^{13}C NMR Study of How the Oxyanion pK_a Values of Subtilisin and Chymotrypsin Tetrahedral Adducts Are Affected by Different Amino Acid Residues Binding in Enzyme Subsites S_1-S_4[†,‡]

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ABSTRACT: A range of substrate-derived chloromethane inhibitors have been synthesized which have one to four amino acid residues. These have been used to inhibit both subtilisin and chymotrypsin. Using ^{13}C NMR, we have shown that all except one of these inhibitors forms a tetrahedral adduct with chymotrypsin, subtilisin, and trypsin. From the pH-dependent changes in the chemical shift of the hemiketal carbon of the tetrahedral adduct, we are able to determine the oxyanion pK_a in the different inhibitor derivatives. Our results suggest that in both the subtilisin and chymotrypsin chloromethane derivatives the oxyanion pK_a is largely determined by the type of amino acid residue occupying the S_1, subsite while binding in the S_2-S_4 subsites only has minor effects on oxyanion pK_a values. Using free energy relationships, we determine that the different R groups of the amino acid residues binding in the S_1 subsite only have minor effects on the oxyanion pK_a values. We propose that the lower polarity of the chymotrypsin active site relative to that of the subtilisin active site explains why the oxyanion pK_a is higher and more sensitive to the type of chloromethane inhibitor used in the chymotrypsin derivatives than in the subtilisin derivatives.

Specific substrate-derived chloromethane inhibitors of trypsin (*1*, *2*), chymotrypsin (*3*, *4*), and subtilisin (*5*−*7*) alkylate N-3 of the imidazole ring of the active site histidine residue in all these enzymes. Using ^{13}C NMR, it has been shown that in trypsin− (*8*−*12*), chymotrypsin− (*13*−*16*), and subtilisin−chloromethane inhibitor complexes (*13*−*18*) the hydroxy group of the active site serine adds to the inhibitor to form a tetrahedral adduct analogous to the tetrahedral intermediate thought to be formed during catalysis by the serine proteases. Through determination of the pH-dependent changes in the chemical shifts of the α-methylene and hemiketal carbon of these tetrahedral adducts, it has been shown that the pK_a of the oxyanion is lowered while the pK_a of the imidazolium cation is increased in these derivatives (*9*, *10*, *13*−*19*). From these studies, it has been concluded that the serine proteases have evolved to stabilize zwitterionic tetrahedral intermediates and that this stabilization is required for optimal catalytic efficiency (*9*, *13*−*15*, *17*). ^{1}H NMR and ^{15}N NMR have also been used to determine oxyanion pK_a values in chymotrypsin and α-lytic protease chloromethane inhibitor derivatives (*20*). Using site-directed mutagenesis, it has been shown that hydrogen bonding in the oxyanion hole makes a significant contribution to the oxyanion pK_a which is equivalent to that expected in aqueous solvents where it hydrogen bonds to water (*18*). This led to the conclusion that it is the interaction between the oxyanion and the imidazolium cation that is the main factor causing the lowering of the oxyanion pK_a relative to its value in water (*18*).

In this work, we synthesize a range of substrate-derived chloromethane inhibitors. We estimate how changing the structure of the chloromethane inhibitor will effect the oxyanion pK_a. As both chymotrypsin and subtilisin have extended active sites capable of binding polypeptides, we have also determined how changing the amino acid residues which bind in the S_1-S_4 subsites of subtilisin and chymotrypsin can affect the oxyanion pK_a values in their chloromethane derivatives.

MATERIALS AND METHODS

The materials and methods for preparing Z-[2-^{13}C]Phe-CH_2-chymotrypsin,[1] Z-Gly-Gly-[2-^{13}C]Phe-CH_2-chymotrypsin, and Z-Gly-Gly-[2-^{13}C]Phe-CH_2-subtilisin BPN′ were like those described previously (*15*). L-Alanyl-glycyl-glycine

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‡ J.P.G.M. dedicates this paper to Neil Mackenzie who died recently. He was a good scientist and friend who helped pioneer these studies by synthesising the first ^{13}C-enriched chloromethane inhibitor which we used in our studies on trypsin.

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[1] Abbreviations: Z, benzyloxycarbonyl; Tos, tosyl.

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and *N*-α-benzyloxycarbonyl-glycyl-L-alanine were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

L-[1-^{13}C]Phenylalanine (99 at. %) and L-[1-^{13}C]leucine (99 at. %) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Some L-[1-^{13}C]phenylalanine (99 at. %) was also obtained from MSD Isotopes (U.K. agents, Cambrian Gases, Croydon, Surrey, U.K.). All other chemicals used were obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.).

Synthesis of [1-^{13}C]Serine and [1-^{13}C]Tryptophan. The conversion of [1-^{13}C]glycine to [1-^{13}C]serine was catalyzed by serine hydroxymethyltransferase, and the conversion of [1-^{13}C]serine to [1-^{13}C]tryptophan was catalyzed by tryptophan synthase (*21*, *22*).

Synthesis of Z-Tryptophan, Z-Leucine, Z-[1-^{13}C]Leucine, and Z-[1-^{13}C]Tryptophan. L-Tryptophan and L-leucine were converted into Z-tryptophan and Z-leucine, respectively, using the Schotten Baumann procedures (*23*, *24*) as described by Greenstein and Winitz (*25*).

White crystals of Z-tryptophan were produced: 77% yield; mp 122−129 °C; ^{1}H NMR ([^{2}H$_6$]DMSO) δ 2.8−3.5 (2H, m, $C_8H_6NCH_2CH$), 4.0−4.5 (1H, m, $C_6H_5CH_2CH$), 5.0 (2H, s, $C_6H_5CH_2O$), 6.7−7.8 (11H, broad m, C_6H_5 and C_8H_6N); ^{13}C NMR ([^{2}H$_6$]DMSO) δ 27.1 ($C_6H_5CH_2CH$), 55.2 ($C_6H_5CH_2CH$), 65.5 ($C_6H_5CH_2O$), 110.2−137.2 (10C, *C*H=*C*H; 4C, CH=*C*=), 156.2 (O-*C*-O-NH), 173.9 (*C*OOH). Anal. Calcd for $C_{19}H_{18}N_2O_4$: C, 67.4; H, 5.4; N, 8.3. Found: C, 67.2; H, 5.3; N, 8.2. The ^{13}C NMR spectrum of Z-[1-^{13}C]-tryptophan in [^{2}H$_6$]DMSO contained a single signal at 173.4 ppm due to the ^{13}C-enriched carbon atom.

The Z-leucine produced was a light yellow oil: 74% yield; ^{13}C NMR ([^{2}H]chloroform) δ 21.6, 22.7 (2C,*C*H$_3$), 24.7 [$CH_2CH(CH_3)_2$], 41.3 [$(CH_3)_2CHCH_2$], 52.4 [$(CH_3)_2CHCH_2CH$], 67.1 ($C_6H_5CH_2O$), 127.9−136.1 (5C, *C*H=*C*H; 1C, CH=*C*=), 156.2 (O-*C*O-NH), 177.6 (*C*OOH). Anal. Calcd for $C_{14}H_{19}NO_4$: C, 63.4; H, 7.2; N, 5.3. Found: C, 61.0; H, 7.2; N, 4.9. The ^{13}C NMR spectrum of Z-[1-^{13}C]leucine in [^{2}H$_6$]chloroform contained a single signal at 177.6 ppm due to the ^{13}C-enriched carbon atom. Both ^{1}H and ^{13}C chemical shifts are quoted relative to tetramethylsilane at 0.00 ppm (see NMR Spectroscopy for further details).

Synthesis of Z-Trp-CH$_2$Cl, Z-Leu-CH$_2$Cl, Z-[2-^{13}C]Leu-CH$_2$Cl, and Z-[2-^{13}C]Trp-CH$_2$Cl. Z-Tryptophan and Z-leucine were converted to Z-Trp-CH_2Cl and Z-Leu-CH_2Cl, respectively, using diazomethane and the mixed anhydride method described by Coggins et al. (*2*).

This procedure provided white crystals of Z-Trp-CH_2Cl: 72% yield; mp 139−143 °C; ^{1}H NMR ([^{2}H$_6$]acetone) δ 3.1−3.4 (2H, m, $C_8H_6NCH_2CH$), 4.7, 4.2−4.7 (3H, overlapping q, J = 7.2 Hz and AB, from $C_8H_6NCH_2CH$ and CH_2Cl, respectively), 5.1 (2H, s, $C_6H_5CH_2O$), 6.7−7.8 (11H, overlapping m, C_6H_5 and C_8H_6N); ^{13}C NMR ([^{2}H$_6$]acetone) δ 27.3 ($C_6H_5CH_2CH$), 48.6 (CH_2Cl), 59.7 ($C_6H_5CH_2CH$), 67.0 ($C_6H_5CH_2O$), 112.2−137.9 (10C, *C*H=*C*H; 4C, CH=*C*=), 157.0 (O-*C*-O-NH), 201.7 (*C*OCH_2Cl). Anal. Calcd for $C_{20}H_{19}ClN_2O_3$: C, 64.8; H, 5.2; Cl, 9.6; N, 7.6. Found: C, 64.8; H, 5.2; Cl, 9.3; N, 7.5. The ^{13}C NMR spectrum of Z-[2-^{13}C]Trp-CH_2Cl in [^{2}H$_6$]acetone contained a single signal at 201.7 ppm due to the ^{13}C-enriched carbon atom.

With Z-Leu, a light yellow oil was produced which was a mixture of ∼20% Z-Leu and ∼80% Z-Leu-CH_2Cl. This was used without further purification. For Z-Leu-CH_2Cl: ^{13}C NMR ([^{2}H]chloroform) δ 21.4, 23.1 (2C,*C*H$_3$), 24.8 [$(CH_3)_2CH$], 56.2 [$(CH_3)_2CHCH_2CH$], 46.5 (CH_2Cl), 40.3 [$(CH_3)_2CHCH_2$], 67.2 ($C_6H_5CH_2O$), 128.0−135.9 (5C,*C*H=*C*H; 1C, CH=*C*=), 156.1 (O-*C*O-NH), 201.9 (*C*OCH_2Cl). The ^{13}C-enriched carbon of Z-[2-^{13}C]Leu-CH_2Cl in [H_6]chloroform gave a signal at 201.8 ppm.

Synthesis of Z-Gly-Ala-Phe-CH$_2$Cl and Z-Gly-Ala-[2-^{13}C]-Phe-CH$_2$Cl. Z-Phe-CH_2Cl was converted into Phe-CH_2Cl using hydrogen bromide as described by Segal et al. (*26*). Z-Gly-Ala-Phe-CH_2Cl was prepared from Phe-CH_2Cl and Z-glycylalanyl using the mixed anhydride method (*2*). White crystals were produced: 76% yield; mp 121−123 °C; ^{1}H NMR ([^{2}H$_6$]DMSO) δ 1.1 [3H, d (J = 7.4 Hz), CH-CH_3], 2.5−3.4 (2H, broad, $C_6H_5CH_2CH$), 3.6−4.3 [2H, d (J = 5.6 Hz), $COCH_2$-NH], 4.1−4.8 (3H, overlapping m, q, and AB, from $C_6H_5CH_2CH$, CH_3CH, and CH_2Cl, respectively), 5.0 (2H, s, $C_6H_5CH_2O$), 7.2, 7.4 (10H, 2 broad s, $2C_6H_5$), 8.0−8.7 (3H, m, N*H*); ^{13}C NMR ([^{2}H$_6$]DMSO) δ 17.8 (CH_3), 34.9 ($C_6H_5CH_2CH$), 43.6 (CO-CH_2-NH), 48.1 (CH_2Cl), 48.5 (CH_3CH), 57.9 ($C_6H_5CH_2CH$), 65.7 ($C_6H_5CH_2O$), 126.5−129.3 (10C, *C*H=*C*H), 137.1, 137.5 (2C, CH=*C*=), 156.7 (O-*C*-O-NH), 169.2 (CH_2-*C*-O-NH), 172.8 (CH-*C*-O-NH), 200.3 (*C*OCH_2Cl). Anal. Calcd for $C_{22}H_{26}ClN_3O_5$: C, 59.0; H, 5.9; Cl, 7.9; N, 9.4. Found: C, 58.6; H, 5.7; Cl, 7.3; N, 9.2. The ^{13}C NMR spectrum of Z-Gly-Ala-[2-^{13}C]Phe-CH_2Cl in [^{2}H$_6$]DMSO contained a single signal at 200.3 ppm due to the ^{13}C-enriched carbon atom.

Synthesis of Formyl-Ala-Gly-Gly. Formyl-L-alanyl-glycyl-glycine was synthesized from formic acid and L-alanyl-glycyl-glycine (*27*). The crude product was used without further purification.

Synthesis of Formyl-Ala-Gly-Gly-Phe-CH$_2$Cl and Formyl-Ala-Gly-Gly-[2-^{13}C]Phe-CH$_2$Cl. Phe-CH_2Cl was coupled to formyl-Ala-Gly-Gly using the mixed anhydride method (*2*). White crystals of formyl-Ala-Gly-Gly-Phe-CH_2Cl were produced: 70% yield; mp 158−161 °C; ^{1}H NMR ([^{2}H$_6$]-DMSO) δ 1.1 [3H, d (J = 7.4 Hz), CH-CH_3], 2.5−3.4 (2H, m, $C_6H_5CH_2CH$), 3.6, 3.7 [4H, d (J = 5.6 Hz), $2COCH_2$-NH], 4.0−4.8 (3H, overlapping m, q, and AB, from $C_6H_5CH_2CH$, CH_3CH, and CH_2Cl, respectively), 7.2 (5H, 1 broad s, C_6H_5), 8.0−8.4 (5H, m, 4N*H* and 1*H*CO-NH); ^{13}C NMR ([^{2}H$_6$]DMSO) δ 18.2 (CH_3), 34.8 ($C_6H_5CH_2CH$), 41.6, 41.7 (2C, CO-CH_2-NH), 48.1 (CH_2Cl), 46.9 (CH_3CH), 57.9 ($C_6H_5CH_2CH$), 126.4−137.3 (5C, *C*H=*C*H), 137.3 (CH=*C*=), 160.9 (H-*C*O-NH), 169.1, 169.2 (2C, CH_2-*C*O-NH), 172.3 (CH-*C*O-NH), 200.2 (*C*OCH_2Cl). Anal. Calcd for $C_{18}H_{23}ClN_4O_5$: C, 52.6; H, 5.7; Cl, 8.6; N, 13.6. Found: C, 52.5; H, 5.7; Cl, 8.8; N, 13.3. The ^{13}C NMR spectrum of formyl-Ala-Gly-Gly-[2-^{13}C]Phe-CH_2Cl in [^{2}H$_6$]DMSO contained a single signal at 200.2 ppm due to the ^{13}C-enriched carbon atom.

Synthesis of Z-Gly-Ala-Leu-CH$_2$Cl and Z-Gly-Ala-[2-^{13}C]-Leu-CH$_2$Cl. L-Leucine methyl ester hydrochloride was prepared from L-leucine using HCl and methanol as described by Greenstein and Winitz (*25*). The mixed anhydride method was used to form Z-Gly-Ala-Leu methyl ester from L-Leu methyl ester and Z-Gly-Ala (*2*). Z-Gly-Ala-Leu methyl ester was saponified to Z-Gly-Ala-Leu using 0.5 M NaOH (*25*). Z-Gly-Ala-Leu-CH_2Cl was prepared from Z-Gly-Ala-Leu using diazomethane and the mixed anhydride method (*2*).

With Z-Gly-Ala-Leu, a light yellow oil was produced which was a mixture of ∼30% Z-Gly-Ala-Leu and ∼70%

Z-Gly-Ala-Leu-CH_2Cl. This was used without further purification. For Z-Gly-Ala-Leu-CH_2Cl: ^{13}C NMR ([^2H]chloroform) δ 17.6 (*C*H_3 Ala), 21.2, 22.9 (2C,*C*H_3 Leu), 24.6 [$(CH_3)_2$*C*H], 38.9 [$(CH_3)_2$*C*H*C*H_2], 44.2 (CO-*C*H_2-NH), 46.9 (*C*H_2Cl), 55.1 [$(CH_3)_2$CHCH_2*C*H], 50.9 (CH_3-*C*H), 66.9 (C_6H_5*C*H_2O), 127.7−128.3 (5C, *C*H=*C*H), 135.9 (CH=*C*=), 156.6 (O-*C*O-NH), 169.9 (CH_2-*C*O-NH), 173.1 (CH-*C*O-NH Ala), 201.3 (*C*OCH_2Cl). The ^{13}C NMR spectrum of Z-Gly-Ala-[2-^{13}C]Leu-CH_2Cl in [^2H]chloroform contained a single signal at 201.3 ppm due to the ^{13}C-enriched carbon atom.

Enzyme Solutions. δ-Chymotrypsin (salt free, from bovine pancreas) and subtilisin BPN′ (crystallized and lyophilized) were obtained from Sigma Chemical Co. and were used without further purification. Protein concentrations of both native and alkylated enzymes were determined using ϵ_{280} values of 50 000 and 32 300 M^{-1} cm^{-1} for chymotrypsin (*28*) and subtilisin (*29*), respectively. The concentrations of the active enzymes were determined by titration with *N-trans*-cinnamoylimidazole as described previously (*13*, *15*). Fifty to seventy percent of the protein in the subtilisin samples and 70−95% of the protein in the chymotrypsin samples were catalytically active when titrated with *N-trans*-cinnamoylimidazole.

Alkylation of Chymotrypsin by Chloromethane Inhibitors. Samples of δ-chymotrypsin were alkylated using the same methods used to alkylate chymotrypsin by Tos-[2-^{13}C]Phe-CH_2Cl (*13*, *30*) except that the alkylating reagents were dissolved in 1,4-dioxane except for formyl-Ala-Gly-Gly-Phe-CH_2Cl which was dissolved in DMSO.

Alkylation of Subtilisin by Chloromethane Inhibitors. Samples of subtilisin BPN′ were alkylated by Z-Gly-Ala-[2-^{13}C]Phe-CH_2Cl, formyl-Ala-Gly-Gly-[2-^{13}C]Phe-CH_2Cl, and Z-Gly-Ala-[2-^{13}C]Leu-CH_2Cl using the same methods used to alkylate subtilisin BPN′ with Z-Gly-Gly-[2-^{13}C]Phe-CH_2Cl (*15*). Due to the low reactivity of subtilisin with Z-[2-^{13}C]Phe-CH_2Cl (*6*), a modified procedure was used for its inhibition. Z-[2-^{13}C]Phe-CH_2Cl (20 mM) was dissolved in 1,4-dioxane, and six 0.5 mL portions were added to 250 mL of 0.06 mM active subtilisin in 0.05 M phosphate buffer (pH 7.0) over a period of 3 days to give a final concentration of 0.24 mM Z-[2-^{13}C]Phe-CH_2Cl. This gave a 60% reduction in catalytic activity. Control experiments showed that in the absence of inhibitor ∼30% of the catalytic activity was lost presumably due to autolysis. Therefore, we estimate that only ∼30% of the enzyme was inhibited by alkylation with Z-[2-^{13}C]Phe-CH_2Cl. The remaining activity (∼40% of catalytic activity) was lowered to 0.04% by treatment of the enzyme solution with Z-Gly-Gly-Phe-CH_2Cl (0.1 M in 1,4-dioxane) to a final concentration of 0.06 mM Z-Gly-Gly-Phe-CH_2Cl. The solution was centrifuged at 15600*g* and concentrated to a volume of approximately 25 mL by ultrafiltration on an Amicon PM10 membrane. The concentrated solution was then dialyzed against 3 × 2 L of 5 mM potassium phosphate buffer (pH 7.0) to remove low-M_r materials. Samples of Z-[2-^{13}C]Trp-CH_2Cl and Z-[2-^{13}C]Leu-CH_2Cl also had low reactivities with subtilisin and were inhibited using the same procedure.

pH Titrations and Analysis of pH-Dependent Changes in the Chemical Shift. These were carried out as described by Malthouse et al. (*9*), except that samples were titrated with 0.8 M KOH or 0.8 M HCl containing 20% (v/v) ^2H_2O.

NMR Spectroscopy. NMR spectra at 1.88 T were recorded with a Bruker WP80 wide-bore spectrometer operating at 20.115 MHz for ^{13}C nuclei. The spectral conditions for the samples of alkylated subtilisin and chymotrypsin at 1.88 T were as follows: 4096 time domain data points, 240 ppm spectral width, 0.426 s acquisition time, 0.22 s relaxation delay time, and 90° pulse angle; 6144−49152 transients were recorded per spectrum and 1 W broad-band ^1H decoupling. All spectra were zero filled to give 8192 data points before being transformed using an exponential weighting factor of 5 Hz. For samples of subtilisin inhibited by Z-[2-^{13}C]Leu-CH_2-subtilisin, Z-Gly-Ala-[2-^{13}C]Leu-CH_2-subtilisin, Z-Gly-Ala-[2-^{13}C]Phe-CH_2-chymotrypsin, Z-[2-^{13}C]Trp-CH_2-chymotrypsin, Z-[2-^{13}C]Leu-CH_2-chymotrypsin, and Z-Gly-Ala-[2-^{13}C]Leu-CH_2-chymotrypsin, a 0.4 s relaxation delay was used and the pulse sequence of Belton et al. (*31*) was used to minimize acoustic ringing. Both ^1H and ^{13}C chemical shifts are quoted relative to tetramethylsilane at 0.00 ppm. In aqueous solutions, the chemical shift of the α-carbon of glycine was used as a chemical reference as described previously (*13*). For nonaqueous solvents, either 10% tetramethylsilane was used as an internal standard or an appropriate solvent signal was used as a secondary reference (*32*).

Sample volumes were 1.0−2.0 mL. All samples contained 1−4.2 mM protein and 20% (v/v) ^2H_2O for obtaining a deuterium lock signal, as well as 50 mM glycine, 50 mM glycylglycine, and 2.5 mM phosphate buffer for maintaining stable pH values during pH titrations. The sample of formyl-Ala-Gly-Gly-Phe-CH_2-chymotrypsin also contained 1 mM [2-^{13}C]glycine to help identify the signal due to C-2 of glycine. Samples containing chymotrypsin and its alkylated forms also contained 0.4 M KCl except for samples of chymotrypsin alkylated by Z-Leu-CH_2Cl and formyl-Ala-Gly-Gly-Phe-CH_2Cl.

RESULTS

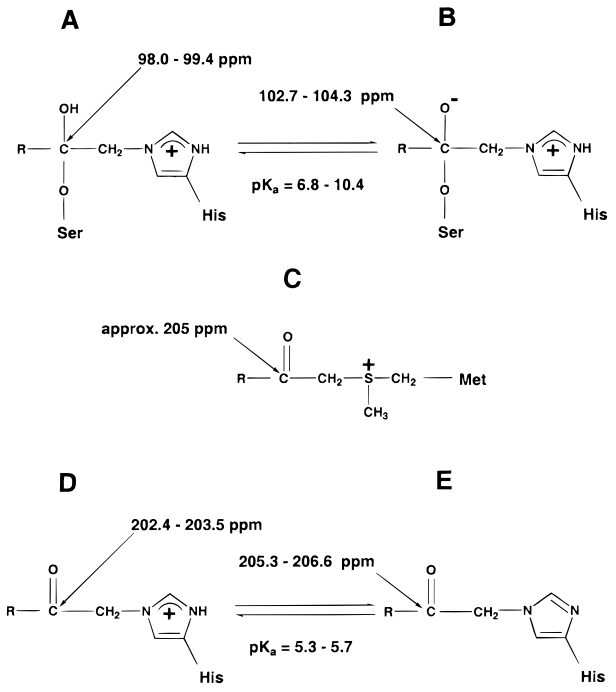
Properties and Assignment of the ^{13}C NMR Signals from Samples of Subtilisin Which Have Been Alkylated with ^{13}C-Enriched Chloromethane Inhibitors. When subtilisin was alkylated by Z-Gly-Gly-[2-^{13}C]Phe-CH_2Cl, a single new signal at 98.9−103.6 ppm (Table 1) was observed (*15*). Similar signals were observed (Table 1) when trypsin was alkylated by Z-[2-^{13}C]Lys-CH_2Cl (*8*, *9*) and when chymotrypsin was alkylated by Z-Gly-Gly-[2-^{13}C]Phe-CH_2Cl, Z-[2-^{13}C]Phe-CH_2Cl, and Tos-[2-^{13}C]Phe-CH_2Cl (*13*−*15*). These signals at ∼100 ppm are characteristic of sp^3 hybridized carbons, and they were assigned (*8*, *9*, *13*−*15*) to hemiketals formed by the addition of the hydroxy group of the active site serine to the ^{13}C-enriched carbonyl carbon of the inhibitor (structures A and B in Scheme 1). Likewise, when subtilisin (Figure 1a) was alkylated by formyl-Ala-Gly-Gly-[2-^{13}C]Phe-CH_2Cl (Figure 1b), Z-Gly-Ala-[2-^{13}C]Phe-CH_2Cl (Figure 1c), Z-[2-^{13}C]Phe-CH_2Cl (Figure 1d), Z-[2-^{13}C]Trp-CH_2Cl (Figure 1e), Z-[2-^{13}C]Leu-CH_2Cl (Figure 1f), and Z-Gly-Ala-[2-^{13}C]Leu-CH_2Cl (Figure 1g), new signals at ∼100 ppm were observed which were not present in the ^{13}C NMR control spectrum of Z-Gly-Gly-Phe-CH_2-subtilisin which was not ^{13}C-enriched (Figure 1a). Only ∼20% of the subtilisin was alkylated by Z-[2-^{13}C]Phe-CH_2Cl and Z-[2-^{13}C]Trp-CH_2-Cl (see Materials and Methods), so the ^{13}C-enriched signal

Table 1: Titration Constants Obtained from ^{13}C NMR Studies of Subtilisin, Trypsin, and δ-Chymotrypsin Chloromethane Inhibitor Derivatives

		chemical shift (ppm)			
enzyme	inhibitor	δ_1[a,b]	δ_2[a,b]	$\delta_1 - \delta_2$[a]	pK_a[b]
subtilisin	formyl-Ala-Gly-Gly-Phe-CH_2Cl	98.88	103.56	4.68	6.79
subtilisin	Z-Gly-Ala-Phe-CH_2Cl	99.41	103.85	4.44	6.94
subtilisin[c]	Z-Gly-Gly-Phe-CH_2Cl	98.87	103.59	4.72	6.92
subtilisin	Z-Phe-CH_2Cl	99.28	103.58	4.30	6.84
subtilisin	Z-Trp-CH_2Cl	99.07	103.47	4.40	7.54
subtilisin	Z-Leu-CH_2Cl	99.01	103.29	4.28	7.63
subtilisin	Z-Gly-Ala-Leu-CH_2Cl	98.37	103.08	4.71	8.01
chymotrypsin	formyl-Ala-Gly-Gly-Phe-CH_2Cl	98.51	103.00	4.49	8.62
chymotrypsin	Z-Gly-Ala-Phe-CH_2Cl	98.58	103.17	4.59	8.97
chymotrypsin[c]	Z-Gly-Gly-Phe-CH_2Cl	98.47	103.17	4.70	8.92
chymotrypsin[d]	Tos-Phe-CH_2Cl	99.10	103.66	4.56	8.85
chymotrypsin[c]	Z-Phe-CH_2Cl	98.79	103.58	4.79	9.42
chymotrypsin	Z-Trp-CH_2Cl	99.27	104.25	4.98	10.38
chymotrypsin	Z-Leu-CH_2Cl		no tetrahedral adduct detected		
chymotrypsin[e]	Z-Gly-Ala-Leu-CH_2Cl	98.03	102.73	4.7	≥9.97
trypsin[f]	Z-Lys-CH_2Cl	98.95	103.08	4.13	7.88

[a] δ_1 and δ_2 are the pH-independent chemical shifts at low and high pH, respectively. [b] Errors were in the ranges of 0.01−0.08 pK_a unit for the pK_a values and 0.01−0.15 ppm for δ_1 and δ_2. [c] Data from ref *15*. [d] From ref *14*. [e] The tetrahedral adduct was not observed above pH 9.0, and we have estimated the pK_a by assuming a titration shift of 4.7 (see the text for details). [f] From refs *8* and *9*.

Scheme 1: Structures and Chemical Shifts of Chloromethane Inhibitor Derivatives



has a lower intensity with these derivatives (Figure 1d,e). The chemical shift of the ^{13}C-enriched carbon of the formyl-Ala-Gly-Gly-[2-^{13}C]Phe-CH_2-subtilisin derivative titrated from 98.88 to 103.56 ppm with increasing pH, corresponding to a pK_a of 6.79 (curve A in Figure 2). Similar titration shifts at 4.28−4.72 ppm (Table 1) were observed with all the other subtilisin chloromethane inhibitor derivatives. These titration shifts are assigned to oxyanion formation (structures A and B in Scheme 1).

Properties and Assignment of the ^{13}C NMR Signals from Samples of Chymotrypsin Which Have Been Alkylated with ^{13}C-Enriched Chloromethane Inhibitors. When chymotrypsin is alkylated by Tos-[2-^{13}C]Phe-CH_2Cl or Z-[2-^{13}C]Phe-CH_2-Cl, two new signals are observed: one at 98.8−103.7 ppm and the other at ∼205 ppm (*13*, *15*). The signals at 98.8−103.7 ppm were assigned to hemiketals formed by the addition of the hydroxy group of the active site serine to the ^{13}C-enriched carbonyl carbon of the inhibitor (structures A and B in Scheme 1), while the signal at ∼205 ppm was assigned to the species (structure C in Scheme 1) resulting from alkylation of methionine 192 (*13*, *15*). When chymotrypsin was alkylated using an excess of the larger inhibitor Z-Gly-Gly-[2-^{13}C]Phe-CH_2Cl, only one new signal at ∼100 ppm was observed (*15*). Likewise, in this work, only one new signal at ∼100 ppm was observed when chymotrypsin (Figure 3a) was alkylated by formyl-Ala-Gly-Gly-[2-^{13}C]Phe-CH_2Cl (Figure 3b), Z-Gly-Ala-[2-^{13}C]Phe-CH_2Cl (Figure 3c), Z-[2-^{13}C]Trp-CH_2Cl (Figure 3d), and Z-Gly-Ala-[2-^{13}C]Leu-CH_2Cl (Figure 3e).

The chemical shift of the ^{13}C-enriched carbon of the formyl-Ala-Gly-Gly-[2-^{13}C]Phe-CH_2-chymotrypsin derivative titrated from 98.51 to 103.00 ppm with increasing pH, corresponding to a pK_a of 8.62 (curve B in Figure 2). Similar titration shifts at 4.28−4.72 ppm (Table 1) were observed with all the other chymotrypsin chloromethane inhibitor derivatives with the exception of the Z-[2-^{13}C]Leu-CH_2-chymotrypsin derivative and the Z-Gly-Ala-[2-^{13}C]Leu-CH_2-chymotrypsin derivative. These titration shifts are assigned to oxyanion formation (structures A and B in Scheme 1). The mean titration shift observed with these chymotrypsin chloromethane derivatives was 4.7 ± 0.2 ppm. The signal from the ^{13}C-enriched carbon of the Z-Gly-Ala-[2-^{13}C]Leu-CH_2-chymotrypsin derivative was not observed above pH 9.0. However, assuming a simple titration curve (as defined by the equation in the legend of Figure 2), we have used the experimental data to estimate that the oxyanion pK_a is 9.97 for this derivative (curve C in Figure 2). This was done by assuming a normal titration shift of 4.7 ppm. If this assumption is correct, then the oxyanion pK_a must be ≥9.97 for the Z-Gly-Ala-[2-^{13}C]Leu-CH_2-chymotrypsin derivative (Table 1).

When chymotrypsin (Figure 4a) was alkylated by Z-[2-^{13}C]Leu-CH_2Cl, a new signal at 206.6 ppm was observed (Figure 4b), but no new signals were detected at ∼100 ppm. The chemical shift of the signal at 206.6 ppm did not change from pH 3.95 (Figure 4b) to 7.90 (Figure 4c). However, after incubation for 4 h at pH 9.76 and lowering of the pH to

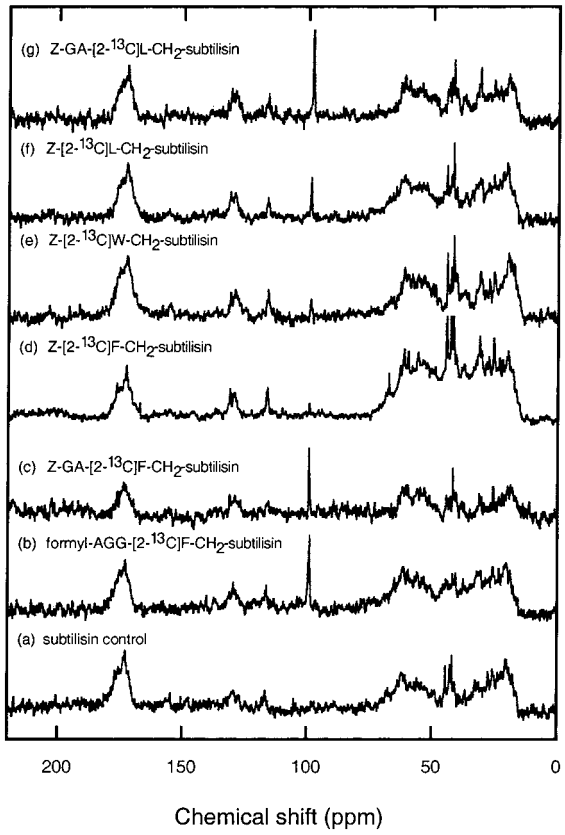


FIGURE 1: ^{13}C NMR spectra of subtilisin inhibited by substrate-derived chloromethane inhibitors. Acquisition parameters were as described in Materials and Methods. All spectra were processed using an exponential weighting factor of 5 Hz. The number of transients recorded per spectrum was 65 536, 12 288, 6144, 116 736, 81 920, 65 536, and 49 152 for spectra a−g, respectively. Sample conditions were as follows: (a) 0.9 mL of 1.6 mM Z-Gly-Gly-Phe-CH_2-subtilisin at pH 5.05, (b) 1.2 mL of 2.66 mM formyl-Ala-Gly-Gly-[2-^{13}C]Phe-CH_2-subtilisin at pH 5.54, (c) 1.6 mL of 1.68 mM Z-Gly-Ala-[2-^{13}C]Phe-CH_2-subtilisin at pH 5.44, (d) 1.6 mL of 1.75 mM Z-[2-^{13}C]Phe-CH_2-subtilisin at pH 4.86, (e) 1.0 mL of 2.76 mM Z-[2-^{13}C]Trp-CH_2-subtilisin at pH 4.94, (f) 1.4 mL of 2.15 mM Z-[2-^{13}C]Leu-CH_2-subtilisin at pH 4.98, and (g) 1.7 mL of 0.95 mM Z-Gly-Ala-[2-^{13}C]Leu-CH_2-subtilisin at pH 5.07. The signals at ∼42.4, ∼41.6, and ∼44.2 ppm are due to glycine and glycylglycine which were added to some samples to ensure stable pH values during the pH titrations. The tops of these buffer signals have been omitted in spectrum d.

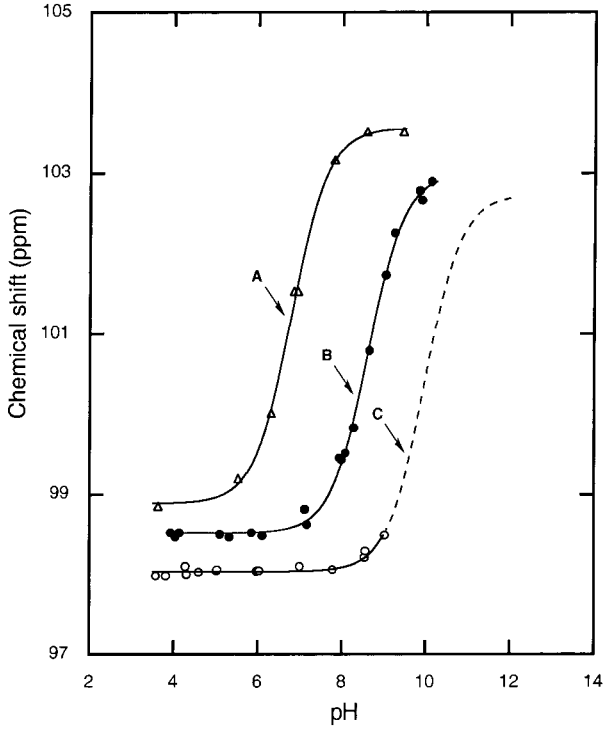


FIGURE 2: pH titration of the signals due to the ^{13}C-enriched carbon atoms in Z-Gly-Ala-[2-^{13}C]Leu-CH_2-chymotrypsin, formyl-Ala-Gly-Gly-[2-^{13}C]Phe-CH_2-chymotrypsin, and formyl-Ala-Gly-Gly-[2-^{13}C]-Phe-CH_2-subtilisin. Acquisition parameters and sample conditions were as described in Materials and Methods. The continuous lines were calculated using the equation $\delta = [S_1/(1 + K_a/[H])] + [S_2/(1 + [H]/K_a)]$. Curve A (△) was calculated for formyl-Ala-Gly-Gly-[2-^{13}C]Phe-CH_2-subtilisin using the following fitted parameters: pK_a = 6.79 ± 0.03, S_1 = 98.88 ± 0.07, and S_2 = 103.56 ± 0.06. Curve B (●) was calculated for formyl-Ala-Gly-Gly-[2-^{13}C]Phe-CH_2-chymotrypsin using the following fitted parameters: pK_a = 8.62 ± 0.02, S_1 = 98.51 ± 0.03, and S_2 = 103.00 ± 0.05. Curve C (○) was calculated for Z-Gly-Ala-[2-^{13}C]Leu-CH_2-chymotrypsin using the following parameters: pK_a = 9.97, S_1 = 98.03, and S_2 = 102.73.

4.04 (Figure 4d), the signal at 206.6 ppm was still present. Signals due to alkylated methionines have similar chemical shifts of ∼205 ppm which do not change from pH 3 to 9 (*13*, *15*). But these signals are irreversibly lost after exposure to pHs of >9 (*13*, *15*). Therefore, this suggests that this signal is not due to methionine. The chemical shift of 206.6 ppm is similar to the signals observed at 202.4−206.6 ppm due to alkali-denatured chloromethane inhibitor derivatives. In such alkali-denatured derivatives, the chemical shift increases from 202.4−203.5 to 205.3−206.6 ppm as the pH increases, corresponding to a pK_a of 5.3−5.7 (*17*). This titration shift has been assigned (*9*, *17*, *19*) to the ionization of the alkylated imidazolium cation in the alkali-denatured species (structures D and E in Scheme 1). No such titration shift was observed with the Z-[2-^{13}C]Leu-CH_2-chymotrypsin derivative. Therefore, we conclude that the signal at 206.6 ppm does not result from an alkali-denatured species (structures D and E in Scheme 1) analogous to those seen in previous studies (*9*, *17*). As this inhibitor caused irreversible inhibition of chymotrypsin, an active site residue must have been alkylated. This residue could be either the active site serine hydroxy group or the imidazole group of the active site histidine. With the native enzyme, alkylation of the active site histidine is expected to raise its pK_a as ionization via the exposed N-3 of its imidazole group is prevented (*14*). Therefore, if it is the active site histidine which is alkylated, then no tetrahedral adduct is formed and the pK_a of the alkylated imidazole group must be >8.0 to explain the fact that we did not observe a titration shift from pH 3.95 (Figure 4b) to 7.90 (Figure 4c). We would also like to point out that we cannot dismiss the possibility that an extremely stable alkylmethionine has been formed.

Calculating How the Intrinsic Substituent Effects of the Different R Groups Affect Oxyanion pK_a Values. The R groups in phenylalanine and tryptophan have Taft substituent constants (σ^*) of 0.22 (*33*) and −0.022 (*34*), respectively. The isopropyl group of leucine has a σ^* of −0.19 (*33*), so allowing a factor of 0.4 (*35*) for attenuation across a methylene group, we estimate that the R group of leucine has a Taft substituent constant of −0.076. Therefore, these different substituents will have different effects on the oxyanion pK_a. After allowing for the attenuation (by a factor of 0.4) of the substituent constants across the α-carbon, we used eq 2 derived from eq 1 (*36*) to calculate how the

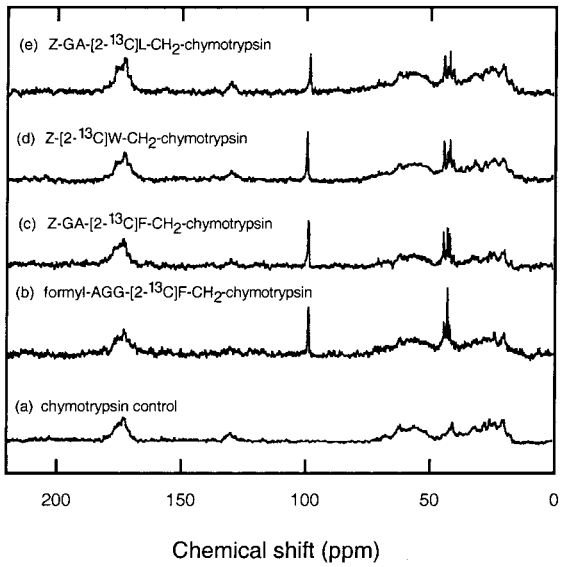


FIGURE 3: ^{13}C NMR spectra of chymotrypsin inhibited by substrate-derived chloromethane inhibitors. Acquisition parameters were as described in Materials and Methods. All spectra were processed using an exponential weighting factor of 5 Hz. The number of transients recorded per spectrum was 81 920, 16 384, 55 296, 61 440, and 65 536 for spectra a−e, respectively. Sample conditions were as follows: (a) 1.0 mL of 4.18 mM chymotrypsin at pH 4.13, (b) 1.0 mL of 2.30 mM formyl-Ala-Gly-Gly-[2-^{13}C]Phe-CH_2-chymotrypsin at pH 3.93, (c) 1.0 mL of 2.23 mM Z-Gly-Ala-[2-^{13}C]Phe-CH_2-chymotrypsin at pH 4.29, (d) 1.0 mL of 2.90 mM Z-[2-^{13}C]Trp-CH_2-chymotrypsin at pH 3.99, and (e) 1.0 mL of 2.39 mM Z-Gly-Ala-[2-^{13}C]Leu-CH_2-chymotrypsin at pH 3.82. The signals at ~42.4, ~41.6, and ~44.0 ppm are due to glycine and glycylglycine which were added to some samples to ensure stable pH values during the pH titrations.

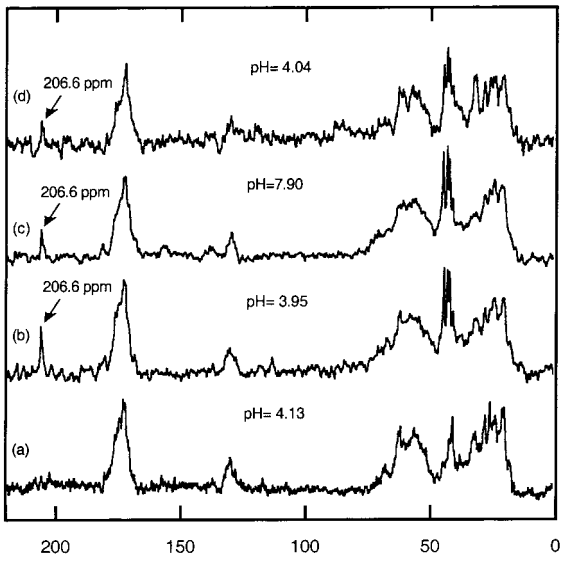


FIGURE 4: ^{13}C NMR spectra of the Z-[2-^{13}C]Leu-CH_2-δ-chymotrypsin derivative. Acquisition parameters were as described in Materials and Methods. All spectra were processed using an exponential weighting factor of 5 Hz. The number of transients recorded per spectrum was 81 920, 32 768, 16 384, and 49 152 for spectra a−d. Sample conditions were as follows: (a) 1.0 mL of 4.18 mM chymotrypsin at pH 4.13, (b) 1.1 mL of 2.62 mM Z-[2-^{13}C]Leu-CH_2-chymotrypsin at pH 3.95, (c) 1.4 mL of 1.94 mM Z-[2-^{13}C]Leu-CH_2-chymotrypsin at pH 7.90, and (d) 0.9 mL of 1.68 mM Z-[2-^{13}C]Leu-CH_2-chymotrypsin at pH 4.04. Spectra a−c were obtained sequentially using the same sample of Z-[2-^{13}C]Leu-CH_2-chymotrypsin. This sample was then lyophilized and dissolved in 0.9 mL of 20% (v/v) ^2H_2O and used to obtain spectrum d. All samples of Z-[2-^{13}C]Leu-CH_2-chymotrypsin contained 50 mM glycine, 50 mM glycylglycine, and 20% (v/v) ^2H_2O.

different R groups affected the oxyanion pK_a.

$$\mathrm{p}K_a = 17.47 - 1.45\sum\sigma^* \quad (1)$$

$$\Delta\,\mathrm{p}K_a = -1.45(\sigma_1^* - \sigma_2^*) \quad (2)$$

Using eq 2, we estimate that replacing phenylalanine at P_1 with tryptophan and leucine will decrease the oxyanion pK_a by 0.14 and 0.17 pK_a unit, respectively. Therefore, for the subtilisin chloromethane inhibitor derivatives (Table 1), the observed increases in their oxyanion pK_a values (0.7 ± 0.09 pK_a unit for Z-Phe-CH_2Cl and Z-Trp-CH_2Cl, 0.79 ± 0.08 pK_a unit for Z-Phe-CH_2Cl and Z-Leu-CH_2Cl, and 1.07 ± 0.05 pK_a units for Z-Gly-Ala-Phe-CH_2Cl and Z-Leu-CH_2-Cl) cannot be attributed to the intrinsic substituent effects of the different R groups.

DISCUSSION

Our results show that phenylalanine and tryptophan residues which bind in the S_1 specificity pocket must fix the P_1 residue in a position which ensures that the active site histidine is alkylated and that the active site serine hydroxy group adds to the inhibitor carbonyl to form a tetrahedral adduct. We suggest that the smaller leucine side chain of the Z-[2-^{13}C]Leu-CH_2-chymotrypsin derivative is unable to do this. But, with the larger Z-Gly-Ala-[2-^{13}C]Leu-CH_2-chymotrypsin derivative, the additional binding in the S_2−S_4 subsites does allow tetrahedral adduct formation.

The oxyanion pK_a values of the subtilisin chloromethane derivatives with a phenylalanine residue in the S_1 subsite were all essentially the same (mean pK_a = 6.87 ± 0.07) irrespective of the amino acid residues binding in S_2−S_4 (Table 1). This shows that for subtilisin chloromethane derivatives with phenylalanine residues binding in the S_1 subsite, the oxyanion pK_a is essentially independent of the amino acid residues binding in the S_2−S_4 subsites. However, when chloromethane derivatives with tryptophan and leucine P_1 residues were used, the oxyanion pK_a values increased (Table 1). This demonstrates that the value of the oxyanion pK_a depends on the type of amino acid residue binding in the S_1 subsite.

With Z-[2-^{13}C]Leu- and Z-[2-^{13}C]Trp-CH_2-subtilisin derivatives, oxyanion pK_a values of 7.5−7.6 were obtained. However, with the Z-Gly-Ala-[2-^{13}C]Leu-CH_2-subtilisin derivative, the oxyanion pK_a was 0.4−0.5 pK_a unit higher (Table 1). This shows that when leucine residues are bound in the S_1 subsite, binding in the S_2−S_4 subsites can increase the oxyanion pK_a value.

In the five chymotrypsin chloromethane derivatives with phenylalanine residues in the S_1 subsite, the oxyanion pK_a values were in the range of 8.62−9.42 (Table 1). However, with the chloromethane derivatives which had leucine or tryptophan in S_1, the oxyanion pK_a values were larger (Table 1). This suggests that in both the subtilisin and chymotrypsin chloromethane derivatives the oxyanion pK_a is largely determined by the type of amino acid residue occupying the S_1 subsite. For four of the chymotrypsin chloromethane derivatives with phenylalanine in the S_1 subsite, the oxyanion

pK_a values were essentially the same (pK_a = 8.62−8.97) as was observed in subtilisin derivatives (pK_a = 6.84−6.94) with phenylalanine in the S_1 subsite (Table 1). However, we are unable to explain why the Z-[2-^{13}C]Phe-CH_2-chymotrypsin derivative, which also had a phenylalanine residue in the S_1 subsite, had an oxyanion pK_a value which was ∼0.5 pK_a unit higher than these values (Table 1).

For amide substrates, acylation is the rate-limiting step with the serine proteases and $K_m = K_s$ (*37*). Therefore, from the K_m values of amide substrates (*37*−*40*), we can conclude that tryptophan residues are bound 2.7−5.1-fold more tightly in the S_1 subsite of chymotrypsin than are phenylalanine residues. Changing from phenylalanine to tryptophan also produced a similar increase of 3.0−5.2 in the specificity constant k_{cat}/K_m with the same amide substrates (*37*−*40*). However, when phenylalanine is replaced by tryptophan in the S_1 subsite of chymotrypsin chloromethane derivatives, the oxyanion pK_a increases by 1−1.5 pK_a units (Table 1) which corresponds to a 10−35-fold increase in the oxyanion dissociation constant. Therefore, better binding in the S_1 subsite could account for some but not all of the increase in the oxyanion pK_a. We conclude that better binding in the S_1 subsite of chymotrypsin does not decrease the oxyanion pK_a and that the effectiveness of binding the P_1 residues in the S_1 subsite is not a major factor in determining the oxyanion pK_a in chymotrypsin chloromethane derivatives.

With subtilisin, changing substrates from Z-Gly-Gly-Phe-*p*-nitroanilide to Z-Gly-Gly-Leu-*p*-nitroanilide increased K_m values 0.25-fold and decreased k_{cat}/K_m values 2-fold (*41*). However, changing from acetyl-Phe-methyl ester to acetyl-Trp-methyl ester increased the k_{cat}/K_m 1.8-fold (*42*). Therefore, when tryptophan, phenylalanine, and leucine bind in the S_1 subsite of subtilisin, their effectiveness in increasing the catalytic specificity of subtilisin is in the following order: tryptophan > phenylalanine > leucine. This shows that for subtilisin the effectiveness of binding in the S_1 subsite is in the following order: tryptophan > phenylalanine > leucine. In the chloromethane derivatives, better binding by phenylalanine relative to leucine could contribute to the lower oxyanion pK_a values in the phenylalanine derivatives (Table 1). However, when phenylalanine was replaced with tryptophan in the subtilisin chloromethane derivatives, the better binding by tryptophan relative to phenylalanine did not lead to a decrease in the oxyanion pK_a; instead, an increase in the oxyanion pK_a was observed (Table 1). This suggests that the oxyanion pK_a is not related to the effectiveness of binding in the S_1 subsite of these subtilisin chloromethane derivatives.

The range of oxyanion pK_a values observed was larger with chymotrypsin derivatives (10.38 − 8.62 = 1.76) than with the subtilisin derivatives (8.01 − 6.79 = 1.22), and the mean oxyanion pK_a values were 9.19 ± 0.64 and 7.24 ± 0.48, respectively. These results show that with the chymotrypsin chloromethane derivatives that were studied the oxyanion pK_a is slightly more sensitive to the type of inhibitor used than it is in the corresponding subtilisin derivatives. However, they also show that oxyanion pK_a values are ∼2 pK_a units greater in the chymotrypsin chloromethane derivatives than in the subtilisin chloromethane derivatives.

In chymotrypsin, the oxyanion is hydrogen bonded to the main chain NH groups of serine 195 and glycine 193, and in the chloromethane derivatives, alkylation of the active site histidine residue could force the oxyanion out of its optimal position for forming these hydrogen bonds (*43*). We have suggested that in subtilisin the side chain of asparagine 155 could move to compensate for this movement of the oxyanion out of the oxyanion hole (*15*). This, we proposed, could explain why the oxyanion pK_a is lower in subtilisin than in chymotrypsin (*15*). However, it has recently been shown that removal of the hydrogen bond between the oxyanion and asparagine 155 in the Z-Gly-Gly-Phe-CH_2-subtilisin derivative only raises the pK_a of the oxyanion by 1.09 pK_a units (*18*). Therefore, the suggestion (*15*) that the movement of asparagine 155 could be responsible for the oxyanion pK_a being ∼2 pK_a units lower in subtilisin chloromethane derivatives than in chymotrypsin chloromethane derivatives is incorrect. This mechanism could only account for about half of the decrease in the oxyanion pK_a with the subtilisin derivatives.

The binding of ligands in the S_1 subsite and chemical modification of active site residues prevent the conformational change associated with the ionization of the isoleucine 16−aspartate 194 ion pair by raising its pK_a to >11 (*13*). Therefore, ionization of the isoleucine 16−aspartate 194 ion pair does not occur up to pH 11 in chloromethane derivatives of chymotrypsin (*13*). The presence of the positively charged amino group of isoleucine 16 should lower the oxyanion pK_a, while the negatively charged carboxylate of aspartate 194 should raise the oxyanion pK_a. If both groups are equally effective, then the isoleucine 16−aspartate 194 ion pair should not have a significant effect on the oxyanion pK_a. Therefore, we believe that it is unlikely that the presence of the isoleucine 16−aspartate 194 ion pair in the chymotrypsin chloromethane derivatives will explain why the oxyanion pK_a values are larger in chymotrypsin chloromethane derivatives than in subtilisin chloromethane derivatives (Table 1).

X-ray crystallographic studies show that while active site residues of chymotrypsin can be described as being buried in a deeply invaginated pocket, the corresponding active site residues of subtilisin are buried in a similar but less well-defined primary active site (S_1S_1') which is best described as a shallow groove or crevice open on one side to solvent (*44*−*46*). Therefore, the active site of chymotrypsin is expected to be less polar than that of subtilisin. We suggest that the lower polarity of the chymotrypsin active site relative to that of the subtilisin active site explains why the oxyanion pK_a is higher and more sensitive to the type of chloromethane inhibitor used in the chymotrypsin derivatives than in the subtilisin derivatives.

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