxal group increases the level of binding by 500−2000-fold.



We show that at pH 7.2 the effective molarities of the catalytic hydroxyl group of the active site serine are 41000−229000 and 101000−159000 for α-chymotrypsin and subtilisin Carlsberg, respectively. It is estimated that oxyanion stabilization and the increased effective molarity of the catalytic serine hydroxyl group can account for the catalytic efficiency of the reaction. We argue that substrate binding induces the formation of a strong hydrogen bond or low-barrier hydrogen bond between histidine-57 and aspartate-102 that increases the pK_a of the active site histidine, allowing it to be an effective general base catalyst for the formation of the tetrahedral intermediate and increasing the effective molarity of the catalytic hydroxyl group of serine-195. A catalytic mechanism for acyl intermediate formation in the serine proteases is proposed.

The catalytic residues serine-195 and histidine-57 of chymotrypsin were first identified by chemical modification studies.[1−3] The third residue of the catalytic triad, aspartate-102, was identified by X-ray crystallographic studies.[4] Kinetic experiments using a highly reactive *p*-nitrophenol substrate showed a rapid release of the *p*-nitrophenol product that was stoichiometric with enzyme, which was followed by a slow turnover the substrate.[5] This provided the first evidence of catalysis proceeding via an acyl intermediate. The minimal kinetic scheme for catalysis involves formation of a Michaelis−Menten complex (ES), an acyl intermediate (ES′), and the first product (P_1), and hydrololysis of the acyl intermediate to give the free enzyme (E) and the second product (P_2). The formation and breakdown of the acyl intermediate are thought to proceed via a tetrahedral intermediate. It is generally accepted that the rate-limiting step in catalysis is either the rate of breakdown or the rate of formation of the tetrahedral intermediate involved in acyl intermediate formation or breakdown. Therefore, the catalytic efficiency of the serine proteases is expected to depend on their ability to catalyze tetrahedral intermediate formation and breakdown. One way they can do this is by transition state stabilization[6] of the tetrahedral intermediate. Therefore, to understand catalysis, we should study the transition state stabilization of the tetrahedral intermediate. However, it is not feasible to study such tetrahedral intermediates because they do not accumulate during catalysis with peptide substrates.[7] Inhibitors that are able to react with chymotrypsin to form transition state analogues mimicking the catalytic tetrahedral intermediate offer a practical method for studying tetrahedral intermediate stabilization in the serine proteases. In this study, we use peptide glyoxal inhibitors to provide insights into how the serine proteases promote tetrahedral intermediate formation.

Specific substrate-derived peptide glyoxals have been shown to be extremely potent competitive inhibitors of the serine proteases chymotrypsin[8−10] and subtilisin.[11,12] Using ^{18}O and ^{2}H isotope shifts, it has been shown that the active site serine hydroxyl group forms a hemiketal with the glyoxal keto group.[13] Therefore, the glyoxal keto carbon should be in the same position as the peptide carbon of the hydrolyzed peptide bond of an analogous substrate. Also, the hemiketal that is formed when the catalytic serine hydroxyl group reacts with the keto carbon of the glyoxal should be a good analogue of the tetrahedral intermediate formed during catalysis. The resonance stabilization of the peptide bond is lost upon formation of catalytic tetrahedral intermediates from peptide substrates. This makes tetrahedral intermediate formation energetically unfavorable, so the amount of tetrahedral intermediate formed during catalysis will be extremely small.[14] Therefore, it is not possible study the tetrahedral intermediates formed during catalysis by techniques such as nuclear magnetic resonance (NMR).[15,16] However, the keto carbons of glyoxal inhibitors readily form tetrahedral adducts with both chymotrypsin[8,9] and subtilisin.[11,12] Oxyanion formation can be followed by ^{13}C NMR,[17,18] and this has made it possible to quantify oxyanion stabilization in both chymotrypsin− and subtilisin−glyoxal inhibitor complexes.[8,9,11,12] However, it was not possible to

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quantify the role of hemiketal formation in glyoxal inhibitor binding. This is because in addition to an inhibitor glyoxal group forming a hemiketal with the active site serine hydroxyl group its peptide chain can also bind in secondary subsites S_1−S_4. To estimate how these two processes contribute to inhibitor binding, we have studied the binding of two derivatives of the N-protected tripeptides Z-Ala-Ala-Phe-COOH and Z-Ala-Pro-Phe-COOH (Z = benzyloxycarbonyl). The glyoxal derivatives were formed by converting the peptide carboxyl group to a glyoxal group, and the Z-Ala-Ala-Phe-H and Z-Ala-Pro-Phe-H derivatives were formed by replacing the peptide carboxyl group with a hydrogen atom. This has also allowed us to determine how the C-terminal α-carboxylate groups of the N-protected tripeptides contribute to binding. The mechanistic significance of these results is discussed.

EXPERIMENTAL PROCEDURES

Materials. All materials were obtained from Sigma-Aldrich Chemical Co. (Gillingham, Dorset, U.K.).

Inhibitor Synthesis. The peptide-derived glyoxal inhibitors Z-Ala-Pro-Phe-COCHO and Z-Ala-Ala-Phe-COCHO were synthesized as described by Djurdjevic-Pahl et al.[8] and Cosgrove et al.[19] Z-Ala-Pro-Phe-H and Z-Ala-Ala-Phe-H were synthesized by coupling 2-phenethylamine to either Z-Ala-Ala or Z-Ala-Pro as required, using 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDCI·HCl) as a coupling reagent.[20,21]

NMR Spectra of Z-Ala-Ala-Phe-H and Z-Ala-Pro-Phe-H. ^{13}C NMR analysis of Z-Ala-Ala-Phe-H gave the folowing data (75.475 MHz, DMSO-d_6): δ 18.11 (1C, CH$\underline{C}$H$_3$), 18.49 (1C, CH$\underline{C}$H$_3$), 35.01 (1C, C$_6$H$_5\underline{C}$H$_2$), 40.14 (1C, C$_6$H$_5$CH$_2\underline{C}$H$_2$), 48.09 (1C, $\underline{C}$HCH$_3$), 50.06 (1C, $\underline{C}$HCH$_3$), 65.37 (1C, O-$\underline{C}$H$_2$Ph), 126.08−128.68 (10C, $\underline{C}$H═CH), 137.02−139.33 (2C, CH═$\underline{C}$═), 155.74 (1C, O-$\underline{C}$O-NH), 171.92−172.00 (2C, $\underline{C}$ONH). ^{13}C NMR analysis of Z-Ala-Pro-Phe-H gave the following data (trans form, 83.5%) ([^{2}H$_6$]-DMSO): δ 16.85 (1C, CH$\underline{C}$H$_3$), 24.41 (1C, CHCH$_2\underline{C}$H$_2$CH$_2$), 29.23 (1C, CH$\underline{C}$H$_2$CH$_2$CH$_2$), 35.13 (1C, C$_6$H$_5\underline{C}$H$_2$CH$_2$), 46.57 (1C, CHCH$_2$CH$_2\underline{C}$H$_2$), 48.15 (1C, $\underline{C}$HCH$_3$), 40.20 (1C, C$_6$H$_5$CH$_2\underline{C}$H$_2$), 59.65 (1C, $\underline{C}$HCH$_2$CH$_2$CH$_2$), 65.34 (1C, C$_6$H$_5\underline{C}$H$_2$), 126.02−128.68 (10C, $\underline{C}$H═$\underline{C}$H), 137.07−139.45 (2C, CH═$\underline{C}$═), 155.69 (1C, O-$\underline{C}$O-NH), 170.84 and 171.43 (2C, $\underline{C}$O-NH). The amount of the cis form present (16.5%) was determined by quantifying the signals from the trans β (29.2 ppm) and γ (24.4 ppm) proline carbons and the cis β (31.31 ppm) and cis γ (21.62 ppm) carbons.

Enzyme Solutions. α-Chymotrypsin and subtilisin Carlsberg were obtained from Sigma as crystallized and lyophilized powders. The amounts of fully active chymotrypsin (83%), subtilisin Carlsberg (45−65%), and subtilisin BPN (73%) were determined as described by Finucane et al.[22] and O'Connell et al.[23]

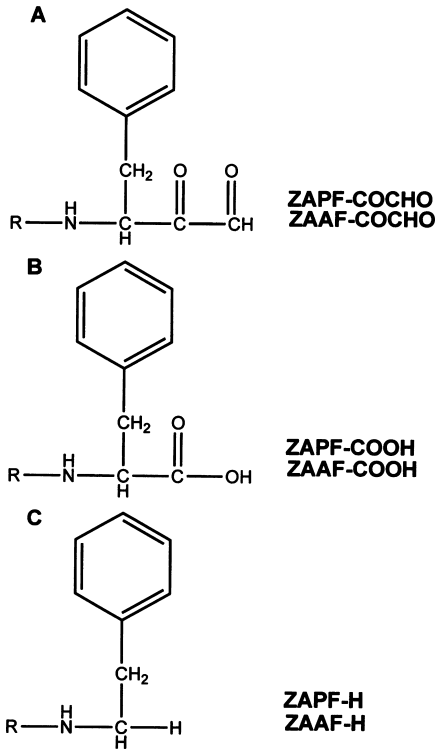
Inhibition of Chymotrypsin and Subtilisin Carlsberg by Z-Ala-Ala-Phe-COOH and Z-Ala-Ala-H. The inhibition of the chymotrypsin-catalyzed hydrolysis of suc-Phe-pNA or suc-Ala-Ala-Pro-Phe-pNA was studied at 25 °C in 3 mL cuvettes containing 0.1 M buffers [pH 7.1−7.3 (potassium phosphate), 3.2 (sodium formate), and 10.6 (potassium carbonate)] and 3.3% (v/v) dimethyl sulfoxide. The initial rate of hydrolysis of suc-Phe-pNA (pH 7.1−7.3 and 10.6) or suc-Ala-Ala-Pro-Phe-pNA (pH 3.2) was followed by the measuring the release of *p*-nitroaniline (E_{410} = 8800 M^{-1}cm^{-1} [24]) over a 5−15 min period. The pH of the reaction mixture was determined. Stock solutions of substrate and inhibitor were dissolved in dimethyl sulfoxide. The same procedure was used with subtilisin except that the substrate suc-Ala-Ala-Pro-Phe-pNA was used.

K_i values were estimated when $[S_0] \ll K_M$. Therefore, the equation for competitive inhibition $\{d[P]/dt = (k_{cat}[E][S])/[[S] + K_M(1 + [I]/K_i)]\}$ is reduced to $d[P]/dt = (k_{cat}/K_M)[E][S]K_i/([I] + K_i)$. K_i values were estimated by using a nonlinear least-squares regression program.[18]

RESULTS

Role of the α-Carboxylate Group in Peptide Binding by α-Chymotrypsin and Subtilisin Carlsberg. The K_i values for inhibition of α-chymotrypsin by Z-Ala-Ala-Phe-COOH (Chart 1B) and Z-Ala-Pro-Phe-H (Chart 1C) were determined by determining how they inhibited the chymotrypsin-catalyzed hydrolysis of suc-Phe-pNA when the substrate concentration was nonsaturating (Figure 1). The K_i values with subtilisin were measured in the same way except that suc-Ala-Ala-Pro-Phe-pNA was used as a substrate.

Chart 1. Structures of Inhibitors[a]



[a]R is either ZAP (Z-Ala-Pro-) or ZAA (Z-Ala-Ala-).

Replacing the α-carboxyl group of Z-Ala-Ala-Phe-COOH and Z-Ala-Pro-Phe-COOH with a hydrogen atom to give Z-Ala-Ala-Phe-H and Z-Ala-Pro-Phe-H, respectively (Chart 1C), led to 3−4-fold tighter binding at pH 7 (Table 1). Therefore, the presence of an α-carboxylate group at pH 7 decreases the effectiveness of peptide binding. This result is expected because for catalytic efficiency we would expect that the enzyme will bind the peptide products of catalysis less effectively than the parent peptide of the substrate. Therefore, when α-chymotrypsin and subtilisin catalyze the hydrolysis of the peptide substrates, the α-carboxylate of the peptide product will help promote its dissociation from the enzyme. This shows that the

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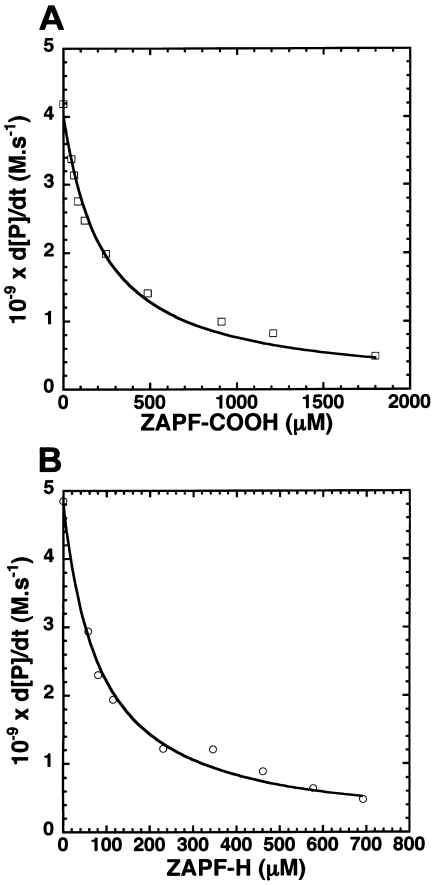


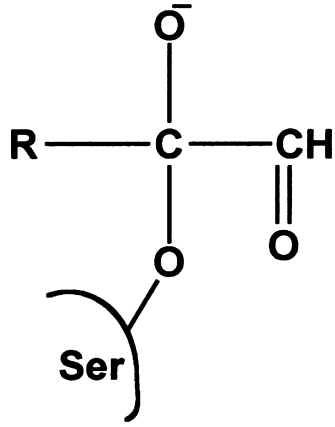
Figure 1. Inhibition of the α-chymotrypsin-catalyzed hydrolysis of suc-Phe-pNA by Z-Ala-Pro-Phe-H (ZAPF-H) and Z-Ala-Pro-Phe-COOH (ZAPF-COOH). All samples contained 3.3% dimethyl sulfoxide and 0.1 M potassium phosphate buffer (pH 7.2). (A) For inhibition by ZAPF-COOH (10 data points), the α-chymotrypsin and suc-Phe-pNA concentrations were 5.2 and 54 μM, respectively. The solid line was calculated using the equation $d[P]/dt = [(k_{cat}/K_M)[E][S]K_i]/([I] + K_i)$ and the fitted values of 14.3 ± 0.4 M^{-1} s^{-1} and 233 ± 24 μM for k_{cat}/K_M and K_i, respectively. (B) For inhibition by ZAPF-H (9 data points), the α-chymotrypsin and suc-Phe-pNA concentrations were 5 and 54 μM, respectively. The solid line was calculated as described for panel A using the fitted values of 17.9 ± 0.5 M^{-1} s^{-1} and 84.2 ± 6.4 μM for k_{cat}/K_M and K_i, respectively. All errors are the standard errors obtained on fitting the experimental data.

α-carboxylate group does not make a positive contribution to binding in α-chymotrypsin and subtilisin.

Binding of Glyoxal Inhibitors. In contrast to the results described above, when the glyoxal groups of Z-Ala-Ala-Phe-COCHO or Z-Ala-Pro-Phe-COCHO (Chart 1A) were replaced with a hydrogen atom to give Z-Ala-Ala-Phe-H and Z-Ala-Pro-Phe-H, respectively (Chart 1C), the K_i values of the inhbitors were ~3 orders of magnitude larger than those of the corresponding glyoxal inhibitor (Table 1).

NMR studies have shown that at pH ~7 the active site catalytic serine residues of both chymotrypsin[8,9,13] and subtilisin[11,12] form negatively charged hemiketals with the glyoxal inhibitors Z-Ala-Ala-Phe-COCHO and Z-Ala-Pro-Phe-COCHO (Chart 2). With subtilisin Carlsberg and Z-Ala-Ala-Phe-COCHO, an equal amount of the fully hydrated form of the structure in Chart 2 is also formed.[12] Therefore, while the negatively charged carboxylate group decreases binding efficiency, the formation of a negatively charged hemiketal with a glyoxal inhibitor facilitates binding. This tight binding demonstrates that hemiketal formation is energeticaly favored and confirms that the negatively charged hemiketal is a good transition state analogue of the catalytic tetrahedral intermediate.

Chart 2. Negatively Charged Hemiketal Formed When Glyoxal Inhibitors React with the Serine Proteases



Scheme 1 represents the minimal scheme that we have used to analyze hemiketal formation when glyoxal inhibitors interact with chymotrypsin or subtilisin. In this model, K_{H1} is the hydration constant for the glyoxal inhibitor (K_{H1} = hydrate/keto group = [GH]/[G]), K_s is the dissociation constant [K_s = ([E][G])/[EG]] of the noncovalent enzyme−glyoxal inhibitor complex (EG), and $K_{HK(obs)}$ is the observed equilibrium constant for hemiketal formation [$K_{HK(obs)}$ = [hemiketal]/[EG]]. The observed binding constant $K_{i(obs)}$ = [E]([G] + [GH])/([EG] + [hemiketal]).

Therefore

Table 1. Binding of Inhibitors to Chymotrypsin and Subtilisin Carlsberg

		$K_{i(obs)}$ (μM)[a]				
enzyme	pH	ZAAF-H	ZAAF-COOH	ZAAF-COCHO	$K_{iZAAF-H}/K_{iZAAF-COOH}$	$K_{iZAAF-H}/K_{iZAAF-COCHO}$
α-chymotrypsin	7.2	166 ± 9	532 ± 50	0.365[b]	0.31	455
subtilisin Carlsberg	7.1	97.6 ± 12.6	419 ± 32	0.0876 ± 0.016	0.23	1110
		$K_{i(obs)}$ (μM)[a]				
enzyme	pH	ZAPF-H	ZAPF-COOH	ZAPF-COCHO	$K_{iZAPF-H}/K_{iZAPF-COOH}$	$K_{iZAPF-H}/K_{iZAPF-COCHO}$
α-chymotrypsin	7.2	77.4 ± 6.4	241 ± 7	0.0335[b]	0.32	2310
subtilisin Carlsberg	7.2	1600 ± 600	ND[c]	1.00 ± 0.03	ND[c]	1600

[a]Errors are the standard deviations of three determinations. [b]From ref 9. [c]Not determined.

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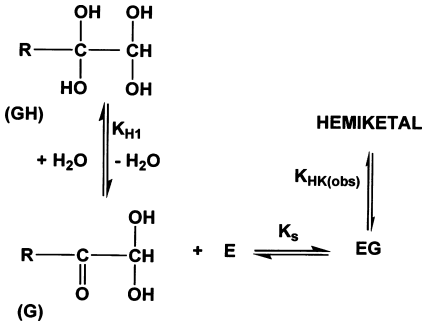
Scheme 1. Minimal Scheme for Hemiketal Formation by the Serine Proteases



$$K_{i(obs)} = K_s[1 + K_{H1(obs)}]/[1 + K_{HK(obs)}] \quad (1)$$

$$K_{HK(obs)} = \{K_s[1 + K_{H1(obs)}] - K_{i(obs)}\}/K_{i(obs)} \quad (2)$$

The hydration constant $[K_{H1(obs)}]$ of the keto carbon of the glyoxal group of Z-Ala-Ala-Phe-COCHO was estimated by quantitative ^{13}C NMR to be 1.58 ± 0.05. It was assumed that the $K_{i(obs)}$ value for Z-Ala-Ala-Phe-H was a good approximation for K_s, the disassociation constant for the noncovalent enzyme−glyoxal inhibitor complex (EG) in Scheme 1.

Therefore, using the values of $K_{i(obs)}$ for Z-Ala-Ala-Phe-COCHO and Z-Ala-Ala-Phe-H (Table 2), we calculated the $K_{HK(obs)}$ values for the glyoxal inhibitors (Table 2). For chymotrypsin and subtilisin, the $K_{HK(obs)}$ values were 1170 and 2870, respectively, for ZAAF-COCHO at pH 7 (Table 2).

The hydration constant $[K_{H1(obs)}]$ of the keto carbon of the glyoxal group of Z-Ala-Pro-Phe-COCHO is 1.28 ± 0.05.[13] Therefore, we could estimate $K_{HK(obs)}$ for Z-Ala-Pro-Phe-COCHO inhibiting both chymotrypsin and subtilisin. In this case, even larger values of $K_{HK(obs)}$ were obtained for chymotrypsin and subtilisin Carlsberg (Table 2). However, the hydration constant $[K_{H1(obs)}]$ is not a true thermodynamic constant and must be divided by the water concentration to give the correct thermodynamic association constant (K_{H1}). For the hydrates of Z-Ala-Pro-Phe-COCHO and Z-Ala-Ala-Phe-COCHO, the values of K_{H1} were 0.0230 and 0.0284 M^{-1}, respectively (Table 2).

Effective Molarity of the Catalytic Serine in α-Chymotrypsin and Subtilisin. Hemiketal formation within the enzyme−inhibitor complex is a unimolecular process [$K_{HK(obs)}$ = [hemiketal]/[EG]], while the hydration of the unbound glyoxal inhibitor is a bimolecular process involving the reaction of water with the glyoxal group [K_{H1} = [hemiketal]/([glyoxal][H_2O])]. Therefore, the $K_{HK(obs)}/K_{H1}$ ratio gives the molarity of water required to be as effective as the enzyme's serine hydroxyl group in hemiketal formation. At pH 7.2 with Z-Ala-Ala-Phe-COCHO, the effective concentration of the active site serine hydroxyl group of chymotrypsin relative to a water hydroxyl group is 1170/0.0284 = 41200 M. For subtilisin Carlsberg at pH 7.1, the effective concentration is even larger (2870/0.0284 = 101000 M). These correspond to stabilizations of 26.3 and 28.6 kJ/mol for Z-Ala-Ala-Phe-COCHO with chymotrypsin and subtilisin Carlsberg, respectively (Table 2).

With Z-Ala-Pro-Phe-COCHO at pH 7.2, the effective concentration of the active site serine hydroxyl group relative to a water hydroxyl group is larger for chymotrypsin (229000 M) and subtilisin Carlsberg (159000 M). These correspond to stabilizations of 30.6 and 29.7 kJ/mol for Z-Ala-Pro-Phe-COCHO with chymotrypsin and subtilisin Carlsberg, respectively (Table 2). For subtlisin BPN, the effective concentration of the active site serine hydroxyl group is ∼20 times lower than that for subtilisin Carlsberg (Table 2).

Effect of pH on Hemiketal Formation. From pH 7.2 to 3.2, there is an ∼17-fold decrease in the level of binding of Z-Ala-Pro-Phe-COCHO and a similar ∼5-fold decrease in the level of binding of Z-Ala-Pro-Phe-H (Table 3). This demonstrates that protonation of the hemiketal oxyanion weakens binding by only a small amount (∼3-fold). The fact that the K_i values for both Z-Ala-Pro-Phe-COCHO and Z-Ala-Pro-Phe-H increase from pH 7.2 to 10.6 by similar factors of ∼6 and ∼3, respectively (Table 3), supports the earlier suggestion[9] that this is due to a conformational change resulting from the ionization of the ion pair between isoleucine-16 and aspartate-194 in chymotrypsin.

Table 3. Effect of pH on Hemiketal Formation in Glyoxal Inhibitor Complexes with Chymotrypsin

	$K_{i(obs)}$[a] (μM)		
pH	ZAPF-H	ZAPF-COCHO	$K_{HK(obs)}$
3.2	381 ± 41	0.581[b]	1494
7.2	77.4 ± 6.4	0.0335[b]	5267
10.6	242 ± 16	0.201[b]	2744

[a]Errors are the standard deviations of three determinations. [b]From ref 9.

How the Effective Molarity of the Catalytic Serine and Oxyanion Stabilization Contribute to Catalysis. During catalysis by chymotrypsin, formation of the acyl intermediate is expected to proceed via a tetrahedral intermediate (Scheme 2)

Table 2. Effective Molarities of the Catalytic Serine of the Serine Proteases upon Formation of Hemiketals with Glyoxal Inhibitors

		$K_{i(obs)}$[a] (μM)					
enzyme	pH	ZAAF-H	ZAAF-COCHO	$K_{HK(obs)}$	K_{H1} (M^{-1})	effective molarity (M)	ΔG at 25 °C (kJ/mol)
α-chymotrypsin	7.2	166 ± 9	0.365[b]	1170	0.0284	41200	−26.3
subtilisin Carlsberg	7.1	97.6 ± 12.6	0.0876 ± 0.016	2870	0.0284	101000	−28.6
		$K_{i(obs)}$[a] (μM)					
enzyme	pH	ZAPF-H	ZAPF-COCHO	$K_{HK(obs)}$	K_{H1} (M^{-1})	effective molarity (M)	ΔG at 25 °C (kJ/mol)
α-chymotrypsin	7.2	77.4 ± 6.4	0.0335[b]	5270	0.023	229000	−30.6
subtilisin Carlsberg	7.2	1600 ± 600	1.00 ± 0.03	3650	0.023	159000	−29.7
subtilisin BPN	7.0	451 ± 47[d]	5.32[c]	192	0.023	8390	−22.4

[a]Errors are the standard deviations of three determinations. [b]From ref 9. [c]From ref 11. [d]pH 7.3.

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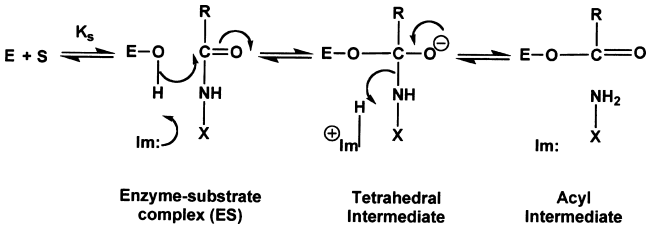
Scheme 2. Formation of the Acyl Intermediate by the Serine Proteases



analogous to the hemiketal formed when Z-Ala-Ala-Phe-COCHO or Z-Ala-Pro-Phe-COCHO reacts with chymotrypsin. It is therefore reasonable to assume that both tetrahedral species will be formed and stabilized in the same way with the active site histidine acting as a general base catalyst for tetrahedral intermediate formation and a general acid catalyst for tetrahedral intermediate breakdown (Scheme 2). Therefore, we would expect that the enzyme-catalyzed formation of the tetrahedral intermediate would be ~100000 times more effective than the uncatalyzed reaction in water (Table 2).

It has been determined that the rate of hydrolysis of a peptide bond in water at 25 °C and pH 7 is 3×10^{-9} s^{-1}.[25] In the enzyme-catalyzed reaction, the greater effective molarity of the serine hydroxyl group should increase this rate by at least 100000 to a value of 3×10^{-5} s^{-1}. This rate is still considerably slower than the k_{cat} values usually observed with chymotrypsin and similar substrates such as Ac-Ala-Pro-Phe-NH_2,[26] for which k_{cat} is 2.3 s^{-1} at pH 8. Therefore, k_{cat} would be expected to be ~1 s^{-1} at pH 7 as k_{cat} depends on a pK_a of ~7.

However, we have also shown that in chymotrypsin[8,9] and subtilisin[11,12] the pK_a for the formation of an oxyanion by the glyoxal hemiketal is reduced by ~5 pK_a units. If the catalytic rate is directly proportional to the amount of oxyanion formed, then this would increase the catalytic rate from 3×10^{-5} to 3 s^{-1}. This is very similar to the expected value of ~1 s^{-1}.

DISCUSSION

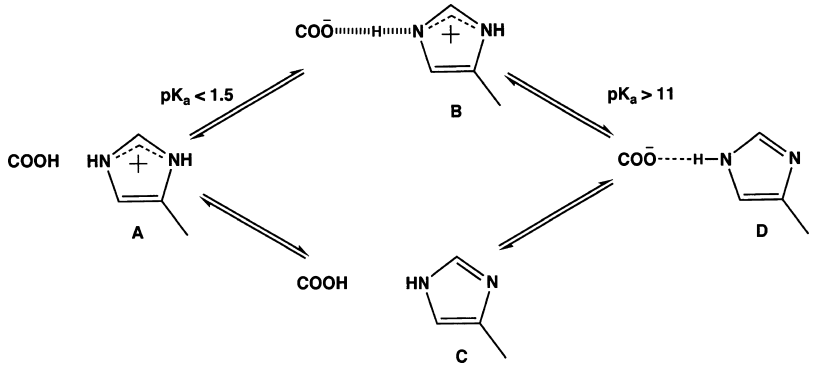
It is accepted that for chemical reactions in aqueous solutions reactions proceed via neutral tetrahedral intermediates.[27] However, it is clear from studies with both reversible[8,9,11,12,15,28] and irreversible inhibitors[17,18,23,29] that the serine proteases preferentially stabilize tetrahedral adducts with a negatively charged oxyanion.

Protonation of the hemiacetal oxyanion and hemiketal oxyanion had only a small effect (an 5−17-fold increase in K_i) on inhibitor binding (Table 3). Therefore, the oxyanion and its conjugate acid are bound with similar affinities. Both the oxyanion and its conjugate acid should be hydrogen bonded within the oxyanion hole.[30,31] Tighter binding of the negatively charged oxyanion relative to its neutral conjugate acid is expected because hydrogen bonds involving charged groups are stronger than those involving uncharged groups.[32,33] However, it has been shown that in glyoxal inhibitor complexes the oxyanion pK_a is reduced by ~5 pK_a units.[8,9,11,12] It is clear that this large decrease in pK_a cannot be attributed to better binding of the oxyanion but must reflect destabilization of its conjugate acid.

We have shown that the binding of glyoxal inhibitors to chymotrypsin[9] and subtilisin[12] increases the pK_a of the active site histidine to a value of >11. This led to the suggestion[9,12] that this increase in pK_a would allow the positively charged imidazolium ring of the active site histidine to lower the hemiketal oxyanion pK_a and also lower the pK_a of the active site serine hydroxyl, increasing its nucleophilicity. The hydroxide ion is solvated in water and so not very reactive.[27] However, in nonaqueous solvents, its reactivity may be increased by as much as 14 orders of magnitude.[34] Therefore, in the enzyme−inhibitor complex, the entropic advantage of fixing the reactive groups within the Michaelis complex[35] combined with desolvation, a lower pK_a for the active site serine hydroxyl, and general base catalysis by the imidazole group of histidine-57 can easily explain the effective concentration of ~100000 M for the active site serine hydroxyl group relative to the hydroxyl group of water.

Blow[4] first identified the catalytic triad of serine-195, histidine-57, and aspartate-102, with aspartate-102 forming a hydrogen bond to Nδ1 of histidine-57. His canonical forms are still be being incorrectly quoted as suggesting a neutral aspartate and histidine even though this possibility was not considered likely by the authors.[36] Recently, it has been proposed that the catalytic aspartate is protonated in Michaelis−Menten complexes.[37] However, subsequent studies contradict this as they claim to show that aspartate-102 has a low pK_a of ≤1.5 that they propose is compatible with the reaction-driven ring flip mechanism but not mechanisms that require a strong hydrogen bond between histidine-57 and aspartate-102.[38] However, ^{1}H NMR has been used to observe the Nδ1 proton shared between histidine-57 and aspartate-102.[39,40] Bachovchin[41] used ^{15}N enrichment of the Nδ1 atom of histidine-57 to confirm that the NMR signal at 14−18 ppm is due to the Nδ1 proton of histidine-57. Pioneering studies of peptidyl trifluoromethyl ketone complexes of chymotrypsin showed the presence of a ^{1}H NMR signal at 18.7 ppm that was

Scheme 3. Microscopic Ionization States of Histidine-57 and Aspartate-102 in the Serine Proteases



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attributed to a hydrogen strongly hydrogen bonded between histidine-57 and aspartate-102.[28] It was proposed that such proton chemical shifts of ∼16−20 ppm show the presence of strong hydrogen bonds called low-barrier hydrogen bonds.[42] This has been confirmed by D/H fractionation factors and activation energies[43] and also by deuterium[44] and tritium[45] isotope shifts in peptidyl trifluoromethyl ketone complexes of chymotrypsin. Therefore, a chemical shift of 16−18 ppm for Nδ1 of histidine-57 shows that histidine-57 is fully protonated and confirms that a strong hydrogen bond exists between aspartate-102 and Nδ1 of histidine-57.

We have shown that in glyoxal inhibitor complexes proton signals at 18.7 and 17.4 ppm can be observed at pH 3.5 and 10.9, respectively, showing that the pK_a of the active site histidine has been increased to a value of >11 (structure B in Scheme 3) on binding the glyoxal inhibitor.[9,12] The fact that the signal at 18.7 ppm is present at low pH when the oxyanion is present as its conjugate acid shows that the increase in pK_a must be due to the interaction of aspatate-102 with histidine-57 and not with the oxyanion. However, it has been argued that because aspartate-102 has a low pK_a a strong hydrogen bond cannot exist between histidine-57 and aspartate-102.[38] However, as a strong hydrogen bond is formed between the negatively charged carboxylate group and the Nδ1 proton of the positively charged histidine, then it will be difficult to protonate the aspartate as this will result in disruption of the low-barrier hydrogen bond. Consequently, the aspartate is expected to have a low pK_a of <1.5 (structure B in Scheme 3) as recently reported.[38] Therefore, when inhibitors or substrates are bound to chymotrypsin, the zwitterionic species (structure B in Scheme 3) is the predominant species formed from pH 1.5 to 11.The strong hydrogen bond between the aspartate and the histidine shows that reaction-driven ring flip mechanism is not possible. The fact that the low-barrier hydrogen bond is present even when the oxyanion has been protonated suggests that the binding of inhibitors and substrates must increase the pK_a of histidine-57. This, as our work shows, can increase the effective molarity of the active site hydroxyl group by more than 5 orders of magnitude (Table 2). The catalytic serine is expected to have a pK_a of ∼15, so the increase in the pK_a of the active site histidine will allow it to enhance the nucleophilicity of the serine hydroxyl group so that it can react at the substrate peptide carbonyl to form a tetrahedral intermediate. The strengthening of the hydrogen bond between histidine-57 and aspartate-102 may result from a decrease in the dielectric constant due to the substrate excluding water from the active site[17] and or by the induction of steric compression between histidine-57 and aspartate-102.[46] Therefore, when substrate binds, water is excluded from the active site and the pK_a of the histidine is increased, allowing it to abstract the proton from the serine hydroxyl, which then reacts with the peptide carbonyl of the substrate to form a tetrahedral intermediate (Scheme 2). A small reorientation of the imidazole ring[47] will then allow histidine-57 to act as a general acid catalyst protonating the nitrogen of the leaving group (Scheme 2), which is expected to have a pK_a of ∼10.[17,48] The formation of the acyl intermediate will then complete the first half of the catalytic cycle.

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