Chymotrypsin inhibitory conformation induced by amino acid side chain–side chain intramolecular CH/π interaction

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Dipeptide amides H-D-Leu-Phe-NH-R have been found to assume a conformation induced by the CH/π interaction and to inhibit chymotrypsin strongly. A series of benzyl amide derivatives H-D-Leu-Phe-NH-$[CH_2]_n$-C_6H_5 (n = 0–4) have been assayed for chymotrypsin. They inhibit the enzyme in a competitive manner and the highest inhibition is achieved by the amide of n = 1 (K_i = 3.6 × 10^{-6} M). The activity enhancement is dependent upon the length of methylene chain, not upon the increase in molecular hydrophobicity, indicating the presence of an optimal distance between dipeptide backbone and C-terminal phenyl group for chymotrypsin inhibition. The C-terminal phenyl group has been found to interact with chymotrypsin stereospecifically. The *R*-isomer of H-D-Leu-Phe-NH-$CH(CH_3)$-C_6H_5 is as active as the benzyl amide, while the *S*-isomer is about twenty-fold less active. When the fluorine atom is introduced at a *para*-position of the C-terminal phenyl group, the resulting dipeptide H-D-Leu-Phe-NH-CH_2-C_6H_4F-*p* exhibits about six-times increased inhibitory activity (K_i = 6.1 × 10^{-7} M; this dipeptide is one of the most potent chymotrypsin inhibitors to date). ^{1}H NMR conformational analyses of these dipeptide amide derivatives show the CH/π interaction between D-Leu-isobutyl and Phe-phenyl as a key structural element for chymotrypsin inhibition. These structural examinations strongly suggest that in the inhibitory conformation the C-terminal phenyl group fits the chymotrypsin S_1 site, while the hydrophobic core constructed by D-Leu-Phe CH/π interaction fits the chymotrypsin S_2 or S_1' site.

Introduction

The bioactive conformation of a peptide ligand is the steric arrangement that the peptide adopts in order to fit the binding site of acceptor molecules. In general, the stabilization or fixation of bioactive conformation reinforces the ligand–acceptor interaction, resulting in enhancement of biological activity. For peptidic ligands, cyclization, for instance, is an effective way to fix the conformation.[1] The validity of cyclization has been exemplified in such receptor agonists as enkephalin,[2] dynorphin[3] and substance P.[4]

Another way to substantiate a specific interaction is to load structural elements into a bioactive structure. For example, Ac-Pro-Ala-Pro-phenylalaninal and boro-Phe derivatives were found to be effective chymotrypsin inhibitors.[5,6] In addition to the substrate structure for chymotrypsin, these inhibitors contain an aldehyde group and boric acid, respectively, as a key functional element for inhibition.

Several chymotrypsin-like proteases have recently been found in cells from lesions such as those present in muscular dystrophy[7] and rheumatoid arthritis,[8] leukaemia[9] and allergy.[10] Although inhibitors of these chymotrypsin-like enzymes are expected to be therapeutics, only a limited number of compounds are known as specific inhibitors of chymotrypsin or chymotrypsin-like enzymes.[11] A difficulty in designing the chymotrypsin inhibitors is attributed to the fact that the inhibitor–enzyme interactions at the catalytic site are mainly hydrophobic.

Recently, we have found that H-D-Leu-Phe-OBzl inhibits chymotrypsin very effectively (K_i = 22 × 10^{-6} M).[12] Although this dipeptide benzyl ester seemed to be devoid of an apparent key functional group for inhibition, it was found from the ^{1}H NMR conformational analyses that the side chains of D-Leu-Phe are in close proximity. This hydrophobic interaction appeared to induce a conformation which led to a structure that fits the enzyme active centre. The design of such dipeptide inhibitors originated from our previous studies in which a structurally constrained amino acid α,β-cyclopropylphenylalanine was incorporated into dipeptides.[13–15] The phenyl group of α,β-cyclopropylphenylalanine was found to make a hydrophobic interaction with the C-terminal methyl group. The recent novel idea of 'CH/π interaction' by Nishio *et al.* (1995) has characterized these hydrophobic interactions as a kind of hydrogen bonding between C–H groups and the phenyl π-electron system.[16] In the present study, in order to ascertain the inhibitory conformation of D-Leu-Phe dipeptides against chymotrypsin, we have carried out several structural examinations of their benzyl amide derivatives.

Results

Resistance of dipeptides to chymotrypsin hydrolysis

The stability of synthetic dipeptides against chymotrypsin was assessed by monitoring on reversed-phase HPLC. Peaks corresponding to the starting esters or amides and their free acid derivatives were integrated at certain intervals. Ac-Tyr-OEt (ATEE) was completely degraded within 20 min by chymotrypsin. Although H-D-Leu-Phe-OBzl was fairly stable, it was also completely hydrolysed to H-D-Leu-Phe-OH within 90 min (Fig. 1). This indicated that H-D-Leu-Phe-OBzl is a partial inhibitor, being also a substrate of chymotrypsin.

When the benzyl amide H-D-Leu-Phe-NHBzl was tested for

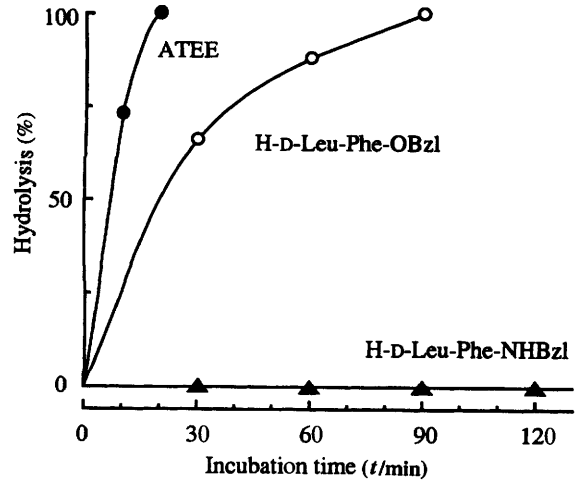

Fig. 1 Chymotrypsin hydrolysis of H-D-Leu-Phe dipeptide benzyl amide (▲) and ester (○) along with Ac-Tyr-OEt (ATEE) (●). The percentage of residual compound was estimated from the HPLC (ODS C_{18} column) peak integration against the amount at 0 min.

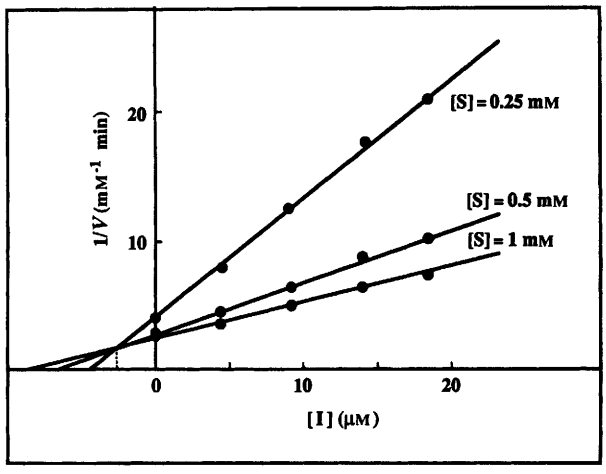

Fig. 2 Dixon plot for chymotrypsin hydrolysis of ATEE in the presence of H-D-Leu-L-Phe-NHBzl at pH 7.0 (50 mM phosphate buffer) and 25 °C. The substrate concentrations used are indicated in the Figure, and the K_i-value was estimated from the cross-point of three lines as 3.6×10^{-6} M.

Table 1 Inhibitory potency of Leu-Phe dipeptides against chymotrypsin

Dipeptide	Inhibitory potency	
	K_i (μM)	Relative potency
D-Leu-Phe-O-CH_2-C_6H_5	22 ± 1.5	100
D-Leu-Phe-NH-CH_2-C_6H_5	3.6 ± 0.24	610
D-Leu-Ala-NH-CH_2-C_6H_5	64 ± 0.6	44
D-Leu-Phe-NH-CH_3	Inactive	0
D-Leu-Phe-NH_2	Inactive	0
D-Leu-Phe-NH-(*R*)-CH(CH_3)-C_6H_5	4.7 ± 1.2	480
D-Leu-Phe-NH-(*S*)-CH(CH_3)-C_6H_5	210 ± 21	10
D-Leu-Phe-NH-CH_2-C_6H_4F-*p*	0.61 ± 0.05	3600
L-Leu-Phe-NH-CH_2-C_6H_5	140 ± 22	16

hydrolysis by chymotrypsin, no hydrolysis was observed after 2 h (Fig. 1). Even much more prolonged incubation times (24 and 48 h) did not in the least affect its enzyme stability. None of the dipeptide amides having the D-L configurational sequence were degraded by chymotrypsin, and even H-Leu-Phe-NHBzl in the L-L configurational sequence was not degraded at all.

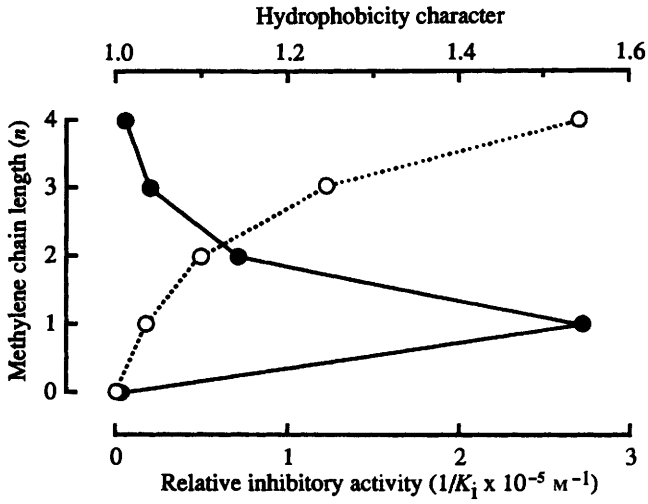

Fig. 3 Chymotrypsin inhibitory activity (●) and molecular hydrophobicity (○) of H-D-Leu-Phe-NH-$[CH_2]_n$-C_6H_5 as a function of methylene chain length *n*. The inhibitory potency was evaluated as the reciprocal of the inhibitory constants. The molecular hydrophobicity was estimated as the ratio of the retention time relative to that of H-D-Leu-Phe-NH_2, which has no phenyl group on the amide.

Inhibitory activity of dipeptides against chymotrypsin

(A) Inhibitory potency. Inhibitory activity of dipeptide amides against chymotrypsin was examined by using ATEE as a substrate. It was found that dipeptide benzyl amide H-D-Leu-Phe-NHBzl can inhibit chymotrypsin strongly. Analyses by Dixon plot[17] (Fig. 2) and Lineweaver–Burk plot[18] indicated the inhibition to be competitive. The K_i-value was calculated to be 3.6×10^{-6} M (Fig. 2), indicating that this benzyl amide is about six-fold more effective than the corresponding benzyl ester ($K_i = 22 \times 10^{-6}$ M).

The inhibitory constants (K_i) of dipeptide derivatives are shown in Table 1. Dipeptide amides lacking the C-terminal benzene ring, namely D-Leu-Phe-$NHCH_3$ and D-Leu-Phe-NH_2, were completely inactive. On the other hand, the removal of phenyl from Phe-2, forming D-Leu-Ala-NHBzl, caused a considerable inhibition. These results indicated that the C-terminal phenyl group is essential for inhibition of chymotrypsin, probably by binding to the specific binding site of chymotrypsin. The importance of the Phe-2 phenyl group should also be noted, because a sharp drop (18-fold) in inhibitory activity by the removal of Phe-2 phenyl was very prominent. Furthermore, the importance of the D-L configurational sequence should be emphasized with weak inhibitory activity of the L-L isomer. Although the L-L isomer of H-D-Leu-Phe-NHBzl also inhibited chymotrypsin, it was extremely weak (only 2.5% of D-isomer) (Table 1).

(B) Structural essentials for inhibition. A series of derivatives of the dipeptide benzyl amide, H-D-Leu-Phe-NH-$[CH_2]_n$-C_6H_5 (n = 0–4), were tested to examine the effect of the methylene chain length between dipeptide backbone and phenyl group. If two phenyl groups in H-D-Leu-Phe-NH-CH_2-C_6H_5 are spatially arranged for specific interaction with chymotrypsin, there would be an optimal spacer length to make both phenyls fit the respective binding sites. When inhibitory potencies were compared in terms of the reciprocal of inhibitory constants, $1/K_i$, as shown in Fig. 3, a sharp peak emerged at $n = 1$. The benzyl amide ($n = 1$) was most potent, indicating that the length with $n = 1$ is an optimum intramolecular methylene chain length.

When the number of methylene chains increases, the molecular hydrophobicity of dipeptides would also increase. This may result in the enhancement of nonspecific or/and specific hydrophobic interaction between dipeptides and enzyme, reinforcing the enzyme inhibition. In this study, the

molecular hydrophobicity was evaluated by the retention time in analytical HPLC equipped with a reversed-phase ODS-C_{18} column. As expected, dipeptides with an increasing number of methylene chains emerged later with larger retention times, indicating that they were retained longer in a column due to their stronger hydrophobic interaction with C_{18} silica gel. It should be noted, however, that this increase in hydrophobicity is completely independent of the inhibitory potency of dipeptides. As shown in Fig. 2, no relationship between two curves of inhibitory potency and molecular hydrophobicity is obvious. Thus, it was concluded that the maximal inhibitory activity by the benzyl amide ($n = 1$) is due to its structural optimization to inhibit the enzyme. The optimal distance $n = 1$ between the C-terminal phenyl group and the dipeptide backbone would correspond to the distance between the binding sites in chymotrypsin.

(C) Stereospecificity in inhibition. If the C-terminal phenyl group of H-D-Leu-Phe-NH-CH_2-C_6H_5 ($n = 1$) fits the chymotrypsin S_1 site, the interaction of this phenyl group with the S_1 site would be stereospecific. By introducing a methyl group into the methylene group of the C-terminal benzyl moiety, two stereoisomers of (*R*)- and (*S*)-1-phenylethyl amides are feasible. Although both isomers inhibited chymotrypsin in a competitive manner, they exhibited considerably different inhibitory potencies. The *R*-isomer H-D-Leu-Phe-NH-(*R*)-$CH(CH_3)$-C_6H_5 was found to be potent, its K_i value (4.7×10^{-6} M) being comparable to that of the benzyl amide (Table 1). In sharp contrast, the *S*-isomer was very weak ($K_i = 2.1 \times 10^{-4}$ M), approximately 20-fold less active than the *R*-isomer and benzyl amide.

(D) Activity enhancement by *p*-fluorophenyl derivative. If the C-terminal phenyl group fits the S_1 site, the head of this group would face toward Ser-189 of the chymotrypsin S_1 pocket. Expecting hydrogen bonding with the hydroxy group of Ser-189, we prepared the *p*-fluorophenyl derivative. It was found that H-D-Leu-Phe-NH-CH_2-C_6H_4F-*p* inhibits chymotrypsin very strongly, its inhibitory constant (6.1×10^{-7} M) being one order of magnitude smaller than that of H-D-Leu-Phe-NH-CH_2-C_6H_5.

Conformational analysis by ^{1}H NMR spectroscopy in $(CD_3)_2SO$ solution

(A) CH/π side chain–side chain interaction. For a series of benzyl amide analogues, ^{1}H NMR measurements were carried out in [$^2H_6$]dimethyl sulfoxide ([$^2H_6$]DMSO) at room temperature. Signal assignments were accomplished basically on 2D COSY spectra and 2D long-range COSY spectra (COSY = chemical-shift correlation spectroscopy). When the chemical shifts in [$^2H_6$]DMSO of Leu side-chain protons of H-D-Leu-Phe-NHBzl were compared with those of H-D-Leu-Ala-NHBzl which lacks the Phe phenyl group, considerably larger upfield shifts were observed for D-Leu βCH$_2$ (0.372 ppm), γCH (0.572 ppm) and δCH$_3$ (0.212 ppm) (Fig. 4 and Table 2). All dipeptide amides having a D-Leu-Phe backbone structure exhibited large upfield shifts of proton signals of the D-Leu side chain; βCH$_2$ (0.37–0.41 ppm), γCH (0.39–0.57 ppm) and δCH$_3$ (0.17–0.22 ppm) (Table 2). These upfield shifts are certainly

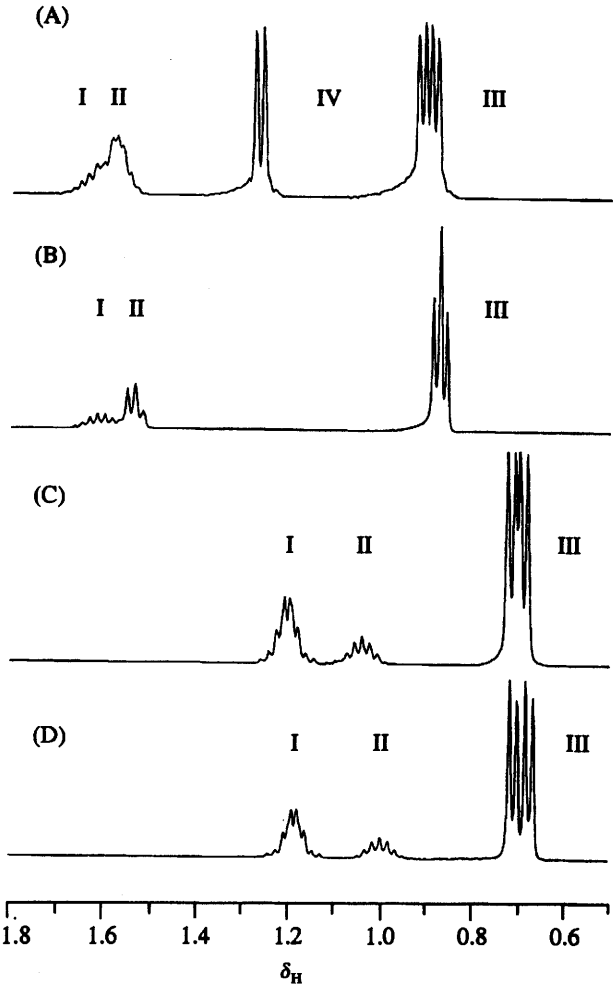

Fig. 4 Comparison of chemical shifts of the Leu isobutyl group in 400 MHz ^{1}H NMR 1D spectra of Leu-containing dipeptides. (A) H-D-Leu-Ala-NHBzl, (B) H-L-Leu-Phe-NHBzl, (C) H-D-Leu-Phe-NHBzl and (D) H-D-Leu-Phe-NHCH$_3$. The spectra were recorded in [$^2H_6$]-DMSO at 25 °C, and each signal was assigned by 2D COSY and 2D long-range COSY measurements. Signals were assigned as follows: I, Leu βCH_2; II, Leu γ*CH*; III, Leu δCH_3 and IV in the spectrum A, Ala βCH_3.

Table 2 Upfield shifts of proton chemical signals of D-Leu isobutyl in 400 MHz ^{1}H NMR 1D measurements related to those of D-Leu-Ala-NHBzl

Dipeptide	Upfield shifts (ppm)		
	Leu βCH_2	Leu γCH	Leu δCH_3
D-Leu-Ala-NH-CH_2-C_6H_5	0.0 (1.567)[a]	0.0 (1.567)	0.0 (0.904)
D-Leu-Phe-NH-C_6H_5	0.371	0.391	0.172
D-Leu-Phe-NH-CH_2-C_6H_5	0.372	0.572	0.212
D-Leu-Phe-NH-[CH_2]$_2$-C_6H_5	0.395	0.521	0.205
D-Leu-Phe-NH-[CH_2]$_3$-C_6H_5	0.383	0.505	0.198
D-Leu-Phe-NH-[CH_2]$_4$-C_6H_5	0.392	0.492	0.199
D-Leu-Phe-NH-CH_3	0.387	0.576	0.212
D-Leu-Phe-NH_2	0.390	0.567	0.215
D-Leu-Phe-NH-(*R*)-$CH(CH_3)$-C_6H_5	0.397	0.405	0.188
D-Leu-Phe-NH-(*S*)-$CH(CH_3)$-C_6H_5	0.397	0.405	0.188
D-Leu-Phe-NH-CH_2-C_6H_4F-*p*	0.378	0.561	0.209
L-Leu-Phe-NH-CH_2-C_6H_5	0.048	−0.024	0.037

[a] Values in parentheses show the chemical shifts (ppm) of protons of D-Leu isobutyl in D-Leu-Ala-NH-CH_2-C_6H_5.

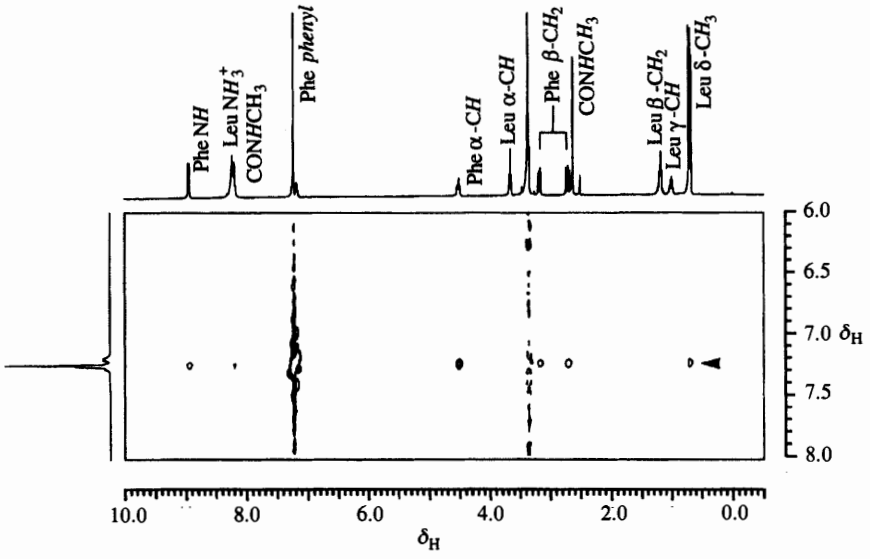

Fig. 5 2D NOESY spectrum for pairs of the Phe-2 aromatic ring protons and aliphatic protons in D-Leu-Phe-$NHCH_3$, which possesses only one aromatic ring (Phe-2). The cross-peak indicated with an arrowhead was assigned to the NOE between the Phe phenyl and the D-Leu δCH_3 groups.

attributed to the shielding effect from the Phe phenyl group. The fact that D-Leu isobutyl proton signals of H-D-Leu-Phe-NH_2 and H-D-Leu-Phe-$NHCH_3$ also showed similar upfield shifts confirms this conclusion. These dipeptides lack the C-terminal amide-phenyl group, and thus they possess only one benzene ring of Phe-2 in the molecule. These results implied that the Phe phenyl group is in proximity to D-Leu isobutyl.

(B) Nuclear Overhauser effect NOE enhancements between side chains. The presence of a side chain–side chain interaction between Leu isobutyl and Phe phenyl groups was further evidenced by measurements of two-dimensional NOESY spectra and by the nuclear Overhauser effect (NOE) for H-D-Leu-Phe-NHBzl and H-D-Leu-Phe-$NHCH_3$. The NOESY spectrum of H-D-Leu-Phe-NHBzl showed a cross-peak between D-Leu δ-CH_3 and an aromatic envelope, but no such peak was observed for H-L-Leu-L-Phe-NHBzl. Since the signals of two benzene rings from Phe-2 and C-terminal phenyl emerge in the same aromatic envelope, it is hard to identify which phenyl is in an interaction with D-Leu δ-CH_3. However, the cross-peak between D-Leu δ-CH_3 and the aromatic envelope was also clearly observed in the NOESY spectrum of H-D-Leu-Phe-$NHCH_3$ (Fig. 5). Since H-D-Leu-Phe-$NHCH_3$ lacks the C-terminal phenyl group, this result clearly indicates that the side chains of D-Leu and Phe are in proximity.

H–{H} NOE difference spectra were obtained by irradiation of each selected proton signal (data not shown). When proton signals of the D-Leu β-CH_2, γ-CH and δ-CH_3 in H-D-Leu-Phe-$NHCH_3$ were irradiated separately, distinct NOE enhancements were found for signals at the aromatic envelope (δ 7.2–7.4) in addition to those observed for signals of D-Leu protons, and *vice versa*. No enhancement was observed for those of H-D-Leu-Ala-NHBzl which lacks Phe phenyl. These results further confirm that the side chains of D-Leu and Phe are spatially in proximity to each other.

(C) Conformational rigidity. The signals (double doublets) of protons of Phe βCH_2 of H-D-Leu-Phe-NHBzl were non-equivalent, showing a splitting signal pattern ($\Delta\delta$ = 0.464 ppm). The H^{α}–H^{β} vicinal coupling constants of Phe were ~4.8 and 10.2 Hz. When the fractional population P_{I}, P_{II}, P_{III} for three staggered rotamers about the C^{α}–C^{β} bond of Phe were calculated using these *J*-couplings for Pachler's equation,[19] the obtained average P_X-values were 0.83, 0.11, 0.06 (the values of P_{I} and P_{II} are exchangeable) (Table 3). These data indicate that H-D-Leu-Phe-NHBzl has a substantial rigidity of the Phe side chain around the χ angle. This is certainly an indication that the Phe phenyl group is in close proximity to Leu isobutyl.

Table 3 Pachler's coefficient (P_X) for three staggered rotamers about the C^{α}–C^{β} bond of Phe-2 in Leu-Phe dipeptide benzyl amides and esters

Dipeptide	P_{I}	P_{II}	P_{III}
D-Leu-Phe-O-CH_2-C_6H_5	0.68	0.19	0.16
D-Leu-Phe-NH-CH_2-C_6H_5	0.83	0.11	0.06
D-Leu-Phe-NH-CH_3	0.80	0.12	0.08
L-Leu-Phe-O-CH_2-C_6H_5	0.48	0.33	0.19
L-Leu-Phe-NH-CH_2-C_6H_5	0.51	0.36	0.13

Since reported P_X values for H-D-Leu-Phe-OBzl were 0.68, 0.19, 0.16, it is evident that H-D-Leu-Phe-NHBzl is in a conformation much more rigid than that of benzyl amide. Other amide analogues also showed P_X-values similar to those of H-D-Leu-Phe-NHBzl (Table 3). No particular difference in ^{1}H NMR spectra was found between those of H-D-Leu-Phe-NH-(*R*)-CH(CH_3)-C_6H_5 and H-D-Leu-Phe-NH-(*S*)-CH(CH_3)-C_6H_5.

Conformation in water

Similar conformational analyses by 400 MHz ^{1}H NMR measurements were performed for H-D-Leu-Phe-NHBzl, H-D-Leu-Ala-NHBzl, H-D-Leu-Phe-$NHCH_3$ and H-L-Leu-L-Phe-NHBzl. Large upfield shifts for D-Leu βCH_2 (0.32–0.39 ppm), γCH (0.50–0.55 ppm) and δCH_3 (0.16–0.20 ppm) were observed, but not those in the L-Leu residue. Distinct NOESY cross-peaks between D-Leu δCH_3 and Phe phenyl were observed for H-D-Leu-Phe-$NHCH_3$ (data not shown), indicating that these groups are in proximity. Calculated average P_X values were 0.80, 0.11, 0.09 for three staggered rotamers about the C^{α}–C^{β} bond of Phe in H-D-Leu-Phe-$NHCH_3$. All these results indicated that dipeptides with a D-Leu-Phe backbone structure adopt essentially the same conformation in both water and [$^2H_6$]DMSO.

Discussion

Enzyme inhibitors often possess a structure similar to that of substrates. When H-D-Leu-Phe-OBzl was found to interact with chymotrypsin in a competitive manner, we thought that the Phe phenyl group might be directed toward the chymotrypsin S_1 site. Indeed, this residue fitted the S_1 site, since chymotrypsin eventually hydrolysed the ester bond. However, the results from

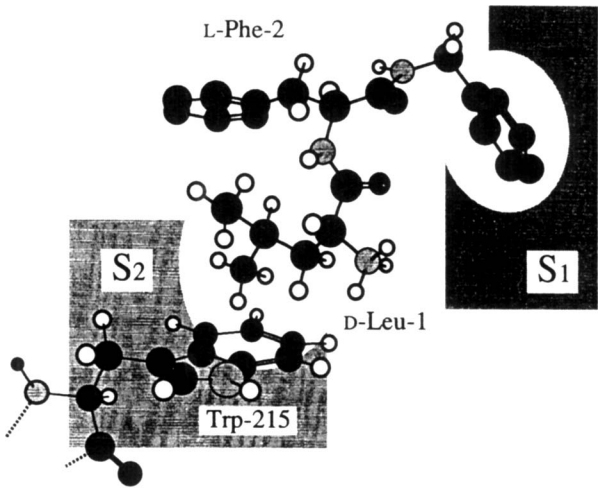

Fig. 6 Possible binding models of dipeptide inhibitor H-D-Leu-Phe-NHBzl in the chymotrypsin active centre. The C-terminal phenyl group fits the S_1 site, while the hydrophobic core constructed by side chain–side chain CH/π interaction fits the S_2 or $S_{1'}$ site.

^{1}H NMR measurements suggested that H-D-Leu-Phe-OBzl adopts a conformation in which D-Leu-Phe side chains are in proximity, and led to an assumption that this conformation is responsible for chymotrypsin inhibition. Formation of such a unique conformation has been confirmed in the present study by detailed NMR conformational analyses for a series of dipeptide amide derivatives.

The side chain–side chain complexation between D-Leu isobutyl and Phe phenyl has been demonstrated by different types of NMR measurements using various dipeptides with a D-Leu-Phe backbone structure. First, the proximity of Phe phenyl to D-Leu isobutyl was shown by observation of the upshifts of chemical shifts of the D-Leu isobutyl (Table 2 and Fig. 4). Such upfield shifts were first shown by Deber and Joshua (1972) for a series of dipeptides of the type of H-Phe-Xxx-OH, where Xxx represents various amino acids.[20] They noted distinct upfield shifts (0.2–0.7 ppm) of D-Xxx β- and γ-methylene proton signals in the 100 MHz NMR spectra. Although D-Leu-Phe dipeptides in this study contained the C-terminal phenyl group, the upfield shifts of the D-Leu isobutyl were evidenced as a result of the shielding effect from the benzene ring of the Phe phenyl group. H-D-Leu-Phe-NHCH$_3$ and H-D-Leu-Phe-NH$_2$, both of which lack the C-terminal phenyl group, exhibited similar upfield shifts of D-Leu isobutyl, while D-Leu isobutyl in H-D-Leu-Ala-NHBzl which lacks a Phe phenyl showed no upshift. Further evidence of side chain–side chain proximity was obtained from measurements of NOE enhancements. Proton signals of D-Leu δCH$_3$ and Phe phenyl exhibited enhancements in NOE difference spectra when each signal was irradiated separately. NOESY spectra clearly showed their cross-peak (Fig. 5). Thus, it was concluded that D-Leu-Phe dipeptides bear a conformation in which the side chains stick to each other. Such conformational fixation was further characterized by a splitting of NMR signals of Phe β-methylene ($\Delta\delta$ between double doublets = 0.46 ppm), which was caused by different anisotropic effects from the Phe phenyl fixed in a certain orientation.

The interaction between D-Leu isobutyl and Phe phenyl is well characterized in terms of CH/π interaction. The concept of the CH/π interaction has recently been documented in detail by Nishio *et al.* (1995).[16] Several characteristics of CH/π interaction are described: (i) it can play a role in polar media such as biological environments, (ii) multiple CH groups as in methyl, isopropyl and isobutyl groups can participate simultaneously in interactions with a π group, and (iii) the π-electron density of the aromatic ring increases. Although enthalpy for one unit of CH/π interaction was reported to be small (~1 kcal mol^{-1}; 1 cal = 4.184 J), the total enthalpy becomes sizeable when the CH/π interaction is multiple. Thus, in the case of D-Leu-Phe dipeptides, the CH/π interaction between D-Leu isobutyl and Phe phenyl appears to be quite strong.

One of the characteristics of the CH/π interaction is a relatively large contribution from delocalization (charge transfer from π to σ*) and dispersive interaction as compared with the normal H-bonding.[16] This provides a crucial point that CH/π interaction can play its role in polar media, the interaction being hardly disturbed by the presence of water. It should be noted that the CH/π interaction in H-D-Leu-Phe dipeptides was firmly retained in water.

In these arguments, however, it is not clear yet whether such a hydrophobic core induced by the CH/π interaction is responsible for chymotrypsin inhibition. The most important structural requirement for chymotrypsin inhibition is the C-terminal phenyl group. Elimination of the benzyl or phenyl group from H-D-Leu-Phe-NH-CH$_2$-C$_6$H$_5$ resulted in inactivity. Furthermore, this C-terminal phenyl group should be separated from the amide NH group with only one methylene (n = 1) (Fig. 3). The phenyl group at a position shorter or longer than n = 1 diminished the inhibitory activity drastically. The presence of such an optimum spacer length apparently relates to the enzyme structure. Since the inhibition manner of H-D-Leu-Phe-NHBzl was competitive (Fig. 2), the C-terminal phenyl group appears to interact directly with chymotrypsin at a certain binding site. The interactions between chymotrypsin and substrates or inhibitors at the S_1, $S_{1'}$ and S_2 are all hydrophobic,[21] and the S_1 site is formed as a hydrophobic pocket. The size of this S_1 pocket is 10–12 Å in depth and (5.5–6.5 Å) × (3.5–4.0 Å) in section,[22] into which the benzene ring (6 Å in width and 3.5 Å in thickness) can fit very well. The recognition of the S_1 site by substrates or inhibitors is usually stereospecific. When two stereoisomers of H-D-Leu-Phe-NH-(*R* or *S*)-CH(CH$_3$)-C$_6$H$_5$ were tested for chymotrypsin inhibition, only the *R*-isomer was potent. The *S*-isomer was extremely weak. Judging from all these observations, the C-terminal phenyl group is highly likely to point toward the S_1 site. In this interaction mode, the hydrophobic core constructed by side chains would fit the S_2 or $S_{1'}$ site. Fig. 6 depicts such a possible interaction mode between chymotrypsin S_1 and S_2 sites and H-D-Leu-Phe-NHBzl.

When a fluorine atom was introduced into the *para*-position of the C-terminal phenyl group, the inhibitory potency of the resulting H-D-Leu-Phe-NH-CH$_2$-C$_6$H$_4$F-*p* considerably increased. It became almost as potent as chymostatin.[23] If the C-terminal phenyl group fits the S_1 site, this fluorine would face toward chymotrypsin Ser-189 of the S_1 pocket. It is then likely that hydrogen bonding between fluorine and Ser-189-OH reinforces the enzyme–inhibitor interaction, resulting in an increase in inhibitory activity.

Nishio *et al.* (1995) have exemplified several protein–ligand complexes substantiated by CH/π interactions.[16] In addition, they indicated the presence of a CH/π network in proteins, which plays a role in stabilizing the structure of ligand-binding sites. The present study has shown another structural ability of CH/π interaction, which is to stabilize a specific conformation of a small ligand molecule. This emphasizes the usefulness of CH/π interaction for designing molecules which interact specifically with acceptors.

In spite of the assumption described above, totally different inhibition modes may also be feasible. As mentioned, H-D-Leu-Phe-OBzl was eventually hydrolysed by chymotrypsin. This was due to the occupation of chymotrypsin S_1 site by Phe-2. Since D-Leu-Phe dipeptides are assumed to be in a conformational equilibrium between the structure having the side chain–side chain CH/π interaction and the extended structure without CH/π interaction, Phe-2 may occupy the chymotrypsin S_1 site. In their case, the C-terminal phenyl group, which is essential for inhibition, should interact with

Table 4 Analytical data for Boc-dipeptide amides

Compound (Formula)	Yield (%)	Mp (*T*/°C)	$[\alpha]_D$	R_f[a]	Found (%) (Required) C	H	N
Boc-D-Leu-Phe-NHBzl ($C_{27}H_{37}N_3O_4$)	82	179–181	−21.8	0.72	69.3 (69.35)	7.85 (7.98)	8.9 (8.99)
Boc-D-Leu-Ala-NHBzl ($C_{21}H_{33}N_3O_4$)	88	109–111	−11.6	0.41	64.5 (64.43)	8.3 (8.50)	10.5 (10.73)
Boc-D-Leu-Phe-NHCH$_3$ ($C_{21}H_{33}N_3O_4$)	81	137–138	+6.5	0.37	64.3 (64.43)	8.5 (8.50)	10.65 (10.73)
Boc-D-Leu-Phe-NH$_2$ ($C_{20}H_{31}N_3O_4$)	92	177–179	+11.6	0.36	63.6 (63.63)	8.4 (8.28)	10.8 (11.13)
Boc-D-Leu-Phe-NH-(*R*)-CH(CH$_3$)-C$_6$H$_5$ ($C_{28}H_{39}N_3O_4$)	86	141–143	−3.9	0.63	69.5 (69.83)	8.1 (8.16)	8.6 (8.72)
Boc-D-Leu-Phe-NH-(*S*)-CH(CH$_3$)-C$_6$H$_5$ ($C_{28}H_{39}N_3O_4$)	93	179–181	−13.5	0.74	69.7 (69.83)	8.2 (8.16)	8.7 (8.72)
Boc-D-Leu-Phe-NH-C$_6$H$_4$F-*p* ($C_{27}H_{36}FN_3O_4$)	81	141–144	−1.6	0.63	67.7 (67.97)	7.1 (7.21)	8.6 (8.35)
Boc-Leu-Phe-NHBzl ($C_{27}H_{37}N_3O_4$)	89	150–152	−25.4	0.64	69.3 (69.35)	8.0 (7.98)	8.9 (8.99)
Boc-D-Leu-Phe-NH-C$_6$H$_5$ ($C_{26}H_{35}N_3O_4$)	83	151–152	−26.4	0.72	68.8 (68.84)	7.8 (7.79)	9.2 (9.27)
Boc-D-Leu-Phe-NH-[CH$_2$]$_2$-C$_6$H$_5$ ($C_{28}H_{39}N_3O_4$)	84	140–142	−15.5	0.74	69.8 (69.83)	8.0 (8.16)	8.6 (8.72)
Boc-D-Leu-Phe-NH-[CH$_2$]$_3$-C$_6$H$_5$ ($C_{29}H_{41}N_3O_4$)	87	147–148	−10.9	0.67	70.25 (70.27)	8.3 (8.34)	8.45 (8.48)
Boc-D-Leu-Phe-NH-[CH$_2$]$_4$-C$_6$H$_5$ ($C_{30}H_{43}N_3O_4$)	93	118–119	−9.4	0.70	70.75 (70.69)	8.5 (8.50)	8.3 (8.25)

[a] $CHCl_3$–MeOH–AcOH = 95:5:1 (v/v).

Table 5 Analytical data for dipeptide amides

Compound (Formula)	Yield (%)	Mp (*T*/°C)	$[\alpha]_D$	R_f[a]	Found (%) (Required) C	H	N
H-D-Leu-Phe-NH-C$_6$H$_5$·HCl ($C_{22}H_{30}ClN_3O_2$)	95	235–237	−5.8	0.82	65.3 (65.42)	7.5 (7.49)	10.2 (10.40)
H-D-Leu-Ala-NH-C$_6$H$_5$·HCl ($C_{16}H_{26}ClN_3O_2$)	93	91–93	−10.2	0.81	58.9 (58.62)	7.8 (7.99)	12.7 (12.82)
H-D-Leu-Phe-NH-CH$_3$·HCl ($C_{16}H_{26}ClN_3O_2$)	94	232–233 (decomp.)	−12.9	0.75	58.3 (58.60)	7.9 (8.01)	12.7 (12.82)
H-D-Leu-Phe-NH$_2$·HCl ($C_{15}H_{24}ClN_3O_2$)	84	202–204	−6.2	0.76	57.3 (57.41)	7.4 (7.71)	13.4 (13.39)
H-D-Leu-Phe-NH-(*R*)-CH(CH$_3$)-C$_6$H$_5$·HCl·H$_2$O ($C_{23}H_{34}ClN_3O_3$)	88	210–212	+36.7	0.79	63.65 (63.36)	7.75 (7.86)	9.5 (9.64)
H-D-Leu-Phe-NH-(*S*)-CH(CH$_3$)-C$_6$H$_5$·HCl·H$_2$O ($C_{23}H_{34}ClN_3O_3$)	84	202–204	−44.4	0.77	63.6 (63.36)	7.8 (7.86)	9.35 (9.64)
H-D-Leu-Phe-NH-C$_6$H$_4$F-*p*·HCl ($C_{22}H_{29}ClFN_3O_2$)	89	249–253	−10.8	0.80	62.6 (62.62)	6.9 (6.87)	10.0 (9.95)
H-Leu-Phe-NH-C$_6$H$_5$·HCl ($C_{22}H_{30}ClN_3O_2$)	97	229–230	+25.1	0.81	65.65 (65.42)	7.5 (7.49)	10.15 (10.40)
H-D-Leu-Phe-NH-C$_6$H$_5$·HCl ($C_{21}H_{28}ClN_3O_2$)	95	223–224 (decomp.)	+25.4	0.82	64.5 (64.68)	7.3 (7.25)	10.5 (10.78)
H-D-Leu-Phe-NH-[CH$_2$]$_2$-C$_6$H$_5$·HCl ($C_{23}H_{32}ClN_3O_2$)	92	234–236 (decomp.)	−7.7	0.85	66.3 (66.09)	7.8 (7.72)	10.0 (10.05)
H-D-Leu-Phe-NH-[CH$_2$]$_3$-C$_6$H$_5$·HCl ($C_{24}H_{34}ClN_3O_2$)	97	218–220 (decomp.)	−3.0	0.84	66.9 (66.72)	7.85 (7.95)	9.6 (9.73)
H-D-Leu-Phe-NH-[CH$_2$]$_4$-C$_6$H$_5$·HCl ($C_{25}H_{36}ClN_3O_2$)	96	188–189	−12.3	0.82	67.5 (67.31)	8.4 (8.15)	9.45 (9.42)

[a] BuOH–AcOH–pyridine–water = 4:1:1:2 (v/v).

a site other than S_1. These interactions might be sufficient to prevent the substrate reaching the sites, and the non-susceptibility of dipeptide amides to chymotrypsin hydrolysis would sustain this inhibiting action. However, a preliminary result from X-ray analysis of dipeptide–chymotrypsin complex indicated that the C-terminal phenyl group actually occupies the S_1 site. These analytical data will be soon reported elsewhere.

Experimental

Peptide synthesis

A series of dipeptide benzyl amides was synthesized by the two-step coupling procedure: *i.e.* (1) the coupling of Boc-Phe-OH with various amines by water-soluble carbodiimide, and (2) the coupling of Boc-D-Leu-OH with the resulting H-Phe- amides. The removal of the Boc group was carried out by treatment with 4.6 M HCl in 1,4-dioxane for Boc-D-Leu-Phe amides. Purification of the final amino-liberated dipeptide amides was carried out by recrystallization from methanol–diethyl ether. The purity was verified by high-performance thin-layer chromatography and HPLC. Analytical data are shown in Tables 4 and 5.

Hydrolysis of dipeptide benzyl esters by chymotrypsin

The stability of dipeptide benzyl esters and amides toward

chymotrypsin hydrolysis was monitored by HPLC at certain intervals (t = 5–120 min). The peptide (1 mM) was incubated in phosphate buffer (pH 7.0; total volume 3.0 ml) with chymotrypsin (1.3 μg ml^{-1}) at 25 °C. An aliquot (10 μl) of the incubation mixture was added to acetic acid (10 μl), and the solution was injected into the HPLC machine. The percentage hydrolysis was calculated from the peak integration ratio between each compound remained at t min and that (= 100) at 0 min. Analytical HPLC was performed on a Wakosil-II 5C18 HG reversed-phase column (4.6 × 150 mm) (Wako Pure Chem. Ind., Osaka, Japan) in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile (10–80%) for 30 min.

Enzyme assay

Dipeptides synthesized were tested for their inhibitory activity against bovine chymotrypsin (Worthington Biochemical Co., Freehold, NJ, USA). Substrate Ac-Tyr-OEt (ATEE) was purchased from the Peptide Institute Inc. (Osaka, Japan). For the kinetic analyses of interactions between dipeptides and chymotrypsin, the standard method was carried out. Briefly, the substrate ATEE was dissolved in 50 mM phosphate buffer (pH 7.0) with or without inhibitors. The enzyme reaction was initiated by adding an aliquot (30 μl) of the chymotrypsin solution (final concentration, 1.3 μg ml^{-1}) to the solution of substrate and inhibitor (1470 μl) at 25 °C. The final concentration of the inhibitors was varied, depending on the inhibitory potency. Dipeptides were first dissolved in MeOH (30 μl) and then diluted with 50 mM phosphate buffer (970 μl). The rate of hydrolysis of the substrates was determined in duplicate by the spectroscopic measurement of the change in absorbance at 237 nm by using a Hitachi 124 spectrometer. Inhibition constants, K_i, were determined by the Dixon plot analysis (Dixon, 1953).[17]

^{1}H NMR measurements

Samples were prepared by dissolving 5 mg of the peptide in 0.5 ml of [$^2H_6$]DMSO (99.96% ^{2}H; Aldrich, Milwaukee, WI, USA). For measurements in D_2O (99.9% ^{2}H; Isotec Inc., Miamisburg, OH, USA), 2.5 mg of each sample was dissolved in 0.5 ml solvent. All ^{1}H NMR spectra were recorded on a Bruker AM-400 spectrometer operating at 400 MHz ^{1}H frequency at 25 °C. The chemical shifts were determined using tetramethylsilane and 3-(trimethylsilyl)propanesulfonic acid sodium salt as internal standards in [$^2H_6$]DMSO and D_2O, respectively. The signals were assigned by the 2D phase-sensitive DQF COSY. The COSY spectra were obtained by using the standard method.[24–26] Five-hundred and twelve free-induction decays (FIDs) were acquired with successive t_1-values. The digital resolution was 9.76 Hz per point.

The H-{H} nuclear Overhauser effect (NOE) data were recorded by a gated irradiation pulse sequence. The NOE difference spectra were obtained by subtracting the control spectrum from an original NOE spectrum produced by presaturation of selected proton signals with a low decoupling power for 3 s before pulse. The NOESY spectra were obtained with 1024 FIDs and a mixing time of 500 ms.

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