to that given above (bp 95–99° (0.3 mm)): pmr ($CDCl_3$) δ 1.3–1.8 (multiplet, 4 H), 1.8–2.4 (complex multiplet, 2 H), 2.5–3.0 (broad triplet, 2 H), 7.46, and 7.94 (AB quartet, J = 8 Hz, 4 H).

Titration of Biebrich Scarlet with Chymotrypsin. Stock chymotrypsin solutions were prepared by dissolving the enzyme (5–30 mg per ml) in 0.05 M phosphate buffer at pH 7.0. Varying amounts of such solutions were then promptly added to vials containing a fixed amount of dye (*e.g.*, 200 μl of 3.70×10^{-4} M Biebrich Scarlet) and an amount of buffer to bring each sample to an appropriate volume. The range of enzyme concentration was $0.1–5.0 \times 10^{-4}$ M after correcting for the percentage of active sites. Spectrophotometric experiments were always begun within an hour after the solutions were prepared, the time being allowed for the samples to come to thermal equilibrium. Difference spectra in the range of 650–450 nm were recorded by comparing the absorption of Biebrich Scarlet in the chymotrypsin solutions with that in the reference solution containing no protein. The same experiments were repeated for 0.05 M phosphate buffer containing various amounts of 2-propanol.

Kinetics. Kinetic experiments were initiated by adding 100 μl of a concentrated solution (10–40 mM) of sulfonyl fluoride inhibitor in 2-propanol to 3.0 ml of *ca.* 0.05 M phosphate buffer (pH 7.0) containing 1.83% 2-propanol, chymotrypsin ($2–6 \times 10^{-5}$ M), and Biebrich Scarlet (9.0×10^{-5} M). The final concentration of 2-propanol was 5.0%. Rates were determined by following decreasing absorbance at the wavelength of maximum $\Delta\epsilon$ (553 nm). Since 2-propanol perturbs the binding of the dye to the enzyme, a control containing only this cosolvent was also run in each case, and the decreasing absorbance was corrected for this perturbation. An attempt was made to maintain the inhibitor concentration in large excess of that of the enzyme, but due to the limited solubility of the inhibitors even in 5% 2-propanol, this excess was only 5–20-fold.

Reaction Stoichiometry. 4-Fluorobenzenesulfonylchymotrypsin was formed under conditions essentially identical with those used for the kinetic experiments using a fivefold molar excess of the irreversible inhibitor. 4-Fluorobenzenesulfonic acid was eliminated from a 0.88-g sample of this protein by the procedure of Weiner, *et al.*[16] A known amount of 4-fluorobenzoic acid was added to the reaction mixture and the solution concentrated. The fluorine-19 spectrum of the concentrate was recorded in the region 30–32 ppm upfield from external trifluoroacetic acid and, by using the relative peak areas of the two trifluoromethyl-substituted compounds, it was determined that 1.0 ± 0.05 mol of inhibitor was bound per mol of enzyme.

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Inhibition of Chymotrypsin A_α with N-Acyl- and N-Peptidyl-2-phenylethylamines. Subsite Binding Free Energies[1]

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Abstract: The dissociation constants for complexes of chymotrypsin A_α with a series of N-acyl- and N-peptidyl-2-phenylethylamine competitive inhibitors were measured in a 9.5% ethanol, 0.10 M $CaCl_2$ solution at pH 7.80 at 25°. Individual subsite binding free energies for the extended substrate binding site in chymotrypsin were calculated. The free energies of binding in this series of N-acyl- and N-peptidyl-2-phenylethylamines become more negative with increasing number of interactions with enzyme as predicted from the crystallographic model. The major contributors to the binding free energy are hydrophobic interactions, the S_1–P_1 tosyl pocket–phenylethyl group interaction (2 kcal/mol) and the interaction of an aliphatic side chain of a P_2 residue with Ile-99 in the S_2 binding subsite of the enzyme. The individual subsite binding free energies are not additive. Comparison of the dissociation constants of N-acyl- and N-peptidyl-2-phenylethylamines with the K_M values for the corresponding substrates indicates that the inhibitors are more tightly bound. This is consistent with the hypothesis that serine proteases place the scissionable peptide bond of a substrate on a stereoelectronic "rack" favoring formation of a tetrahedral intermediate.

Recent X-ray studies on chymotrypsin A_γ crystals inhibited with peptide chloromethyl ketone inhibitors (**1**) have shown the existence of an extended substrate binding site in this enzyme.[2] These active site directed irreversible inhibitors are bound to the enzyme *via* a covalent linkage between the imidazole ring of His-57 and the methylene group of the inhibitor (**2**). The benzyl group of the inhibitor occupies the so-called "tosyl pocket," a slit in the surface of the enzyme near the catalytic residues (Asp-105, His-57, and Ser-195)[3] and the peptide chain of the inhibitor forms an extended antiparallel β-sheet hydrogen bonding structure with the peptide backbone of residues Ser-214, Trp-215, and Gly-216 of the enzyme. Subsequent solution studies confirmed the relevance of the crystallographic results to the behavior of the enzyme in solution. Segal[4] showed that the K_M values derived from the rates of hydrolysis of seven peptide esters by chymotrypsin A_α were in accord with the crystal binding scheme. In addition, the rates of inhibition of chymotrypsin A_α with peptide chloromethyl ketones in solution were shown to correlate positively with ob-

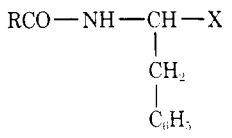
(1) This investigation was supported by U. S. Public Health Service Grant No. GM-18292 and by the Research Corporation. A National Science Foundation undergraduate summer research fellowship (1972) is gratefully acknowledged (B. K. C.).

(2) D. M. Segal, G. H. Cohen, D. R. Davies, J. C. Powers, and P. E. Wilcox, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 85 (1971); D. M. Segal, J. C. Powers, G. H. Cohen, D. R. Davies, and P. E. Wilcox, *Biochemistry*, **10**, 3728 (1971).

(3) D. M. Blow, *Enzymes, 3rd Ed.*, **3**, 185 (1971).

(4) D. M. Segal, *Biochemistry*, **11**, 349 (1972).

served enzyme–inhibitor interactions in chymotrypsin crystals.[5]



1. X = $COCH_2Cl$
2. X = $COCH_2$—N(imidazole)—Enz
3. X = $CONHR'$
4. X = H

Several questions may be posed concerning the extended substrate binding site in chymotrypsin. First, one might ask the relative contribution of each of the enzyme's subsites to the overall free energy of binding of a peptide substrate to chymotrypsin. Neither of the above kinetic studies were able to measure K_S (or K_I), the dissociation constant for the enzyme–substrate (or inhibitor) complex, and thus free energies of binding could not be obtained. Secondly, the question arises whether the subsite binding free energies in an enzyme–substrate complex are additive. If this were the case, once individual subsite binding free energies were known, the K_S (or K_I) for any substrate (or inhibitor) could be calculated simply by examining the contacts it would make with the enzyme.

In this paper, we report one approach toward measuring the individual subsite binding free energies by the use of a series of competitive inhibitors of chymotrypsin A_α.[6] The inhibitors chosen for study were *N*-acyl- or *N*-peptidyl-2-phenylethylamines (**4**), which can be considered to be derivatives of phenylalanine related to natural substrates (**3**), however, with the scissionable peptide bond replaced by a hydrogen atom. Since at present there is no evidence that the NH or the carbonyl group of the peptide bond of a substrate cleaved by chymotrypsin has any interaction with the enzyme prior to bond cleavage (although two hydrogen bonds are probably formed between the oxygen atom of the peptide bond and the enzyme as a tetrahedral intermediate is generated[7]), the free energies of binding for these inhibitors (**4**) were anticipated to closely approximate those expected for the corresponding substrates.

Experimental Section

Chymotrypsin A_α was obtained from Worthington Biochemical Co. (Lot CDIOBK) and was used without further purification. The substrate, *N*-acetyl-L-tyrosine ethyl ester monohydrate, was synthesized in our laboratory and recrystallized from ethanol–water to a constant melting point (79.5–81.0°). All other chemicals were analytical grade. Thin layer chromatography (tlc) was done on silica gel G plates (E. Merck). All amino acids were optically active and had the L configuration unless otherwise specified.

Synthesis of the Inhibitors. Inhibitors were prepared using the mixed anhydride coupling method.[8] One equivalent of isobutyl chloroformate was added to a solution of 1 equiv of the amino blocked amino acid or dipeptide acid and 1 equiv of *N*-methylmorpholine dissolved in anhydrous tetrahydrofuran at −15°. This mixture was stirred for approximately 30 min, 1 equiv of 2-phenylethylamine was added, and the mixture was stirred for at least 30 min while being allowed to warm to room temperature. The tetrahydrofuran was then evaporated, water added to the residue, and the water solution extracted three times with ethyl acetate. The combined ethyl acetate extracts were washed with 0.5 *M* citric acid and 1.0 *M* $NaHCO_3$ and then dried over anhydrous magnesium sulfate. Evaporation of the ethyl acetate resulted in a product, which was purified as described below for each individual compound. All compounds were analyzed by tlc, nmr and mass spectra, and combustion analysis. The peak positions in the nmr spectra are reported in ppm relative to tetramethylsilane. The following abbreviations are used: s, singlet; d, doublet; t, triplet; m, multiplet; b, broad.

***N*-(Acetylglycyl)-2-phenylethylamine (Ac-Gly-NHCH$_2$CH$_2$Ph).** The resultant yellow crystals were recrystallized from hot ethyl acetate to yield white crystals (9.1% yield): mp 149.5–151°; nmr ($CDCl_3$) δ 7.25 (5 H, s, C_6H_5), 3.85 (2 H, d, $NHCH_2CO$), 3.47 (2 H, m, CH_2CH_2Ph), 2.85 (2 H, t, CH_2Ph), and 2.00 (3 H, s, CH_3CO); mass spectrum *m/e* 220 (M^+), 177 (M − CH_3CO), 148 ($CONHCH_2CH_2Ph$), 129 (M − C_7H_7), 120 ($NHCH_2CH_2Ph$), 105 (CH_2CH_2Ph), 100 (M − $NHCH_2CH_2Ph$), 91 (C_7H_7), 77 (C_6H_5), and 72 ($CH_3CONHCH_2$).

Anal. Calcd for $C_{12}H_{16}N_2O_2$: C, 65.43; H, 7.32; N, 12.72. Found: C, 65.56; H, 7.41; N, 12.77.

***N*-(Acetylalanyl)-2-phenylethylamine (Ac-Ala-NHCH$_2$CH$_2$Ph).** The product was recrystallized from ethyl acetate–cyclohexane to yield crystals (33%): mp 137–139°; nmr ($CDCl_3$) δ 7.25 (5 H, s, C_6H_5), 6.40 (2 H, b, NH), 4.40 (1 H, m, CH), 3.45 (2 H, m, CH_2CH_2Ph), 2.78 (2 H, t, CH_2Ph), 1.95 (3 H, s, CH_3CO), 1.30 (3 H, d, CH_3CH); mass spectrum *m/e* 234 (M^+), 219 (M − CH_3), 191 (M − CH_3CO), 143 (M − C_7H_7), 120 ($NHCH_2CH_2Ph$), 114 (M − $NHCH_2CH_2Ph$), 105 (CH_2CH_2Ph), 91 (C_7H_7), 86 ($CH_3CONHCHCH_3$), and 77 (C_6H_5).

Anal. Calcd for $C_{13}H_{18}N_2O_2$: C, 66.64; H, 7.74; N, 11.96. Found: C, 66.81; H, 7.82; N, 12.04.

***N*-(Acetylleucyl)-2-phenylethylamine (Ac-Leu-NHCH$_2$CH$_2$Ph).** The crude product was recrystallized from ethyl acetate–cyclohexane. Further purification was necessary and involved column chromatography on silica gel. The product eluted with 2% CH_3OH in $CHCl_3$ and was recrystallized from ethyl acetate–cyclohexane to yield white crystals (20%): mp 136–137°; $[\alpha]^{25}D$ 40.18° (*c* 1.66, EtOH); nmr ($CDCl_3$) δ 7.23 (5 H, s, C_6H_5), 6.85 (2 H, b, NH), 4.45 (1 H, m, $NHCHCO$), 3.47 (2 H, m, CH_2CH_2Ph), 2.78 (2 H, t, CH_2Ph), 1.96 (3 H, s, CH_3CO), and 0.90 (6 H, d, $CH(CH_3)_2$); mass spectrum *m/e* 276 (M^+), 261 (M − CH_3), 233 (M − CH_3CO), 185 (M − C_7H_7), 156 (M − $NHCH_2CH_2Ph$), 148 ($CONHCH_2CH_2Ph$), 128 (M − $CONHCH_2CH_2Ph$), 120 ($NHCH_2CH_2Ph$), 105 (CH_2CH_2Ph), and 91 (C_7H_7).

Anal. Calcd for $C_{16}H_{24}N_2O_2$: C, 69.53; H, 8.75; N, 10.14. Found: C, 69.42; H, 8.85; N, 10.16.

***N*-(Acetylglycylglycyl)-2-phenylethylamine (Ac-Gly-Gly-NHCH$_2$CH$_2$Ph).** The crude product was recrystallized from ethyl acetate–methanol and yielded white crystals (50%): mp 230.5–232.5°; nmr (DMSO-d_6) δ 7.25 (5 H, s, C_6H_5) and 1.90 (3 H, s, CH_3CO); mass spectrum *m/e* 277 (M^+), 262 (M − CH_3), 219 (M − CH_3CONH), 205 (M − $CH_3CONHCH_2$), 186 (M − C_7H_7), 174 (M − CH_2CH_2Ph), 177 (M − $CH_3CONHCH_2CO$), 157 (M − $NHCH_2CH_2Ph$), 148 ($CONHCH_2CH_2Ph$), and 129 (M − $CONHCH_2CH_2Ph$).

Anal. Calcd for $C_{14}H_{19}N_3O_3$: C, 60.63; H, 6.91; N, 15.15. Found: C, 60.65; H, 7.07; N, 15.29.

***N*-(Acetylglycylleucyl)-2-phenylethylamine (Ac-Gly-Leu-NHCH$_2$CH$_2$Ph).** The crude product was recrystallized from ethyl acetate–petroleum ether and yielded white crystals (40%): mp 160–162°; nmr ($CDCl_3$) δ 7.25 (5 H, s, C_6H_5), 2.05 (3 H, s, CH_3CO), and 0.90 (6 H, d, $CH(CH_3)_2$); mass spectrum *m/e* 333 (M^+), 318 (M − CH_3), 290 (M − CH_3CO), 261 (M − $CH_3CONHCH_2$), 242 (M − C_7H_7), 218 (M − $CH_3CONHCH_2CONH$), 213 (M − $NHCH_2CH_2Ph$), 185 (M − $CONHCH_2CH_2Ph$), 176 ($NHCHCONHCH_2CH_2Ph$), and 170 ($NHCH_2CONHCH(C_4H_9)CO$).

Anal. Calcd for $C_{18}H_{29}N_3O_3$: C, 64.84; H, 8.16; N, 12.60. Found: C, 65.09; H, 7.93; N, 12.60.

***N*-(Benzyloxycarbonylglycylleucyl)-2-phenylethylamine (Z-Gly-Leu-NHCH$_2$CH$_2$Ph).** The crude product was recrystallized from cyclohexane–ethyl acetate and yielded white crystals (5%): mp 95–97°; nmr ($CDCl_3$) δ 7.40 (5 H, s, C_6H_5), 2.2 (3 H, s, CH_3CO),

(5) K. Kurachi, J. C. Powers, and P. E. Wilcox, *Biochemistry*, **12**, 771 (1973).

(6) No significant differences have been observed between chymotrypsin A_α and chymotrypsin A_γ upon direct comparison of their 5.5-Å resolution electron density maps (G. H. Cohen, B. W. Matthews, and D. R. Davies, *Acta Crystallogr., Sect. B*, **26**, 1062 (1970)) or upon examination of models of their active site regions.

(7) R. Henderson, *J. Mol. Biol.*, **54**, 341 (1970).

(8) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **89**, 5012 (1967).

and 1.00 (6 H, d, $CH(CH_3)_2$; mass spectrum *m/e* 425 (M^+), 268, 192 ($PhCH_2OCONHCH_2CO$), 148 ($CONHCH_2CH_2Ph$), 135 ($PhCH_2OCO$), 120 ($NHCH_2CH_2Ph$), 105 (CH_2CH_2Ph), 91 (C_7H_7), and 77 (C_6H_5).

Anal. Calcd for $C_{24}H_{31}N_3O_4$: C, 67.74, H, 7.34; N, 9.87. Found: C, 67.58; H, 7.43; N, 10.17.

***N*-Acetyl-2-phenylethylamine (Ac-NHCH$_2$CH$_2$Ph).** This compound was prepared using the procedure of Bischler and Napieralski.[9] The product was vacuum distilled (water aspirator) and the fraction with bp 188° was collected. The clear oil crystallized upon cooling to yield crystals with mp 51–52°.

***N*-Acetyl-*N*-methyl-2-phenylethylamine (Ac-N(CH$_3$)CH$_2$CH$_2$Ph).** This compound was prepared using the procedure of Baumgarten, *et al.*[10] To 1 equiv of *N*-methyl-2-phenylethylamine and 1 equiv of *N*-methylmorpholine in tetrahydrofuran at 0° was added 1 equiv of acetyl chloride. After evaporation of the THF, water was added and the water mixture was extracted three times with chloroform. The chloroform was dried over anhydrous magnesium sulfate and evaporated leaving a yellow oil. The oil was vacuum distilled and the product had bp 80–90° (0.1–0.25 mm).

Kinetic Assays. The kinetic assays involved the measurement of the rate of hydrolysis of acetyl tyrosine ethyl ester by chymotrypsin. The rate was measured potentiometrically using a pH-stat composed of a Radiometer Titrator TTT 11b, pH Meter PHM 26c, Autoburette ABU 13, and Recorder SBR 2c. All assays were carried out in a thermostated vessel at 25.0 ± 0.25° and at pH 7.80 ± 0.10; the titrant used was 0.4687 *N* NaOH. The solutions were prepared by dissolving a weighed amount of acetyl tyrosine ethyl ester and a weighed amount of inhibitor in 1.0 ml of ethanol (95%) and diluting to 10.0 ml with 0.11 *M* $CaCl_2$ resulting in a 9.5% ethanol, 0.10 *M* $CaCl_2$ assay solution. The reaction was initiated by the addition of a small aliquot of a stock solution of chymotrypsin A_α dissolved in 1 m*M* HCl. The acetyl tyrosine ethyl ester concentrations were varied between 1 and 10 m*M* for each inhibitor concentration which were varied from 0.2 to 10 m*M*. At least five substrate concentrations were used for each inhibitor concentration with each point being replicated at least three times. The maximum range of initial velocities observed at any single substrate and inhibitor concentration was 8%. The enzyme concentrations were determined by active site titrations at periodic intervals, using the *5-nitro-3H-1,2-benzoxathiole 2,2-dioxide method.*[11] Autolysis of the enzyme during the course of the assay was negligible. Initial velocities were obtained from the pH-stat recorder tracings by the method of Henderson[12] and were divided by the amount of enzyme added to the assay mixture to give the initial velocities in units of micromoles of substrate hydrolyzed min^{-1} mg of $enzyme^{-1}$.

Results

The rates of hydrolysis of Ac-Tyr-OEt in the presence of a series of *N*-acyl- and *N*-peptidyl-2-phenylethylamines by chymotrypsin A_α were studied in a 9.5% ethanol, 0.10 *M* $CaCl_2$ solution at pH 7.80 at 25°. The data were treated by using a *v vs. v*/[S] plot (Eadie–Hofstee form of the Michaelis–Menten equation)[13] where *v* is the initial velocity of Ac-Tyr-OEt hydrolysis and [S] is the substrate (Ac-Tyr-OEt) concentration. The slope and intercept of the line which best fits the data were calculated by the least-squares method using a computer program with all fits having correlation coefficients of greater than 0.96.

For simple competitive inhibition (eq 1), the velocity

$$E{\cdot}I \underset{-I}{\overset{+I}{\rightleftharpoons}} E \overset{+S}{\rightleftharpoons} E{\cdot}S \longrightarrow P + E \qquad (1)$$

of the reaction of an enzyme and its substrate in the presence of an inhibitor is described by eq 2 where K_I

$$v = \frac{V_{max}[S]}{K_M(1 + I/K_I) + [S]} \qquad (2)$$

is the dissociation constant (eq 3) of the enzyme–

$$K_I = \frac{[E][I]}{[E{\cdot}I]} \qquad (3)$$

inhibitor complex. It can be shown (from eq 2) that the slopes from the *v vs. v*/[S] plots in the presence and absence of inhibitor are related by eq 4 from which

$$\text{slope}_{\text{inhibitor}} = \text{slope}_{\text{no inhibitor}}(1 + I/K_I) \qquad (4)$$

K_I for each inhibitor was calculated. The K_M for the hydrolysis of Ac-Tyr-OEt by chymotrypsin in the absence of inhibitor under the conditions of our experiments was 3.71 m*M* and the V_{max} = 544 μmol of substrate hydrolyzed min^{-1} mg of $enzyme^{-1}$. Our results are shown in Table I.

Table I. Inhibition of Chymotrypsin A_α with *N*-Acyl- and *N*-Peptidyl-2-phenylethylamines[a]

Inhibitor	K_I, m*M* (std dev)	V_{max}[b] (std dev)
None	3.71[c] (0.17)	544 (13)
Ac-NCH$_2$CH$_2$C$_6$H$_5$ (CH$_3$)	36.5 (0.33)	530 (24)
Ac-NHCH$_2$CH$_2$C$_6$H$_5$	20.3 (0.52)	526 (33)
Ac-Gly-NHCH$_2$CH$_2$C$_6$H$_5$	9.74 (0.42)	554 (25)
Ac-Ala-NHCH$_2$CH$_2$C$_6$H$_5$	8.77 (0.47)	600 (33)
Ac-Leu-NHCH$_2$CH$_2$C$_6$H$_5$	4.77 (0.31)	526 (22)
Ac-Gly-Gly-NHCH$_2$CH$_2$C$_6$H$_5$	8.23 (0.14)	530 (10)
Ac-Gly-Leu-NHCH$_2$CH$_2$C$_6$H$_5$	1.93 (0.42)	532 (26)
Z-Gly-Leu-NHCH$_2$CH$_2$C$_6$H$_5$	4.38 (0.12)	530 (9)

[a] 9.5% ethanol, 0.10 *M* $CaCl_2$, pH 7.80, 25.0°. [b] Micromoles of substrate hydrolyzed min^{-1} mg of $enzyme^{-1}$. [c] K_M for the hydrolysis of acetyl tyrosine ethyl ester.

Discussion

Recent X-ray crystallographic and solution kinetic studies have demonstrated the existence of an extended substrate binding site in chymotrypsin.[2,4,5] Substrates and substrate related inhibitors are bound to the enzyme *via* a series of hydrophobic contacts and hydrogen bonds. The extended peptide chain of a substrate or substrate related inhibitor forms an antiparallel β-sheet structure with the peptide backbone of residues Ser-214, Trp-215, and Gly-216 of the enzyme. Examination of models of substrates or inhibitors complexed with chymotrypsin has shown that the extended substrate binding site can conveniently be discussed in terms of at least three binding subsites.[14] The primary specificity site S_1, which gives chymotrypsin its specificity for aromatic amino acid residues, consists of the tosyl pocket and the carbonyl group of Ser-214 which can form a hydrogen bond with a substrate. The tosyl pocket, so named since the tosyl

(9) A. Bischler and B. Napieralski, *Chem. Ber.*, **26**, 1905 (1893).

(10) H. E. Baumgarten, F. A. Bower, R. A. Setterquist, and R. E. Allen, *J. Amer. Chem. Soc.*, **80**, 4588 (1958).

(11) F. J. Kezdy and E. T. Kaiser, *Methods Enzymol.*, **19**, 3 (1970).

(12) A. R. Henderson, *Anal. Biochem.*, **42**, 143 (1971).

(13) Of the various linear transformations of the Michaelis–Menten equation this transformation has been shown to give more reliable results than the standard Lineweaver–Burk plot: J. E. Dowd and D. S. Riggs, *J. Biol. Chem.*, **240**, 863 (1965).

(14) In the discussion of binding of various substrates and inhibitors to chymotrypsin, we have adopted the notation originally proposed by I. Schechter and A. Berger, *Biochem. Biophys. Res. Commun.*, **27**, 157 (1967). The individual amino acid residues of a substrate (or inhibitor) are designated P_1, P_2, etc., numbering from the amino acid which supplies the carbonyl group of the peptide bond which is cleaved by the enzyme and numbering in the direction of the amino-terminal end of the substrate. The corresponding subsites of the enzyme which interact with the substrate are designated S_1, S_2, etc. For *N*-acyl- and *N*-peptidyl-2-phenylethylamines, the NHCH$_2$CH$_2$C$_6$H$_5$ group would be designated the P_1 residue, and the tosyl pocket and the carbonyl group of Ser-214 which interact with it would be called the S_1 subsite.

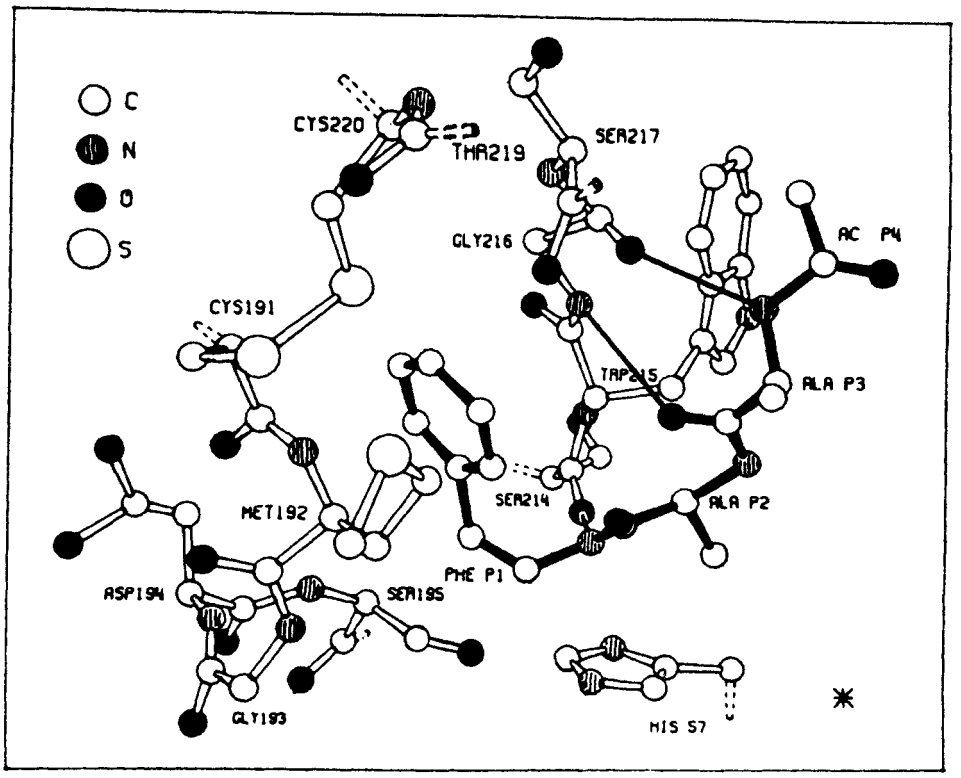


Figure 1. The productive binding mode of Ac-Ala-Ala-$NHCH_2CH_2C_6H_5$ with chymotrypsin.

Table II. Free Energies of Binding of *N*-Acyl- and *N*-Peptidyl-2-phenylethylamines to Chymotrypsin A_α[a]

Inhibitor				$\Delta G°$, kcal mol^{-1}	$\Delta\Delta G°$, kcal mol^{-1}[b]	S_3		S_2 Hydrophobic contact[e]	S_1	
P_4	P_3	P_2	P_1			H-Bond[c]	H-Bond[d]		H-Bond[f]	Contact[g]
			$Ac\text{-}N(CH_3)CH_2CH_2C_6H_5$	−1.96 (0.006)[h]	0					+
			$Ac\text{-}NHCH_2CH_2C_6H_5$	−2.31 (0.016)	−0.35				+	+
		Gly	$Ac\text{-}NHCH_2CH_2C_6H_5$	−2.74 (0.026)	−0.8		+		+	+
		Ala	$Ac\text{-}NHCH_2CH_2C_6H_5$	−2.80 (0.031)	−0.8		+		+	+
		Leu	$Ac\text{-}NHCH_2CH_2C_6H_5$	−3.16 (0.038)	−1.2		+	+	+	+
	Gly	Gly	$Ac\text{-}NHCH_2CH_2C_6H_5$	−2.84 (0.010)	−0.9	+	+		+	+
	Gly	Leu	$Ac\text{-}NHCH_2CH_2C_6H_5$	−3.70 (0.12)	−1.7	+	+	+	+	+
Z	Gly	Leu	$NHCH_2CH_2C_6H_5$	−3.21 (0.017)	−1.3	+	+	+	+	+

[a] 9.5% ethanol, 0.10 *M* $CaCl_2$, pH 7.80, 25.0°, standard state 1 *M*. [b] Differences in binding free energies using $AcN(CH_3)CH_2CH_2C_6H_5$ as the reference compound. [c] Hydrogen bond with the carbonyl group of Gly-216. [d] Hydrogen bond with the NH of Gly-216. [e] Hydrophobic contact with the side chain of Ile-99. [f] Hydrogen bond with the carbonyl group of Ser-214. [g] Hydrophobic contact with the "tosyl pocket." [h] Values in parentheses are standard deviations.

group in tosyl–chymotrypsin A_α was first found located in this region of the enzyme, is a *ca.* 12 × 6.5 × 4.0-Å hole in the enzyme surface formed mainly by sections of peptide backbone of chymotrypsin. The interactions at the S_2 binding site are mainly hydrophobic. The P_2 residue of a substrate (or inhibitor) would make van der Waals contact with the backbone and C_α–C_β bond of Trp-215 and also with Ile-99 if the P_2 residue had an appropriate side chain. The S_3 subsite consists of two hydrogen bonding groups (NH and C=O of Gly-216).

The primary purpose of the present study was to obtain free energies of binding for a series of substrate related competitive inhibitors, *N*-acyl- and *N*-peptidyl-2-phenylethylamines (**4**) in order to measure the importance of each of the subsite binding interactions. A drawing of the productive mode of binding for a *N*-peptidylphenylethylamine is shown in Figure 1 and the free energies of binding for all the inhibitors investigated and their interactions with chymotrypsin are shown in Table II.

The free energy of binding of an inhibitor (or substrate) to an enzyme in solution represents the difference in free energy between the solvated enzyme–inhibitor complex and the sum of the free energies for the solvated enzyme and the solvated inhibitor. A negative free energy of binding could result from a favorable interaction between the enzyme and inhibitor, from a favorable entropy change due to the release of solvent molecules upon complex formation, or from a combination of both. In this study we made no attempt to distinguish between the various possibilities since that must await measurement of the ΔS and ΔH of binding of the inhibitors to chymotrypsin and the ΔS and ΔH of solvation for the enzyme and all of the inhibitors.

Examination of the data listed in Table II shows that

the free energy of binding ranges from −1.96 kcal/mol for Ac-N(CH_3)$CH_2CH_2C_6H_5$ to a high of −3.70 for Ac-Gly-Leu-N$HCH_2CH_2C_6H_5$. In general, the free energy of binding in this series of *N*-acyl- and *N*-peptidyl-2-phenylethylamines increases with increasing number of positive interactions with the enzyme, as predicted from the crystallographic model. This is certainly evidence that we are observing productive binding.[15] The two major contributors to the binding of these inhibitors are hydrophobic interactions. The first is the S_1–P_1 tosyl pocket–phenylethyl group interaction (2 kcal/mol, a cratic contribution is included in this value but is canceled in the $\Delta\Delta G°$ values) and the second is the interaction of the side chain of a P_2 leucine residue with Ile-99 in the S_2 subsite of chymotrypsin.

Hydrogen bonding apparently contributes very little to the overall free energy of binding. The difference between Ac-N(CH_3)$CH_2CH_2C_6H_5$ which cannot hydrogen bond and Ac-N$HCH_2CH_2C_6H_5$ which can form the S_1–P_1 hydrogen bond is −0.35 kcal/mol. With the assumption that the methyl group of Ac-N(CH_3)$CH_2CH_2C_6H_5$ does not affect the free energy of binding to the enzyme, −0.35 kcal/mol represents the difference between an inhibitor–enzyme hydrogen bond and an inhibitor–solvent hydrogen bond. If, however, the transfer of the methyl group of Ac-N(CH_3)$CH_2CH_2C_6H_5$ from the aqueous solution to the enzymes surface in the enzyme–inhibitor complex is a favorable change, then the actual difference between the inhibitor–enzyme and inhibitor–solvent hydrogen bond could be substantially higher than −0.35 kcal/mol. The S_3–P_3 hydrogen bond involving the NH of Gly-216 contributes at least −0.46 kcal/mol (compare Ac-N$HCH_2CH_2C_6H_5$ with Ac-Gly-N$HCH_2CH_2C_6H_5$ or Ac-Ala-N$HCH_2CH_2C_6H_5$) while the S_3–P_3 hydrogen bond involving the C=O of Gly-216 contributes somewhat less (−0.10 kcal/mol, compare Ac-Gly-N$HCH_2CH_2C_6H_5$ with Ac-Gly-Gly-N$HCH_2CH_2C_6H_5$) to the overall binding free energy of an inhibitor. These free energy differences are not simply the differences between an inhibitor–enzyme hydrogen bond and an inhibitor–solvent hydrogen bond, but also represent contributions to the binding free energy from additional contacts, such as the hydrophobic contact of the P_2 residue with the S_2 subsite between the longer inhibitors and chymotrypsin. In addition there is certainly an entropy loss upon binding the longer inhibitors relative to the shorter ones due to rigidification of the additional amino acid residues which would effectively reduce the binding free energy due to the additional hydrogen bonds or hydrophobic interactions.

Our conclusions in respect to the importance of the S_1–P_1 hydrophobic interaction are consistent with earlier observations. Ingles and Knowles[17] showed that dissociation constants (K_I) of complexes of chymotrypsin with a series of inhibitors related to acetylphenylalanine are largely independent of the existence of an acylamino group in the inhibitor. Recently, Caplow and Harper[18] have found little difference in the kinetically derived dissociation constants (K_S) of complexes of N-methylated or nonmethylated tyrosine ethyl ester (and hydroxamic acid) with chymotrypsin. Both studies show that the S_1–P_1 hydrophobic interaction is primarily responsible for binding simple substrates to chymotrypsin.

Our data also indicate that the contributions from individual subsite interactions to the overall binding free energy are not strictly additive. This is evident when the contribution of a P_2 leucine residue is examined. The leucine contributes *ca.* −0.4 kcal/mol to the binding of a dipeptide inhibitor (compare Ac-Gly-N$HCH_2CH_2C_6H_5$ or Ac-Ala-N$HCH_2CH_2C_6H_5$ with Ac-Leu-N$HCH_2CH_2C_6H_5$) while it contributes *ca.* −0.9 kcal/mol to the binding of a tripeptide (compare Ac-Gly-Gly-N$HCH_2CH_2C_6H_5$ with Ac-Gly-Leu-N$HCH_2CH_2C_6H_5$). Possibly, the additional hydrogen bond in the tripeptide inhibitor changes the orientation of the inhibitor such that the P_2 leucine makes a more favorable contact with the side chain of Ile-99 of the enzyme.

Examination of a model of chymotrypsin led to the hypothesis that a fourth binding subsite (S_4) favorable for aromatic groups might exist, since the side chains of Trp-172 and Trp-215 lie on the surface of the protein in this region. However, our data show that Z-Gly-Leu-N$HCH_2CH_2C_6H_5$, which contains a P_4 aromatic residue, is bound less tightly than Ac-Gly-Leu-N$HCH_2CH_2C_6H_5$ by *ca.* 0.4 kcal/mol. An analogous reactivity difference was observed in inhibition of chymotrypsin with peptide chloro ketones containing either a benzyloxycarbonyl group (Z) or an acetyl group as the P_4 residue.[5] Models of enzyme–inhibitor complexes show that the benzyloxycarbonyl group of the inhibitor cannot make a good contact with Trp-215 or Trp-172 in the S_4 binding site of the enzyme. It is probable that an inhibitor with a shorter distance separating the aromatic group of the P_4 residue from the P_3 residue would make better contact with the surface of the enzyme at the S_4 subsite.

Using kinetically determined K_M values,[19] Segal[4] has

(15) The one difficulty with the use of competitive inhibitors is the possibility of observing nonproductive binding modes relative to those expected for the corresponding substrate. An example of nonproductive binding with the serine protease subtilisin has recently been reported by Kraut and his coworkers.[16] Among a series of peptide acids whose binding to subtilisin was investigated crystallographically, Z-Ala-Phe was found to bind in reverse sense with the benzyloxycarbonyl group pointing toward the catalytic residues. With subtilisin such nonproductive binding modes are not unexpected since both the S_1 and S_4 subsites effectively bind aromatic amino acid residues and in this one case the phenylalanine residue of Z-Ala-Phe is bound at the S_4 subsite rather than at the S_1 subsite as expected. With the possible exception of Z-Gly-Leu-N$HCH_2CH_2C_6H_5$ which has a phenyl group at both ends of the inhibitor, none of the *N*-acyl- or *N*-peptidyl-2-phenylethylamines which we investigated would be expected to bind to any substantial extent in nonproductive modes. There are several reasons for such an expectation. First, all X-ray studies of complexes of chymotrypsin with inhibitors such as indole or formyl tryptophan have shown the aromatic group of the inhibitor to be located in the "tosyl pocket" of the enzyme near the catalytic groups (ref 3). Secondly, the major contributor to the overall free energy of binding of an extended inhibitor is the contribution (2 kcal/mol) of the $C_6H_5CH_2CH_2$– group of the inhibitor. None of the other interactions between the inhibitor and the enzyme is as significant. Thus by analogy with the crystallographic results and the known substrate specificity of chymotrypsin, the $C_6H_5CH_2CH_2$– group of each inhibitor should at least be bound in the tosyl pocket of the enzyme. Furthermore, the bonding free energies of the various inhibitors are in line with those expected from the crystallographic model.

(16) J. D. Robertus, J. Kraut, R. A. Alden and J. J. Birktoft, *Biochemistry*, **11**, 4293 (1972).

(17) D. M. Ingles and J. R. Knowles, *Biochem. J.*, **108**, 561 (1968).

(18) M. Caplow and C. Harper, *J. Amer. Chem. Soc.*, **94**, 6508 (1972).

(19) For the following kinetic scheme which has been shown to be

$$E + S \overset{K_S}{\rightleftharpoons} E\cdot S \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2$$

valid for the chymotrypsin hydrolysis of peptide esters and amides, the true dissociation constant (K_S) of the enzyme–substrate complex is related to the Michaelis–Menten constant (K_M) by

$$K_S = [(k_2 + k_3)/k_3]K_M$$

calculated relative free energy changes for the binding of a series of peptide esters to chymotrypsin A_γ, and Izumiya and Yamashita[20] in a series of papers have obtained the K_M values for a large number of peptide amides and esters containing tyrosine as the P_1 residue by measuring their rates of chymotryptic hydrolysis. On the basis of these studies we were able to make several comparisons of individual subsite binding free energies for different systems. Table III lists the free energy changes as affected by chain length and Table IV

Table III. Effect of Chain Extension on Relative Free Energies of Binding to Chymotrypsin[a]

	X = $NHCH_2$-$CH_2C_6H_5$[b]	X = Phe-OMe[c]	X = Tyr-NH_2[d]
Ac-X	0	0	0
Ac-Gly-X	−0.4	−0.3	−2.2
Ac-Gly-Gly-X	−0.5	−1.3	

[a] The free energy of binding for the first compound in the series is used as the reference to which the others are compared. All values are in kcal/mol. [b] 9.5% ethanol, pH 7.8, 25°. [c] 5% dimethylformamide, pH 7.0, 25°, data of Segal.[4] [d] pH 7.8, 30°, data of Yamashita.[20c]

certainty whether the differences are due to experimental variations or to subtle differences in the orientation of each series of substrates or inhibitors with chymotrypsin.

One final point should be made concerning the magnitude of the dissociation constant (K_I) of chymotrypsin complexes with *N*-acyl- and *N*-peptidyl-2-phenylethylamines compared to the K_M values for the corresponding substrates. Several comparisons can be made: Ac-Tyr-NH_2 (32 m*M*) and Ac-Phe-NH_2 (31 m*M*)[22] both have higher K_M values than the K_I for Ac-$NHCH_2CH_2C_6H_5$ (20.3 m*M*), the K_M value of Gly-Gly-Tyr-NH_2 (41 m*M*)[20d] is higher than the K_I value of Ac-Gly-Gly-$NHCH_2CH_2C_6H_5$ (8.2 m*M*), and the K_M value for Gly-Leu-Tyr-NH_2 (21 m*M*)[20d] is higher than the K_I value of Ac-Gly-Leu-$NHCH_2CH_2C_6H_5$ (1.9 m*M*). Although the comparisons are not ideal, in each case the K_I had the lower magnitude, indicating that the phenylethylamine derivatives *are more tightly bound* to the enzyme than the corresponding substrates. Since the conditions under which these K_I were measured (9.5% ethanol) would be expected to give higher values than in water solution with no organic cosolvents, the actual differences under exactly comparable conditions

Table IV. Effect of the P_2 Residue on Relative Free Energies of Binding to Chymotrypsin[a]

	AA				
	Gly	Ala	Leu	Pro	Val
Ac-AA-$NHCH_2CH_2C_6H_5$[b]	0	−0.06	−0.42		
Ac-Gly-AA-$NHCH_2CH_2C_6H_5$[b]	0		−0.86		
Ac-Gly-AA-Phe-OMe[c]	0			−0.66	−1.02
AA-Tyr-NH_2[d]	0	+0.11	−1.52	−1.06	+0.26
Gly-AA-Tyr-NH_2[e]	0	+0.50	−0.41		−0.41

[a] The free energy of binding for the first compound in the series is used as the reference to which the others are compared. All values are in kcal/mol. [b] 9.5% ethanol, pH 7.8, 25°. [c] 5% dimethylformamide, pH 7.0, 25°, data of Segal.[4] [d] pH 7.8, 30° (ref 20a and 20b). [e] pH 7.8, 30° (ref 20d).

lists the effect of the P_2 residue on the relative free energies of binding. It is evident from the data in Table III that although the direction of the free energy changes are similar from system to system, the magnitudes vary over a wide range although part of the differences may be due to the slightly differing experimental conditions. It is also clear from Table IV, except for one case, that an amino acid with a large aliphatic side chain at the P_2 position of a substrate or an inhibitor makes a favorable interaction with the enzyme. However, the free energy values range from −0.4 to −1.5 kcal/mol for a P_2 leucine residue[21] and data must be obtained from other systems before one can know with would be much higher. This is consistent with the recent observation by Kraut and his coworkers[16] that the S_1–P_1 H-bond is not formed in complexes of the structurally similar serine protease subtilisin with peptide acids, whereas this S_1–P_1 H-bond is found in complexes of subtilisin with peptide chloro ketones. They postulate that this S_1–P_1 hydrogen bond is formed concurrently with the formation of a tetrahedral intermediate. The formation of this hydrogen bond and movement of the aromatic side chain of the P_1 residue deeper into the S_1 tosyl pocket thus may be one way the serine proteases place the scissionable peptide bond on a stereoelectronic "rack" favoring formation of the tetrahedral intermediate. The fact that *N*-acyl- and *N*-peptidyl-2-phenylethylamines which are analogs of substrates lacking the scissionable peptide bond are bound more tightly to chymotrypsin than the corresponding substrates is certainly consistent with such a stereoelectonic "rack." Interestingly, Ingles and Knowles[17] made a very similar interpretation of data which showed that the dissociation constants of complexes of chymotrypsin with a series of inhibitors were independent of the ability to form the S_1–P_1 hydrogen bond.

B. Zender and M. L. Bender, *J. Amer. Chem. Soc.*, **86**, 3669 (1964). For amide substrates where $k_3 \gg k_2$ then $K_S \approx K_M$ while for ester substrates where this is not generally true, K_M can be considered to be a lower limit estimate of K_S.

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(21) A free energy difference of −2.9 kcal/mol between a D- and L-leucine residue in the P_2 position of a dipeptide (Ac-Leu-Tyr-OMe) has been reported. This can be considered to be the approximate difference between the glycine compound and the L-leucine compound since the side chain in the D compound would point into solution away from the enzyme: N. F. Kazanskaya, E. M. Slobodyanska, V. I. Tsetlin, E. N. Shepel, V. T. Ivanov, and Y. A. Ovchinnikow, *Biokhimiya*, **35**, 1147 (1970).

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