vestigated as well as Triton detergents for their capacity to antagonize the KCl inhibition of activity. In both series, detergents with an HLB number (see Umbreit and Strominger, 1973) of about 14–15 were active. Their activity was not as strong as that of similar detergents in the Triton series or Triton X-305 and Triton X-405 with higher HLB numbers. When Triton X-405 was used together with a less active Brij or Tween detergent, the stimulation of activity was less than when using Triton alone, which suggests that Brij and Tween detergents inhibit the Triton effect.

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Alkyl Isocyanates as Active Site-Specific Reagents for Serine Proteases. Location of Alkyl Binding Site in Chymotrypsin by X-Ray Diffraction†

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ABSTRACT: The structure of octylcarbamoyl-α-chymotrypsin to a resolution of 3.0 Å is described. The *n*-octyl side chain of the active site directed irreversible inactivator octyl isocyanate is bound exclusively in the hydrophobic substrate binding pocket. The *n*-octyl isocyanate forms a planar urethane bond with the Ser-195 O_γ and extends approximately 1 Å deeper into the hydrophobic pocket than the indolyl group of indoleacryloyl-α-chymotrypsin (Henderson, R. (1970), *J. Mol. Biol. 54,* 341). All the structural changes are essentially identical with those observed in indoleacryloyl-α-chymotrypsin including the observation of a hydrogen bonded water molecule between the carbonyl oxygen of the octylcarbamoyl group and the imidazole group of His-57. The observed mode of *n*-octyl alkyl binding to chymotrypsin is consistent with the hypothesis proposed earlier (Brown, W. E. and Wold, F. (1973), *Biochemistry 12,* 828).

The previous papers in this series (Brown and Wold, 1973a,b) have shown that octyl and butyl isocyanates are active site directed irreversible inactivators of chymotrypsin and elastase, respectively. Based on chemical data (Brown and Wold, 1973a), it was postulated that the inactivation reaction proceeded in a two-step manner. First, the enzyme binds the *n*-alkyl side chain of the isocyanate to form a noncovalent intermediate, analogous to an enzyme–substrate complex. This specific binding is followed by a covalent linkage of the isocyanate group to some functional group in the active site of the enzyme. In support of the second step, it was later shown that the modified functional group in each case was the active site serine, serine-195 in chymotrypsin and serine-188 in elastase (Brown and Wold, 1973b).

On the other hand, the first step of this reaction sequence is based on the assumption that the *n*-alkyl side chain of the

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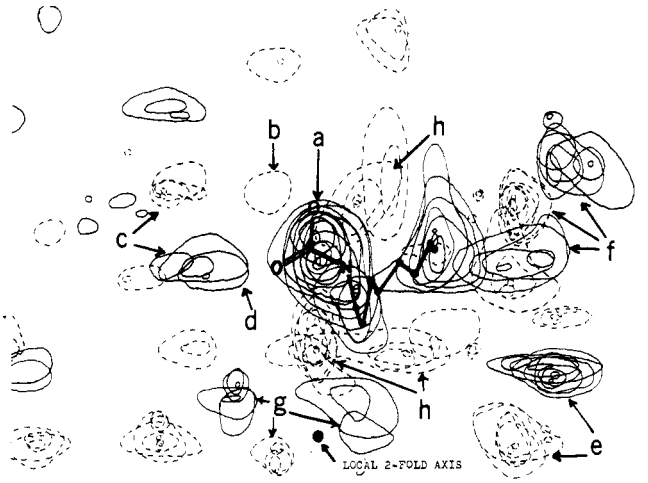

FIGURE 1: The difference in electron density between octylcarbamoyl-α-chymotrypsin and native chymotrypsin at 3.0-Å resolution. The view in this map is looking directly down the local twofold axis illustrated by the dot. The large density at feature (a) is due to the octylcarbamoyl group. The other features are discussed in the text.

Table I: Atomic Coordinates[a] for *n*-Octylcarbamoyl Group in Octylcarbamoyl-α-chymotrypsin.

Atom	X	Y	Z
Ser-195			
OG	16.0	0.2	7.3
Octylcarbamoyl group			
CD	16.0	0.3	6.0
OE	16.0	1.4	5.4
NE	16.0	−0.9	5.4
C1	16.0	−1.2	4.1
C2	17.4	−1.4	3.6
C3	18.6	−1.7	5.0
C4	19.6	−1.9	4.6
C5	20.3	−2.6	5.6
C6	21.7	−3.1	5.2
C7	22.6	−3.6	6.2
C8	23.9	−3.7	6.0
Water[b]			
O	14.0	3.6	5.1

[a] X, Y, Z are measured in Å units according to the Cartesian coordinate system defined by Birktoft et al. (1969). [b] Water molecule hydrogen-bonded between His-57 and carbamoyl group.

isocyanates bind specifically in the hydrophobic binding pockets of the two enzymes. Contrary to this assumption is the appearance of additional binding sites in the α-chymotrypsin crystal which accommodate oversize substrates and still allow substrate attachment to serine-195 (Steitz et al., 1969; Sigler et al., 1966; R. Henderson, personal communication). Furthermore, studies with *n*-alkylboronic acids (Antonov et al., 1970) and with *N*-acetyl-L-amino acid esters with *n*-alkyl side chains (Jones et al., 1965) predict that an *n*-octyl alkyl side chain should be too large for the substrate binding pocket of chymotrypsin. The purpose of the present single crystal X-ray diffraction study is to justify the original *n*-alkyl side chain binding assumption by demonstrating that the *n*-octyl side chain of octyl isocyanate does bind specifically in the tosyl hole of α-chymotrypsin.

Experimental Section

Crystallization α-Chymotrypsin was obtained from Worthington Biochemicals and further purified on CM-Sephadex C-50 by the method of Nakagawa and Bender (1970). Native α-chymotrypsin crystals were grown by the procedure of Sigler et al. (1966), in the presence of 2% dioxane. Prior to use the crystals were washed free of dioxane with 65% ammonium sulfate buffered at pH 4.0 with citrate. Octylcarbamoyl-chymotrypsin crystals were prepared by inactivating the enzyme in solution prior to crystallization. This procedure was required because of rapid isocyanate hydrolysis in aqueous solution and because the isocyanate will not react with chymotrypsin below pH 6. An aliquot of α-chymotrypsin was 99+% inactivated by octyl isocyanate (K&K Labs) according to the procedure described previously (Brown and Wold, 1973a), dialyzed to remove excess reagent, and lyophilized. Crystals of the inactivated chymotrypsin were grown following the same procedure used for native chymotrypsin, however, in the absence of dioxane. The crystals of octylcarbamoyl-chymotrypsin were isomorphous with the native enzyme; however, they grew as large flat plates with a thickness of 0.1–0.3 mm. These crystals were cut to an appropriate size for use on the diffractometer.

X-Ray Analysis. All of the three-dimensional 3-Å data were measured on a modified Picker diffractometer which was interfaced to a PDP/8 computer by Dr. H. W. Wyckoff. Peak intensities were measured by scanning the highest four measurements of a limited ω step scan (Wyckoff et al., 1967) which was dynamically controlled (H. W. Wyckoff, unpublished observations). Background was measured at 1°C intervals in 2θ at nonintegral lattice positions and estimated for each reflection assuming a radial distribution of background. Standard reflections were allowed to decrease by 10% before a crystal was discarded. Each independent reflection in the 3-Å sphere was measured four times, and symmetry related reflections were merged after Lorentz-polarization and absorption corrections were applied (North et al., 1968).

The native chymotrypsin data set was composed of eight sequential data sets with symmetry *R* factors (R_{sym})[1] ranging from 0.019 to 0.039 with an average of 0.029. The octylcarbamoyl-chymotrypsin data set was composed of eight sequential data sets with symmetry *R* factors ranging from 0.028 to 0.043 with an average of 0.037. The difference Fourier technique was used to determine the difference in structure between octylcarbamoyl- and native α-chymotrypsin and was computed using the phases of tosyl-α-chymotrypsin which were kindly provided by Dr. D. M. Blow. The final difference Fourier synthesis contained 8100 independent terms to a resolution of 3 Å with an R_f[2] of 0.16.

Results

The active site region of the difference Fourier map between octylcarbamoyl- and native chymotrypsin is shown in Figure 1. This is not a symmetry averaged map; however, all significant features appear in symmetry related molecules with variations in intensities from molecule to molecule as previously reported (Birktoft and Blow, 1972; Tulinsky et al., 1973). The view in Figure 1 is down the local twofold axis through 9 Å of the molecule from *Z* equals 13 to 22 Å. All significant features are lettered and will be discussed later. Other unlettered contours represent only one contour per map section and were not considered significant

[1] $R_{sym} = 2\Sigma|F_+ - F_-|/\Sigma|F_+ + F_-|$.

[2] $R_f = \Sigma||F_{der}| - |F_{nat}||/\Sigma F_{nat}$.

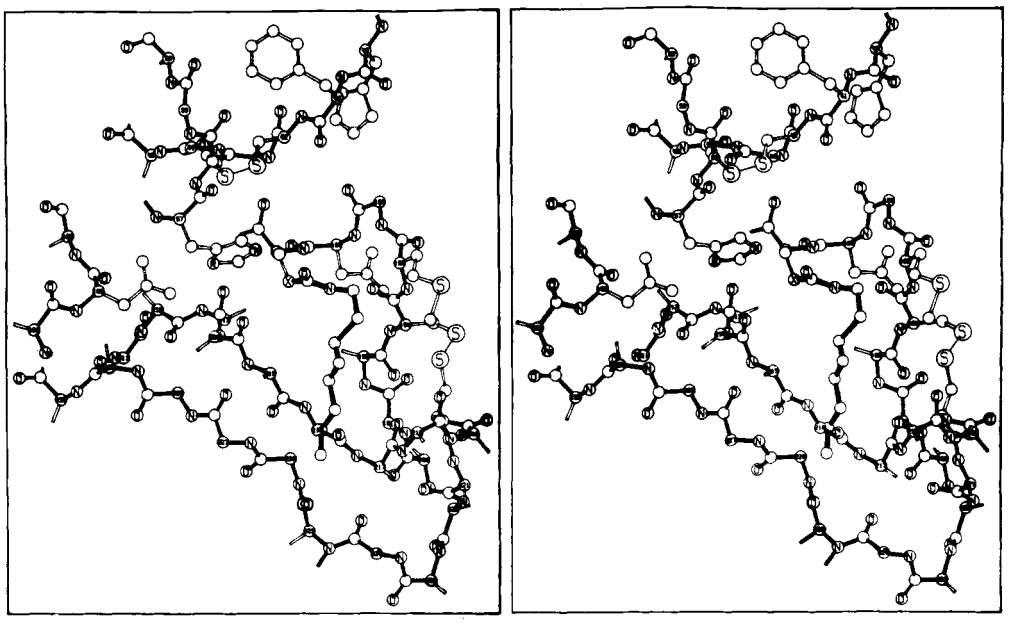

FIGURE 2: Stereo drawing of the active site of octylcarbamoyl-α-chymotrypsin corresponding to the interpretation of the difference map shown in Figure 1. The view is approximately at right angles to the local twofold axis shown in Figure 1.

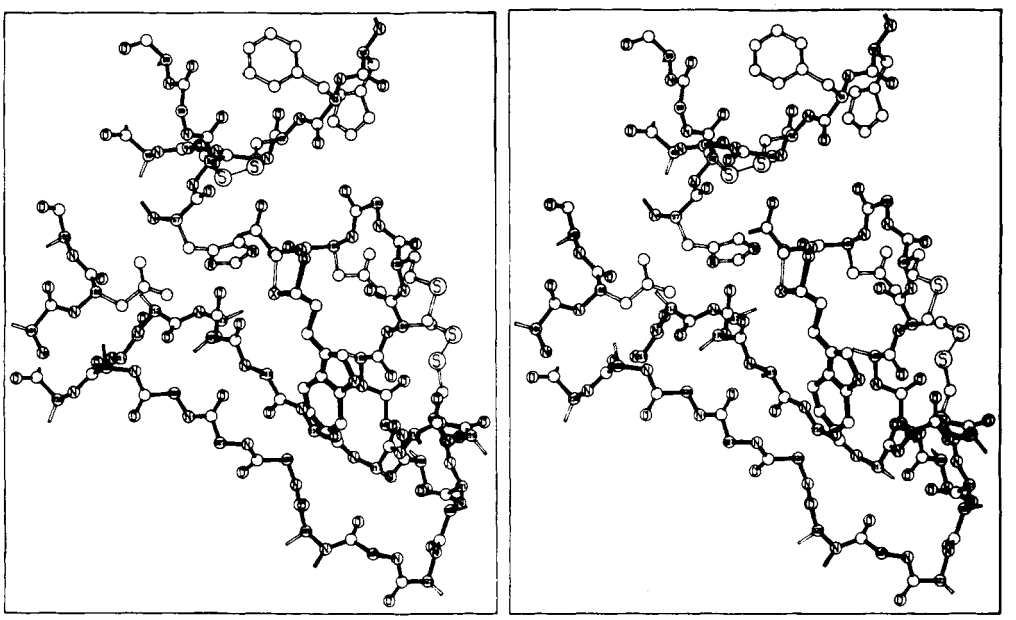

FIGURE 3: Stereo drawing of the active site of indoleacryloyl-α-chymotrypsin. The coordinates used for the drawing are from the work of Henderson (1970) and the view is exactly the same as that given in Figure 2.

at 3-Å resolution. Apart from the region of the map shown in Figure 1, there are no other significant peaks above background level on the remainder of the map.

Figure 2 is a stereo drawing of the active site of octylcarbamoyl-chymotrypsin using the coordinates of Birktoft and Blow (1972) and coordinates for the octylcarbamoyl group derived from the difference map. The coordinates for the octylcarbamoyl group (Table I) were estimated from a model which was optically fitted to the 3-Å difference map. For comparative purposes, the structure of indoleacryloyl-α-chymotrypsin (Henderson, 1970) is shown in Figure 3. All of the structural changes resulting from the modifications of chymotrypsin by octyl isocyanate have also been seen in the 2.5-Å resolution structure of indoleacryloyl-α-chymotrypsin. The main features of the difference Fourier are discussed below and are identified by corresponding letters on the map in Figure 1. Estimates given for specific residue shifts are those described for indoleacryloyl-α-chymotrypsin (Henderson, 1970) and were estimated by observing the superposition of these residues on a difference map between octylcarbamoyl- and indoleacryloyl-α-chymotrypsins.

(a) The largest density, five times the background density in the map, is due to octyl isocyanate which is attached to serine-195. The line overlay shows the best fit of the octylcarbamoyl molecule to the density. The urethane bond between the isocyano group and the Ser-195 O_γ fits as a planar bond as seen in previous studies on urethanes (Bracher and Small, 1967; J. J. Beres and L. E. Alexander, unpublished data, 1974). The broad density corresponding to the tail of the alkyl chain probably indicates an averaging of

density due to free movement of the last four methylene groups in the octyl side chain. The gap in the density at carbons 3 and 4 of the alkyl chain indicates the position of displaced ordered water. This type of density cancellation was noted previously in the structure of formyl-L-tryptophan-chymotrypsin (Steitz et al., 1969) and can be attributed to the close equality in electron density of a water and a methylene group.

(b) This weak negative peak at 3-Å resolution appears in only one of the symmetry related molecules and has an approximate center of gravity at (14.0, 1.5, 7.3).[3] Although not a strong negative peak, its position is in good agreement with the mean position reported for serine-195 O_γ in native chymotrypsin (14.0, 1.8, 7.3) (Birktoft and Blow, 1972). This indicates that the O_γ has moved in a manner similar to that seen in indoleacryloyl- and tosyl-chymotrypsins (Henderson, 1970) and carbamyl-chymotrypsin (Robillard et al., 1972). The new position is illustrated in Figure 1a, and has the approximate coordinates given in Table I.

(c) This positive and associated negative density indicates the movement of the His-57 approximately 0.3 Å toward the solvent area.

(d) The elongation of the His-57 density is attributed to a water molecule hydrogen bonded between $N^{\epsilon 2}$ of His-57 and the carbonyl oxygen O^{ϵ} of the isocyanate group on Ser-195. An electron density map of the octylcarbamoyl structure, produced by using the phases for tosyl-chymotrypsin and subtracting the native structure factor amplitudes $|F_{nat}|$ from twice the octylcarbamoyl structure factor amplitudes $|F_{octyl}|$, shows a definite connective intensity between the two atoms. The center of gravity of the water molecule is given in Table I and agrees nicely with the position of water molecule W2 (13.6, 3.4, 4.7) (Birktoft and Blow, 1972) which is hydrogen bonded between $N^{\epsilon 2}$ of His-57 and $O^{\epsilon 1}$ of the tosyl group on serine-195 in tosyl-chymotrypsin. This similarity in location is not surprising since the position of the tosyl $O^{\epsilon 1}$ (15.9, 1.2, 4.7) (Birktoft and Blow, 1972) in tosyl-chymotrypsin is very close to that of O^{ϵ} (Table I) which is the proposed hydrogen bonding acceptor for the water molecule in octylcarbamoyl-chymotrypsin.

(e) There is a shift in the disulfide bridge between residues 191 and 220. This shift is toward the substrate binding pocket by approximately 0.3–0.4 Å.

(f) A number of positive and negative peaks in this area are due to the backbone chain shifting to accommodate the disulfide movement. At this point there is no evidence to indicate the cause of these movements. Model fitting to the difference map and derivative electron density map indicates that the major changes in chymotrypsin are due to tightening of the main chain from residues 189 to 191 about the substrate. These changes are also noted in tosyl- and indoleacryloyl-chymotrypsin but not in studies of reversibly bound substrates and inhibitors (Steitz et al., 1969).

(g) A number of weak positive and negative peaks grouped around the local twofold axis indicate some slight movement of Met-192 but nothing definitive at 3-Å resolution.

(h) The negative peaks around the octylcarbamoyl group are actually located at the bottom of the tosyl hole and may indicate the position of water molecules which are displaced by the octylcarbamoyl binding. They have no apparent related positive peaks which would indicate a residue shift.

[3] Coordinates are given in ångstrom units according to the Cartesian coordinate system defined by Birktoft et al. (1969).

Discussion

This X-ray diffraction study has shown that octyl isocyanate binds exclusively in the substrate binding pocket of chymotrypsin in what must be considered a "productive binding" since it results in covalent attachment to serine-195. Having shown the octyl side chain to be in the substrate binding pocket, it is of interest to discuss this result in terms of how the active site accommodates a molecule which should be too large (long) and in terms of the stability of the product octylcarbamoyl-chymotrypsin.

The high affinity of chymotrypsin for *n*-alkyl substrates has been demonstrated by Jones et al. (1965) using *N*-acetyl-α-amino acid methyl esters with variable length *n*-alkyl side chains. They showed that as the alkyl chain length increased to C_6 the affinity of the substrate for chymotrypsin became higher than the natural substrate *N*-acetylphenylalanine methyl ester but that the binding was much more "nonproductive". Later, Antonov et al. (1970) introduced *n*-alkylboronic acids as inhibitors of chymotrypsin and showed that their effectiveness as inhibitors increased as the alkyl chain length increased, with a maximum inhibition constant associated with a *n*-hexyl side chain. At the same time, Berezin et al. (1970) showed that the same relationship holds true for aliphatic alcohols with increasing alkyl chain length from C_1 to C_7. Based on these data, it is not surprising that the alkyl isocyanates are highly specific active site directed inactivators of chymotrypsin. But what is surprising is the fact that the *n*-octyl side chain binds in a "productive" manner, that is, it properly aligns the isocyanate reactive group with the serine-195 hydroxyl. Extensive studies have been done to map the topography of the active site of chymotrypsin and particularly the S_1 subsite (notation of Schecter and Berger, 1967) or "tosyl" hole of chymotrypsin. An excellent discussion and summary of these data is presented by Bosshard and Berger (1974). To summarize their discussion, they conclude that the hydrophobic S_1 site of chymotrypsin is rigidly defined and that the binding of many large virtual substrates although of high binding energy (e.g., *p*-iodo-*N*-acetyltyrosine ethyl ester (Garratt and Harrison, 1970) and *O*-methyl-*N*-acetyltyrosine methyl ester (Kunda et al., 1972)) "may lead to some distortion or misfit in the substrate–enzyme interactions at the catalytic locus which, partially or completely, abolishes catalysis." Since the *n*-octyl side chain of octyl isocyanate is longer than the iodotyrosine side chain, approximately as long as a methyl ether derivative, yet binds in a productive (inhibitory) manner to the active site of chymotrypsin; the data presented here define a deeper pocket than previously observed (Steitz et al., 1969). By comparison to indoleacryloyl binding which is directed toward the "bottom" of the pocket at residue 189 (Figure 3), octyl isocyanate binds deeper by approximately 1 Å into the hydrophobic pocket and is directed more toward the middle of the protein and residue 226. The nearly linear alkyl chain extends out toward Met-192 and with rotation about the $N-C_\alpha$ bond, the isocyanate forms the planar urethane link with the Ser-195 O_γ. Based on model-building studies using data presented in the present paper, the limiting binding efficiency should be with *n*-alkyl chains of 8–9 carbons. Chains of greater length would bind with equal efficiency but would extend into the solvent area beyond the active site in a nonproductive manner as proposed for *n*-octyl isocyanate binding to elastase (Brown and Wold, 1973a). Thus specific inactivation by alkyl isocyanates is limited by the length of

the alkyl side chain whose binding dictates the alignment of the reagent reactive group with an active site functional group.

With the confirmation of the specific alkyl binding in the substrate binding pocket of chymotrypsin, it appears that the alkyl isocyanate inactivation mechanism parallels the normal acylation of the enzyme and thus octyl isocyanate is an inactivator because it cannot undergo deacylation. By comparison to other inactivators of chymotrypsin which modify serine-195, this is not surprising for two reasons. First, the results of this study show that the carbonyl oxygen of the isocyano group is directed similar to the $O^{\epsilon 1}$ of the diisopropylphosphoryl group of diisopropylphosphoryl-chymotrypsin and according to the arguments of Robillard et al. (1972) and Henderson (1970), the p orbitals of the carbonyl carbon are not accessible to overlap with the orbitals of the water nucleophile used during deacylation. This is further borne out by the resistance of the octyl carbamoyl group to displacement by strong nucleophiles (Brown, 1971). Although cleavage of a diphenyl carbamoyl (urethane) link to serine-195 has been accomplished by a strong nucleophilic reagent (Erlanger and Cohen, 1963), studies of phosphoryl derivatives have shown that large variations in the susceptibility of the serine–acyl bond to nucleophilic attack exist when the side chains attached to the phosphoryl group are varied (Wilson, 1959; Cohen and Erlanger, 1960).

The second consideration is the natural stability of compounds containing urethane links. As demonstrated in the earlier work with alkyl isocyanates (Brown and Wold, 1973b), with cyanate (Shaw et al., 1964), and with *p*-nitrophenyl cyanate (Robillard et al., 1972), the urethane link to the serine hydroxyl is stable to all normal protein and peptide isolation techniques including performic acid oxidation. This is not surprising since urethanes are known to be very stable compounds with melting points in the range of 50–100°C and boiling points of approximately 200°C. Only at higher temperatures do they decompose to the respective isocyanate and alcohol (Adams and Baron, 1965). Consistent with the protein studies cited above is the finding of Mukaiyama and Hoshino (1956) that decomposition of urethanes at elevated temperatures is catalyzed by both acid and base. All this is in contrast to the normal chymotrypsin substrates which readily undergo deacylation. In those cases the acyl–ester bonds deacylate both in denatured acyl-chymotrypsin and in *O*-acylserine model compounds with mild general base catalysis (Bender et al., 1962; Anderson et al., 1961).

Finally, how can the findings in this study be extrapolated to predict the location of the butyl side chain in butylcarbamoyl-elastase? Atlas (1975) and Thompson (1974) have shown that there are a number of subsites used during peptide binding which would be suitable for binding of short aliphatic carbon chains. This is consistent with the finding that butyl isocyanate is less specific toward elastase (i.e., has more specific "nonproductive" binding sites available) than octyl isocyanate is toward chymotrypsin (Brown and Wold, 1973a). However, the binding which results in inactivation must be within "reach" of the active site serine-188 since the inactivation reaction successfully competes with the reagent hydrolysis in aqueous media (Brown and Wold, 1973a). Therefore, the probable sites of binding are either the S_1 or S_1' subsites since they are adjacent to serine-188 which is acylated in elastase. Because of the homology in the structures of chymotrypsin and elastase (Shotton and Watson, 1970) and by analogy with the structure of octyl-carbamoyl-chymotrypsin, the most probable binding site in elastase is S_1 which binds the amino acid side chain (P_1) on the carboxyl side of the scissle bond.

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Effect of pH on Substrate and Inhibitor Kinetic Constants of Human Liver Alanine Aminopeptidase. Evidence for Two Ionizable Active Center Groups†

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ABSTRACT: The presence of at least two ionizable active center groups has been detected by a study of the effect of pH upon catalysis of hydrolysis of L-alanyl-β-naphthylamide by human liver alanine aminopeptidase and upon the inhibition of hydrolysis by inhibitors and substrate analogs. Octanoic acid, octylamine, and peptide inhibitors have been found to be competitive inhibitors and are therefore thought to bind the active center. L-Phe was previously shown to bind the active center since it was found to be a competitive inhibitor of the hydrolysis of tripeptide substrates (Garner, C. W., and Behal, F. J. (1975), *Biochemistry 14,* 3208). A plot of pK_m vs. pH for the substrate L-Ala-β-naphthylamide showed that binding decreased below pH 5.9 and above 7.5, the points at which the theoretical curve undergoes an integral change in slope. These points are interpreted as the pK_a either of substrate ionizable groups or binding-dependent enzyme active center groups. Similar plots of pK_m vs. pH for L-alanyl-*p*-nitroanilide (as substrate) and pK_i vs. pH for L-Leu-L-Leu-L-Leu and D-Leu-L-Tyr (as inhibitors) gave pairs of pK_a values of 5.8 and 7.4, 6.0 and 7.5, and 5.7 and 7.5, respectively. All the above substrates (and D-Leu-L-Tyr) have pK_a values near 7.5; therefore, the binding-dependent group with a pK_a value near 7.5 is possibly this substrate group. Similar plots of pK_i vs. pH for the inhibitors L-Phe, L-Met, L-Leu, octylamine, and octanoic acid had only one bending point at 7.7, 7.6, 7.4, 6.3, and 5.9, respectively. Amino acid inhibitors, octylamine, and octanoic acid have no groups with pK_a values between 5 and 9. These data indicate that there are two active center ionizable groups with pK_a values of approximately 6.0 and 7.5 which are involved in substrate binding or inhibitory amino acid binding but not in catalysis since V_{max} was constant at all pH values tested.

Human liver alanine aminopeptidase is an important member of that class of aminopeptidases which act upon peptides as well as chromogenic substrates like aminoacyl-β-naphthylamides. This enzyme cleaves those aminoacyl-β-naphthylamides having nonpolar side chains. L-Alanyl-β-naphthylamide is the substrate most rapidly hydrolyzed. The enzyme has been purified and some of its properties have been reported (Little, 1970; Little and Behal, 1971; Starnes and Behal, 1974; Garner and Behal, 1974). The enzyme contains 17.5% carbohydrate and has a monomeric molecular weight of 118000. One atom of zinc is present per monomer.

Attempts to identify active center residues of this aminopeptidase have recently been initiated in an effort to understand its mechanism of action. Similar studies have been performed with other aminopeptidases. Through a study of the kinetics of hydrolysis and chemical modifications, a cooperative tyrosine–histidine system has been proposed for aminopeptidase M (Pfleiderer and Femfert, 1969; Femfert and Pfleiderer, 1969; Femfert, 1971; Femfert et al., 1972). A hydrophobic region was also proposed (Femfert and Cochocki, 1974). Cysteine and histidine have been proposed as active center residues in aminopeptidase B (Makinen and Hopsu-Havu, 1967a,b).

Since several inhibitors and substrates of the human liver enzyme are available, a study of the effect of pH on binding was made to detect and possibly identify active center ionizable groups. Two enzyme ionizable groups are indicated with pK_a values of 6.0 and 7.5.

Experimental Section

Materials. Human liver alanine aminopeptidase was prepared by a procedure described previously (Garner and Behal, 1975). The preparations used were greater than 92% pure by polyacrylamide gel electrophoresis. Peptides, substrates, and inhibitors were obtained from ICN, Sigma, or Aldrich as the highest purity available and were used without further purification. All were shown to be homogeneous on an amino acid analyzer or by thin-layer chromatography (TLC). Water was doubly deionized on a mixed-bed ion exchange column before use. Buffers varying in pH from 5.0 to 9.5 were prepared from a mixture of potassium phosphate, maleic acid, and boric acid (0.1 *M* each). The pH

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