Specificity in the Alkylation of Serine at the Active Site of α-Chymotrypsin by Aromatic α-Bromo Amides†

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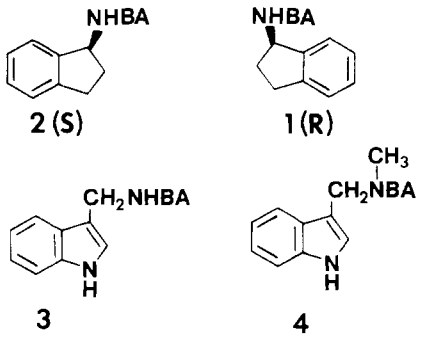
ABSTRACT: A number of aromatic α-bromo amides inactivate α-chymotrypsin by affinity labeling of either Met-192 or Ser-195. While the reaction with Met-192 is independent of pH [cf. Lawson, W. B., & Rao, G. J. S. (1980) *Biochemistry*, preceding paper in this issue], that with Ser-195 is governed by a group on the enzyme (presumably His-57) with a pK_a of about 7. Ser-195 is alkylated by those compounds in which the amide group and the aromatic ring are predominantly noncoplanar. Bromoacetyl-(*S*)-(−)-aminoindan was predicted from studies with models to have a higher reactivity toward Ser-195 than its *R*-(+) enantiomer, and the prediction was confirmed by experiment. Further study with models led to the synthesis and testing of bromoacetyl-3-aminomethylindole and -3-methylaminomethylindole. The latter compound proved to be the best to date for alkylation of Ser-195, both in regard to rate and to specificity. It is hypothesized that serine alkylation results from binding of an α-bromo amide in the active site with its amide carbonyl group positioned between the NH groups of Gly-193 and Ser-195. Further specificity and increased rates in the reaction with Ser-195 are gained by the use of *N*-(amino)methyl derivatives. The *N*-methyl group appears to prevent a nonproductive binding mode in which its NH analogue forms a hydrogen bond with the carbonyl group of Ser-214. The reactivity of Ser-195 is discussed in relation to the charge-transfer system, Asp-102:His-57:Ser-195.

During investigation of the stereochemistry of labeling of Met-192 at the active site of chymotrypsin by aromatic α-bromo amides, we observed that some compounds that did not react rapidly with Met-192 alkylated a serine residue, presumably Ser-195 (Lawson & Rao, 1980). In contrast to the alkylation of Met-192, the reaction displayed a marked pH dependence, similar to that observed in the alkylation of serine at the active site of trypsin by (α-bromoacetamide)alkylamines and -guanidines (Lawson et al., 1968).



Since two optical isomers, BA-(*R*)-(+)-1-aminoindan (**1**)[1] and BA-(*S*)-(−)-1-aminoindan (**2**), had been found to alkylate serine to some extent without a pH dependence (Lawson & Rao, 1980), it was decided to reinvestigate their reactions with the enzyme. A further impetus was provided by the active-site model of Clarke (1977). Examination of possible interactions of **1** and **2** with the active site led to a prediction that the *S*-(−) enantiomer, **2**, should react more readily with Ser-195 than the *R*-(+) enantiomer. Repetition of the earlier work confirmed the rate data but, more importantly, showed that the prediction was correct. Further study with models to improve selectivity in the alkylation of Ser-195 led to the synthesis of BA-3-aminomethylindole (**3**) and BA-3-methylaminomethylindole (**4**). The latter proved to be the best α-bromo amide to date for selective alkylation of Ser-195 in chymotrypsin.

Materials and Methods

α-Chymotrypsin (3× recrystallized, salt free) was obtained from Worthington Biochemical Corp. Organic chemicals were products of Aldrich Chemical Co. or Eastman Organic Chemicals. Ches, Mes, Mops, and Taps were obtained from Calbiochem. α-Bromo amides, except for those described below, were the compounds used in the previous paper (Lawson & Rao, 1980). 3-(Aminomethyl)indole and 3-[(methylamino)methyl]indole were prepared as described by Gower & Leete (1963); these amines are rather unstable (cf. Putokhin & Davidova, 1932) and were stored under vacuum at 4 °C. *N*-(Bromoacetyloxy)succinimide was prepared according to Hora (1973). Silica Woelm TSC and nylon tubing for dry-column chromatography were obtained from ICN Pharmaceuticals. Eastman silica gel plates with fluorescent indicators were used for TLC.

Melting points were uncorrected. Ultraviolet spectra and enzyme assays were carried out with a Cary 118 spectrophotometer. A Perkin-Elmer 710A infrared spectrophotometer and a Varian EM 360 NMR spectrometer were used for recording infrared and NMR spectra. Elemental analyses were performed by Instranal Laboratory, Inc., Rensselaer, NY, and results obtained for the elements listed were within ±0.3% of the theoretical values. The active-site model was described previously (Clarke, 1977; Lawson & Rao, 1980).

BA-3-(aminomethyl)indole (**3**). A solution of 1.55 g (7.37 mmol) of *N*-(bromoacetyloxy)succinimide in 5 mL of dimethyl sulfoxide was added to an ice-cold solution of 1.03 g (7.37 mmol) of 3-(aminomethyl)indole in 3 mL of dimethyl sulfoxide, and the solution was allowed to warm to room temperature for 15 min. The reaction mixture was diluted with

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[1] Abbreviations used: BA, bromoacetyl; Ches, cyclohexylaminoethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid; CPK, Corey–Pauling–Koltun (models); CCS, color-coded-skeletal (models); Cm, carboxymethyl.

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ethyl acetate and extracted with H_2O, 0.2 M HCl, H_2O, 0.2 M $KHCO_3$, and saturated NaCl. The organic layer was dried ($MgSO_4$) and evaporated in a rotary evaporator, and the residue was dried thoroughly under vacuum over Drierite to give 0.85 g of a frothy solid. TLC on silica gel in $CHCl_3$ showed an Ehrlich-positive spot (skatole) near the front, an Ehrlich-, Ellman- (Schloss & Hartman, 1977), and ninhydrin-positive spot near the origin, and a spot corresponding to the product, R_f ca. 0.3, that was slowly Ehrlich positive, Ellman and UV positive, and ninhydrin negative. The above solid (0.85 g) was dissolved in 5 mL of ethyl acetate, rotary evaporated with 5 g of Silica Woelm TSC, layered on a 100-g column of the same adsorbent in a nylon tube, and eluted with $CHCl_3$ until the middle (fluorescent) band was well separated from a band at the origin. The middle band was cut out and eluted with ethyl acetate. Evaporation of the solvent gave an oil that crystallized from ethyl acetate–cyclohexane to yield 230 mg (12%) of pale pink crystals, mp 100–102 °C. Further recrystallization gave fine white needles, mp 102–103 °C. The UV spectrum (in ethanol) was that of a typical indole. IR (in KBr) and NMR (in dimethyl-d_6 sulfoxide) spectra were in agreement with the structure of BA-3-aminomethylindole (**3**). Anal. C, H, N. This material should be kept dry in a freezer.

BA-3-[(methylamino)methyl]indole (**4**). 3-[(Methylamino)methyl]indole (3.0 g, 17.8 mmol) was converted to the bromoacetyl derivative in the same manner as **3**; a similar purification on a dry column of Silica Woelm TSC gave 0.78 g of oil. This was crystallized from dry ether to yield 460 mg (9%), mp 84–95 °C, and recrystallized from benzene to give 170 mg (4%) of white crystals, mp 99–101 °C. Anal. C, H, N. This compound is somewhat less stable than its unmethylated analogue **3**, and should be stored dry in a freezer.

Inactivation of Chymotrypsin and Analysis of the Inactivated Enzyme. Rates of inactivation were determined as in the preceding paper (Lawson & Rao, 1980) except that the buffer was 0.1 M Mops at pH 7.0. Amino acid analysis (Lawson et al., 1968; Lawson & Rao, 1980) was used to detect labeled amino acids eluting between cysteic acid and Asp. The shorter (50-cm) column for neutral amino acids now in use was operated between 50 and 60 °C for an optimum separation of methionine sulfone. The use of this column did not, however, permit an adequate separation of homoserine from Glu. Amounts of Ser and Met modification were estimated primarily on the determination of Cm-Ser and Met-sulfone and partially on the determination of *S*-Cm-homocysteine and Met.

pH Dependence of the Inactivation of Chymotrypsin by BA-N-methylbenzylamine (**7**). Rates of inactivation at 23 °C of 60 μM chymotrypsin were determined with a 20-fold molar excess of **7** in 10% ethanol and 0.1 M buffers, which were acetate (pH 5.0), Mes (pH 5.5–6.5), Mops (pH 7.0–7.5), Taps (pH 8.0–9.0), and Ches (pH 9.5–10.0). Enzyme blanks at pH 5, 6, and 7 were stable for 1 day; blanks at pH 8 and 9.5 lost 13% and 46%, respectively, of their activity. The instability of the enzyme at high pH probably does not affect the accuracy of the results appreciably, since the half-life of the enzymatic reaction is about 4–5 h from pH 8.0 to pH 9.5. The pH 10 value might be questioned, however, since no blank was run at that pH. Results were plotted as a function of reciprocal half-lives for inactivation vs. pH.

Results and Discussion

Inhibition of Chymotrypsin. Half-times for the inhibition of chymotrypsin under standardized conditions were determined by semilogarithmic plots and are given in Table I. The final activities were quite low in the faster reactions, but for practical reasons final activities were about 10% for the slower reactions ($t_{1/2}$ > 2000 min). Amino acid analyses show that the compounds alkylate Met-192 and Ser-195, with methionine alkylation more pronounced at pH 5 and serine alkylation greater at pH 7 (Table II gives results for compounds **1**–**4**). The analyses were carried out after performic acid oxidation of the inactivated enzyme. Under these conditions unmodified methionine is oxidized to methionine sulfone ($MetO_2$), while methionine alkylated with haloacetic acid derivatives is converted to a mixture of free methionine, *S*-(carboxymethyl)-homocysteine, homoserine, and homoserine lactone (Gundlach et al., 1959; Neumann et al., 1962; Lawson & Schramm, 1965). The latter two compounds were not resolved by our current analytical system, but the other products give a clear picture of the results. *O*-(Carboxymethyl)serine (Cm-Ser) is easily detected; the amounts listed in Table II are not corrected for the small quantities of glycolic acid produced by decomposition (Lawson et al., 1968). No evidence of histidine alkylation was noted, but a small amount, comparable to that previously observed in the alkylation of serine at the active site of trypsin (ibid.), cannot be excluded.

It can be safely assumed that the compounds discussed in this paper alkylate Ser-195 for the following reasons: (a) *O*-(carboxymethyl)serine is obtained on amino acid analysis of the inactivated enzyme, (b) chymotrypsin has never been shown to contain more than one uniquely reactive serine residue, and (c) the inhibitors were designed as affinity labels for the active site of chymotrypsin. The reactions of BA-*N*-

Table I: Rates of Inactivation of Chymotrypsin by α-Bromo Amides That Alkylate Ser-195

compound		reaction with chymotrypsin,[a] half-time (min)	
no.	*N*-bromoacetyl derivative of	pH 5	pH 7
1	(*R*)-(+)-1-aminoindan	4980	4980
2	(*S*)-(−)-1-aminoindan	2580	2580
3	3-(aminomethyl)indole	1960	1580
4	3-[(methylamino)methyl]indole	420	156
5	*N*-methylaniline	7380	4320
6	benzylamine	3870	2640
7	*N*-methylbenzylamine	1390	430
8	*N*-methyl-β-phenethylamine	540	324

[a] In 0.1 M acetate buffer, pH 5.0, and in 0.1 M Mops buffer, pH 7.0 (compounds 1, 2, 3, 4, and 7), or 0.1 M Tris buffer, pH 7.0 (compounds 5, 6, and 8; data taken from Table I of Lawson & Rao, 1980), 10% ethanol at 20-fold molar excess over chymotrypsin (60 μM). The half-times for loss of enzyme activity were obtained from pseudo-first-order plots.

Table II: Amino Acid Analyses for Constituents of the Active Site of Chymotrypsin after Reaction with Some Irreversible Inhibitors[a]

compound	pH	Cm-Ser	$MetO_2$	*S*-Cm	Met	His
1	7	0.27	1.30	0.17	0.13	1.94
	5	0.11	0.71	0.23	0.18	1.87
2	7	0.78	1.60	0.13	0.14	1.74
	5	0.36	1.37	0.09	0.17	1.82
3	7	0.64	1.42	0.03	trace	1.71
	5	0.13	0.92	0.27	0.16	1.97
4	7	1.18	1.48	trace		1.67
	5	0.58	1.00	0.24	0.13	1.76
none	7		1.77			1.92

[a] The inactivated enzyme preparation (<10% activity) were subjected to amino acid analysis as described under Materials and Methods after performic acid oxidation according to Hirs (1956), but at 25 °C for 2 h. Cm-Ser, *O*-(carboxymethyl)serine; $MetO_2$, methionine sulfone; *S*-Cm, *S*-(carboxymethyl)homocysteine.

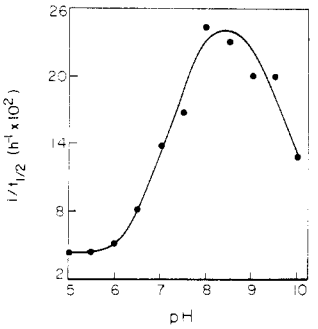

FIGURE 1: pH Dependence of the inactivation of chymotrypsin by BA-*N*-methylbenzylamine (**7**) under conditions described under Materials and Methods.

methylaniline (**5**) and BA-*N*-methylbenzylamine (**7**) were studied (Lawson & Rao, 1980) by the method of Kitz & Wilson (1962) and found to display the typical binding behavior expected of affinity labels (Lawson, 1978).

BA-(*S*)-(−)-aminoindan (**2**) is more selective than its enantiomer (**1**) in the alkylation of serine at the active site (Table I). The most effective and selective compound for the alkylation of serine in chymotrypsin at pH 7 is BA-3-[(methylamino)methyl]indole (**4**); the rate of inactivation (Table I) is the fastest of the series, and methionine alkylation is barely detectable (Table II).

The pH dependencies of the inactivations with compounds **3** and **4** are similar to those observed earlier with most of the compounds found to alkylate serine in chymotrypsin (Lawson & Rao, 1980). Repetition of the inhibitions with the aminoindan derivatives **1** and **2** gave similar half-times, but again no pH dependence could be observed (Table I). It seems likely that solvolysis of the compounds at the higher pH competes with the enzymatic reactions, and if this is true, the pH dependencies of the other slowly reacting inhibitors should be more pronounced than they were found to be. A more detailed study of the pH dependence was carried out with BA-*N*-methylbenzylamine (**7**). The reaction shows one pK_a of approximately 7 and another above pH 9 (Figure 1). The latter is attributable to deprotonation of the N-terminal Ile-16 and the consequent breakup of its hydrogen bond with Asp-194 (Himoe et al., 1967; Blow, 1971).

Stereochemistry of the Inhibitors and Their Interactions with the Active Site of Chymotrypsin. The stereochemistry of aromatic α-bromo amides that inactivate chymotrypsin by affinity labeling was discussed in the previous paper (Lawson & Rao, 1980). The ability of a number of these compounds to alkylate Met-192 correlated well with the likelihood that they exist to a significant extent in conformations with the amide group coplanar with the aromatic ring. Several compounds that should achieve coplanarity with difficulty were found to alkylate Met-192 slowly and to alkylate Ser-195 to some extent. This group of inhibitors is presented in Table I along with two additional ones, compounds **3** and **4**.

All of these compounds should experience some difficulty in achieving coplanar conformations with the exception of BA-*N*-methyl-β-phenethylamine (**8**), which can exist in a coplanar extended conformation. The 1-aminoindan derivatives **1** and **2** can approximate (but not achieve) a coplanar conformation when the bromoacetamido substituent is equatorial to the 5-membered ring. The remainder of the series (**3**–**7**) should exist mostly in noncoplanar conformations due to steric hindrance between the amide groups and the aromatic rings (Lawson & Rao, 1980).

Possible interactions of the compounds with the active site were studied using CPK models and the CCS model of the active site (Clarke, 1977). With the aromatic group in the specificity pocket, two orientations appear attractive for noncoplanar compounds. The first involves hydrogen bonding of the amide NH group (if any) to the carbonyl group of Ser-214. Such binding should be nonproductive for alkylation of Met-192 or Ser-195 because it places the back side of the CH_2Br group at some distance from and incorrectly oriented toward these nucleophiles. The second orientation involves hydrogen bonding of the amide CO group with the NH groups of Gly-193 and Ser-195, similar to the binding of the ester or carboxamide carbonyl group of typical substrates enroute to the tetrahedral intermediate (Blow, 1976, and references therein). In this binding mode the hydroxyl oxygen atom of Ser-195 has easy access to the back side of the CH_2Br group for a nucleophilic attack.

While studying possible interactions of the aminoindan derivatives **1** and **2** with the active site, it appeared that the indan ring of the *S*-(−) enantiomer **2** could fit into the specificity pocket in much the same way as the indole ring of a tryptophan residue while its carbonyl group makes contact with the NH groups of Gly-193 and Ser-195. This orientation allows an attack by the OH group of Ser-195 on the CH_2Br group. The proper conformation of **2** for this orientation can easily be made with a CPK model by placing the carbonyl oxygen of the trans amide group in a position cis to the hydrogen atom on the 1-position of the indan ring. Because of the opposite chirality of compounds **1** and **2**, the same conformation of the *R*-(+) enantiomer **1** places its carbonyl oxygen in a direction opposite from the NH groups of Gly-193 and Ser-195 when the model is inserted into the active site. When the amide group of **1** is turned through 180° to bring the carbonyl group in the right direction, the resulting axial conformation encounters considerable steric hindrance in fitting into the site. These observations led to a prediction that the *S*-(−)-enantiomer **2** should be more effective than **1** in alkylation of Ser-195. The prediction was substantiated in regard to both rate (Table I) and specificity (Table II).

Although BA-(*S*)-(−)-1-aminoindan (**2**) is more rapid and selective in the alkylation of Ser-195 than the *R*-(+)-enantiomer **1**, its rate of reaction with the enzyme is fairly slow ($t_{1/2}$ = 2580 min = 1.5 days). The slow rate could be caused by (1) steric hindrance in the binding pocket due to the CH_2 groups of the indan ring and (2) nonproductive binding due to hydrogen bond formation between the NH group of the amide and the carbonyl group of Ser-214. The first possibility seemed somewhat unlikely since the amldes of acetyl-L-phenylalanine and acetyl-L-hexahydrophenylalanine are hydrolyzed by chymotrypsin at very similar rates (Jennings & Niemann, 1953), as are their methyl esters (Jones & Niemann, 1963). It might have been possible to resolve this question with derivatives of 1-aminoindene. However, it seemed simpler to utilize the sterically similar 3-(aminomethyl)indole system, which is not asymmetric but should become so on binding to the active site. BA-3-(aminomethyl)indole (**3**) inactivates chymotrypsin somewhat faster than does BA-(*S*)-(−)-1-aminoindan (**2**) but in much the same manner (Tables I and II). The indole derivative **3** does not possess bulky CH_2 groups on the ring system but has an amide NH group that could bond to the carbonyl group of Ser-214. The *N*-methyl analogue of **3**, BA-3-[(methylamino)methyl]indole (**4**), which cannot form such a hydrogen bond at the active site, proved to be the best inhibitor of this type to date in regard to both rate of inactivation and specificity for serine alkylation at pH 7 (Tables I and II and Figure 2).

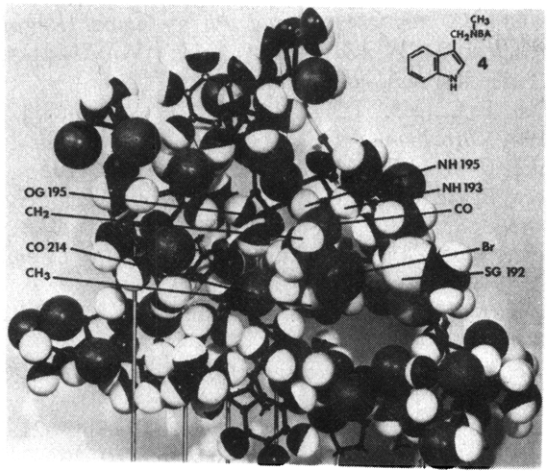

FIGURE 2: BA-3-[(methylamino)methyl]indole (**4**) oriented for alkylation of Ser-195 of chymotrypsin. The *N*-methyl group prevents hydrogen bond formation with the CO group of Ser-214. Abbreviations used: CO 214, carbonyl group of Ser-214; NH 193 and NH 195, backbone NH groups of Gly-193 and Ser-195; OG 195, hydroxyl oxygen atom of Ser-195; SG 192, sulfur atom of Met-192; the other atoms belong to compound **4**.

Our earlier work suggested that the primary effect of an *N*-methyl group in these compounds was to ensure noncoplanarity of the aromatic ring and the amide group and thus to decrease the rate of alkylation of Met-192 (Lawson & Rao, 1980). This view is still valid, especially in the case of BA-aniline (Lawson & Rao, 1980) and BA-*N*-methylaniline (**5**). But the second and no less important effect of an *N*-methyl group that emerged in the present work is that of directing the reaction to Ser-195, most likely by preventing nonproductive hydrogen bonding with Ser-214. The same contrast in rate observed between the indole derivatives **3** and **4** is seen also with the benzylamine derivatives **6** and **7** (Table I; Lawson & Rao, 1980).

Alkylation of Ser-195 and the Charge-Relay System. Perhaps the most interesting result of this study, in relation to the catalytic mechanism of chymotrypsin, is the alkylation of Ser-195 in a reaction that displays a pK_a of 7. In contrast to acylation reactions which could conceivably proceed via transfer of an acyl group from His-57 to Ser-195, alkylation of the serine hydroxyl group must be a direct process, namely, a nucleophilic attack on the CH_2Br group of an inhibitor by the serine anion or its equivalent. An intermediate alkylimidazole would be quite stable, even to acid hydrolysis. But since the pK_a of serine hydroxyl groups is usually around 14 (Bruice et al., 1962), the alkylation of Ser-195 must be mediated by the imidazole ring (pK_a ~7) of His-57. We pointed out some time ago in an analogous situation with trypsin "that the histidine-activated serine residue at the active site is indeed a powerful nucleophile, in contrast to normal serine residues" (Lawson et al., 1968). The present work confirms this conclusion.

Recently it has been argued that the serine need not be a powerful nucleophile and that it reacts with a tetrahedrally distorted carbonyl carbon atom because it is in an ideal position to do so (Kraut, 1977; Matthews et al., 1977). These conclusions are part of a continuing debate on the nature of the charge-relay system, Asp-102:His-57:Ser-195, which was first conceived when Blow and co-workers discovered that His-57 is hydrogen bonded to Asp-102 in the X-ray structure of chymotrypsin (for reviews see Blow, 1976; Kraut, 1977). The system as such exists in a number of serine proteases (Kraut, 1977). Major points of contention are whether the Asp-102 part of the system contributes to the nucleophilicity of Ser-195 or merely orients His-57 correctly with regard to Ser-195 and where the protons in the system are located both in the native enzymes and during substrate hydrolysis.

Hunkapiller et al. (1973) investigated α-lytic protease, enriched in ^{13}C on C-2 of its single histidine residue, by nuclear magnetic resonance. They concluded that the interior proton (located between Asp-102 and His-57) shifts from His-57 to Asp-102 as the imidazole is protonated externally by a decrease in pH (through the pK_a of 6.7) or by a transfer of a proton from Ser-195 during acylation. This interior proton shift to Asp-102 (the ultimate base) leaves the imidazole neutral. Only below pH 4.5 would the imidazole acquire a positive charge by introduction of a second interior proton. The result of this hypothesis is to reverse the pK_a's normally assigned to Asp-102 and His-57.

Bachovchin & Roberts (1978) recently restudied this problem by using α-lytic protease enriched with ^{15}N in the imidazole group of its histidine. Their nuclear magnetic resonance study shows convincingly that the pK_a of the imidazole is 7.0. Consequently, the interior proton remains on His-57, and upon decreasing the pH or receiving a proton from Ser-195 during acylation, the imidazole becomes positively charged while Asp-102 remains as the anion down to its pK_a of about 4.5. Possible roles for the carboxylate anion of Asp-102 were thought to be orientation of the imidazole ring in its proper conformation and tautomer and stabilization of the imidazolium cation. At neutral pH the Asp-102:His-57 couple would remain as an integral unit and only be protonated on the exterior nitrogen of the imidazole.

Bachovchin & Roberts (1978) discussed the mechanistic implications of their findings in some detail. At the same time, they dismissed the possibility of activation of the serine hydroxyl group through hydrogen bonding on the basis that this concept, which involves what might be termed the problem of the exterior proton, is no longer supported by X-ray diffraction studies. Matthews et al. (1977; also see Kraut, 1977) had reexamined the X-ray coordinates of subtilisin, α- and γ-chymotrypsins, β-trypsin, and elastase and concluded that the hydroxyl oxygen (OG) of Ser-195 is too far from the exterior nitrogen (NE2) of His-57 for a hydrogen bond between the two atoms. This is the basis for their proposal that Ser-195 is not intrinsically a strong nucleophile but is reactive because it is precisely in the right place to react. The His-57:Asp-102 couple was assigned the role of transferring a proton from the serine (OG atom) to the leaving group of a substrate (amide nitrogen atom or ester oxygen atom) and was thus considered as a site for proton binding in the transition state of the reaction. Further support for this view was adduced from their previous work on tetrahedral boronic acid complexes of subtilisin, in which the exterior nitrogen of the imidazole is favorably situated for a hydrogen bond between it and the boronic acid oxygen atom that corresponds in position to that of the leaving group of a substrate (Matthews et al., 1975).

The essence of the exterior proton problem is not whether the imidazole plays a role in chymotrypsin-catalyzed reactions, since this concept is now universally accepted. The problem is reduced to a question of timing, namely, whether the imidazole of His-57 removes the proton from OG of Ser-195 before, during, or after its attack on the substrate. Matthews et al. (1977) cautioned against placing "too much reliance on details of the native structure when attempting to postulate mechanisms". In the same paper they speculated that the imidazole might undergo a "bistable flip-flop" between the

two oxygen atoms of the carbonyl group of Asp-102. Without taking a position on this, it seems quite reasonable to hypothesize that the OG of Ser-195 comes close enough to the external nitrogen of His-57 on binding of substrate to allow formation of a hydrogen bond. Subsequent proton removal might well be coordinated with the attack of the oxygen atom on the substrate. This concept is supported by X-ray crystallographic evidence that the hydrogen bond between His-57 and Ser-195 is "perfectly linear and of the expected length" in the complex of trypsin with pancreatic trypsin inhibitor, while it is "rather long" and "deviates appreciably from linearity" in trypsin itself (Bode et al., 1976).

Assignment of an exact mechanism for the chymotryptic hydrolysis of amides (and the reverse reaction, the aminolysis of acyl-enzyme) is difficult because of the numerous tetrahedral intermediates at varying stages and locations of protonation that can occur during the reaction (Satterthwait & Jencks, 1974; Gresser & Jencks, 1977). Alkylation of serine by compounds containing a CH_2Br group is much less complicated, since the reaction should proceed by a bimolecular nucleophilic displacement mechanism (S_N2). A pH dependence is diagnostic of such reactions, exemplified by the solvolysis of α-bromoacetic acid (Hughes & Taher, 1940; Ingold, 1969), and it corresponds here to the increasing concentration of the nucleophile (serine anion or its equivalent) with increasing pH. Since the pK_a of the reaction is about 7, it is difficult to explain the alkylation without aid of the imidazole of His-57. The most probable route at present is analogous to mechanism 2 of Satterthwait & Jencks (1974), namely, concerted general base catalysis by imidazole of the attack of OG of Ser-195 on the alkylating agent. It is not necessary to postulate a free serine alkoxide ion to account for the pronounced nucleophilicity of Ser-195, since its equivalent can be generated with the cooperation of His-57. This mechanism might well serve as the first step in the hydrolysis of amides and esters by chymotrypsin.

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