Relationship between the Hydrophobicity of Dipeptides and the Michaelis–Menten Constant K_m of Their Hydrolysis by Carboxypeptidase-Y and Carboxypeptidase-A

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The enzymatic hydrolysis of dipeptides by carboxypeptidase-Y and carboxypeptidase-A was investigated. In the enzymatic hydrolysis of the dipeptides, a good linear relationship ($r = 0.997$ and 0.999) was found between the Michaelis–Menten constant (K_m) and the hydrophobicity of the substrates evaluated from relative elution volume in reversed-phase HPLC. The correlation suggests that the hydrophobicity of the *C*-terminal amino acid is a major factor in governing the stability of the enzyme–substrate complex. The difference in the slope of the linear-regression lines seems to reflect the degree of relative hydrophobicity of the binding pockets in carboxypeptidase-Y and carboxypeptidase-A.

The hydrophobicity of solutes often influences various biological phenomena as well as chemical properties.[1,2] Hydrophobicity is a characteristic of materials that indicates its disfavor of water. Solutes high in this character tend to be expelled from water structure. Arising from our interest in the relationship between the hydrophobicity of organic compounds and biological or chemical properties,[3,4] we have carried out an examination of the hydrophobicity of several dipeptides and their enzymatic hydrolysis by carboxypeptidase-Y and carboxypeptidase-A. In enzymatic reactions, the hydrophobicity of substrates often avails to incorporate them into enzymes from aqueous media.

Carboxypeptidase-Y (CPD-Y) [EC 3.4.16.5] from bakers yeast is a lysosomal serine exopeptidase, which catalyzes the hydrolysis of the carboxyl-terminal peptide bond of proteins. It has been suggested that a Ser–His–Asp catalytic triad is involved in the process, as found in the trypsin and chymotrypsin families of endopeptidases.[5] An interesting feature is that a peptide bond consisting of a proline residue can be hydrolyzed by the catalysis of CPD-Y, while this bond is resistant to cleavage by α-chymotrypsin and carboxypeptidase-A (CPD-A) [EC 3.4.17.1]. CPD-Y is also a popular model protein for studying protein sorting in yeast.[6] CPD-A from bovine pancreas is a zinc exopeptidase, and is also known to catalyze the hydrolysis of the carboxyl-terminal peptide bond of proteins. The catalysis reaction occurs when the peptide carbonyl group is coordinated to zinc, and a water molecule is delivered to the substrate with catalytic Glu assisting as a general base.[7]

In general, enzyme catalysis is initiated by the interaction between the enzyme and the substrate prior to reaction, which gives rise to what is known as the enzyme–substrate (E–S) complex or Michaelis complex as shown in Scheme 1. The stability of the E–S complex is related to the affinity of the substrate to the enzyme, which is measured by the Michaelis–Menten constant (K_m) for the E–S complex. Generally, the K_m values are affected by various types of weak non-covalent interactions involved in the formation of the E–S complex (electrostatic interactions, hydrogen bonds, and hydrophobic interactions, etc.). Noteworthy among them are usually weak hydrophobic interactions, which have been suggested to be important not only for the formation of the E–S complex (Michaelis complex), but also substrate discrimination.[8] Since the complex between the substrate and the enzyme is formed with weak noncovalent interactions, the reaction between enzyme and substrate is reversible; this phenomenon is very important for product release.

As a step towards understanding the influence of the hydrophobicity of substrates in the hydrolysis of dipeptides by enzymes, we have pursued this issue by comparing the difference in the hydrolytic behavior of peptides catalyzed by CPD-Y and

$$\text{[inhibitor-Enzyme]} \underset{k_{-i}}{\overset{k_{+i}}{\rightleftharpoons}} \text{dipeptide + Enzyme} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{[dipeptide-Enzyme]} \xrightarrow{k_2} \text{product + Enzyme}$$

$$K_i = \frac{k_{-i}}{k_{+i}} \qquad K_m = \frac{k_{-1} + k_2}{k_1}$$

Scheme 1.

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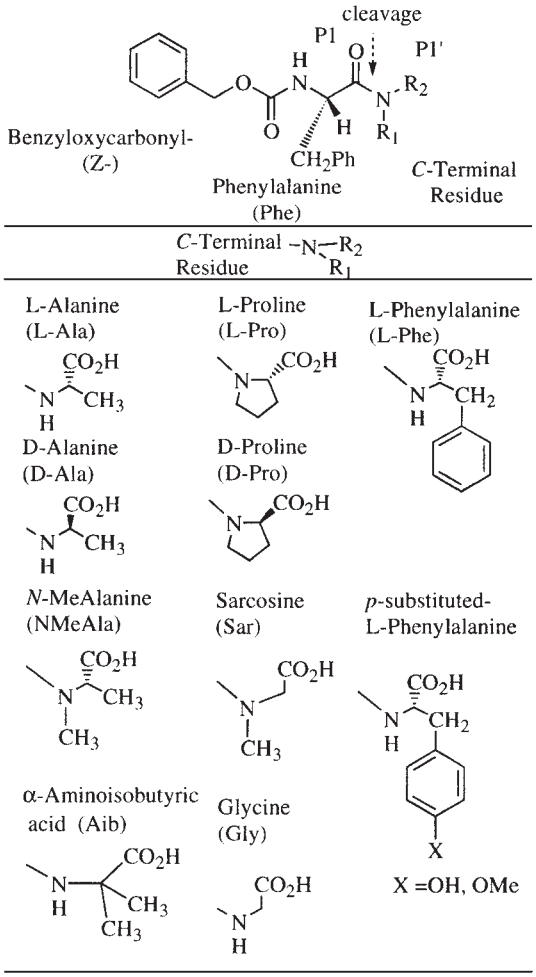


Scheme 2.

CPD-A in relation with substrate hydrophobicity, as described herein.

Results and Discussion

Preparation and Hydrolysis of *N*-Benzyloxycarbonyl Protected Dipeptides. A series of dipeptides [Z–Phe–Gly, Z–Phe–Sar, Z–Phe–D-Ala, Z–Phe–L-Ala, Z–Phe–NMeAla, Z–Phe–Aib, Z–Phe–D-Pro, Z–Phe–L-Pro, and Z–Phe–Phe(*p*-X, X = H, OH, OMe)], as shown in Scheme 2, were prepared by way of typical procedures.

Evaluation of Hydrophobicity by Means of a Chromatographic Method. The hydrophobicities of the dipeptides were evaluated as their affinity towards the stationary phase in reversed-phase HPLC relative to elution volume. In reversed-phase chromatography with ODS columns, the retention of a solute (S), i.e., a dipeptide, is viewed as the reversible association of the solute with an octadecyl function (L) at the chromatographic surface, thus forming a complex (SL). Solute retention is governed by the average value of the contact surface area arising from the plurality of such binding configurations.[9,10] The capacity factor (k') is related to the equilibrium constant (K) for the distribution of the solute between the bulk mobile phase and the stationary phase by

$$k' = K\varphi, \tag{1}$$

where φ is the phase ratio of the column. The energetics of retention is determined by the standard free-energy change (ΔG_{ret}) associated with solute transfer from the mobile phase to the stationary phase, by

$$\Delta G_{ret} = -RT \ln K. \tag{2}$$

The relative free energy ($\delta\Delta G_{ret}$) of partition is derived from

$$\begin{aligned}\delta\Delta G_{ret} &= -RT \ln(K_R/K_S) \\ &= -RT[\ln(V_e - V_0)_R - \ln(V_e - V_0)_S],\end{aligned} \tag{3}$$

where K_R/K_S is the equilibrium constant relative to that (K_S) of the standard compound (in this case, Z–Phe–Gly), V_e is the elution volume, and V_0 is the column volume in HPLC. The values of $V_e - V_0$ can be evaluated from the corresponding retention times, t_r and t_0. Combining Eqs. 1 and 3 yields

$$\delta\Delta G_{ret} = -RT \ln(k'_R/k'_S) = -2.303RT(\delta \log k'), \tag{4}$$

where k'_R/k'_S is the capacity factor relative to that of the standard compound (in this case, Z–Phe–Gly). Thus, $\delta \log k'$ is the difference in the $\log k'$ values with that of the standard compound (in this case, Z–Phe–Gly).

It has been demonstrated by Pliska and his co-workers that the hydrophobic parameters, $\log P$ for α-amino acids, are highly correlated to the R_f values of thin-layer chromatography on silica gel and cellulose plates,[11] which has been revealed likewise by others for ODS columns.[12]

Regarding conditions, the column used was a 250 × 4.6 mm ODS reversed-phase column (Wakosil II 5C18HG), and the mobile phase (0.5 mL/min) consisted of acetonitrile–water (3:7) containing 60 mM AcONa buffer (pH 6.0). In order to confirm the validity of using $\delta\Delta G_{ret}$, we first examined the relationship between $\delta\Delta G_{ret}$ and $\log P$ for Gly, Ala, Pro, Tyr, and Phe, and observed good linearity, as follows:

$$\delta\Delta G_{ret} = -0.503 \log P - 3.01 \quad (r = 0.954,\ n = 5). \tag{5}$$

$\delta\Delta G_{ret}$ values were also measured with a slightly different solvent composition of acetonitrile–water = 23:77. Although the absolute retention times were much longer for this mobile phase (50–70 min compared with 15–25 min), a good relationship (correlation factor $r = 0.983$) was found between the two sets of $\delta\Delta G_{ret}$ values for the two mobile phases of differing composition. Thus, although the absolute retention time could change with differing mobile-phase composition, the relative free-energy relationship between the $\delta\Delta G_{ret}$ and K_m values to be evaluated (vide infra) are expected to be the same. The relative free-energy values of partition ($\delta\Delta G_{ret}$) for the dipeptides are summarized in Table 1.

Kinetics. All of the kinetic experiments were performed in pH 6.5 solutions at 37 °C. The progress of the reactions was determined by spectrophotomerically (230–240 nm) monitoring the relative amount of the released products. The kinetic parameters (K_m, K_i, and k_2), as designated in Scheme 1 for the hydrolysis of these dipeptides or for the inhibition of Z–Phe–L-Pro hydrolysis by peptides bearing a D-amino acid by CPD-Y and CPD-A, are summarized in Table 1. The listed values are the average of 3–5 runs. The kinetic data was calculated by non-linear fitting of the Michaelis–Menten equation to plotted experimental data.

Table 1. Kinetic Parameters for Hydrolysis of Dipeptides by CPD-Y or CPD-A

Dipeptide	$\delta\Delta G_{ret}$ [a)]	CPD-Y		CPD-A	
Z–Phe–Xaa		K_m/mM	k_2/s^{-1}	K_m/mM	k_2/s^{-1}
–NMeAla	−0.677	0.274 ± 0.033	0.657 ± 0.027	—	—
–L-Pro	−0.604	0.339 ± 0.018	45.0 ± 0.8	—	—
–L-Ala	−0.293	0.939 ± 0.099	187.7 ± 11.3	0.238 ± 0.030	13.9 ± 0.63
–Sar	−0.152	1.94 ± 0.06	25.9 ± 0.6	—	—
–Gly	0.00	3.36 ± 0.12	189.4 ± 5.5	—	—
–Aib	−1.28	2.86 ± 0.15	147.3 ± 6.0	—	—
–L-Phe	−2.91	0.180 ± 0.009	194.4 ± 3.0	0.143 ± 0.007	608.3 ± 7.7
–L-Phe(*p*-OH)	−0.854	0.360 ± 0.024	313.9 ± 9.1	0.196 ± 0.005	626.1 ± 2.9
–L-Phe(*p*-OMe)	−3.22	0.435 ± 0.010	143.1 ± 1.6	0.132 ± 0.005	673.2 ± 6.3
	$\delta\Delta G_{ret}$ [a)]	K_i/mM	Inhibition type		
–D-Pro	−0.286	0.680 ± 0.013	Competitive		
–D-Ala	−0.444	1.02 ± 0.05	Competitive		

a) $\delta\Delta G_{ret}$ was calculated from the retention time in HPLC (ODS reversed-phase column).

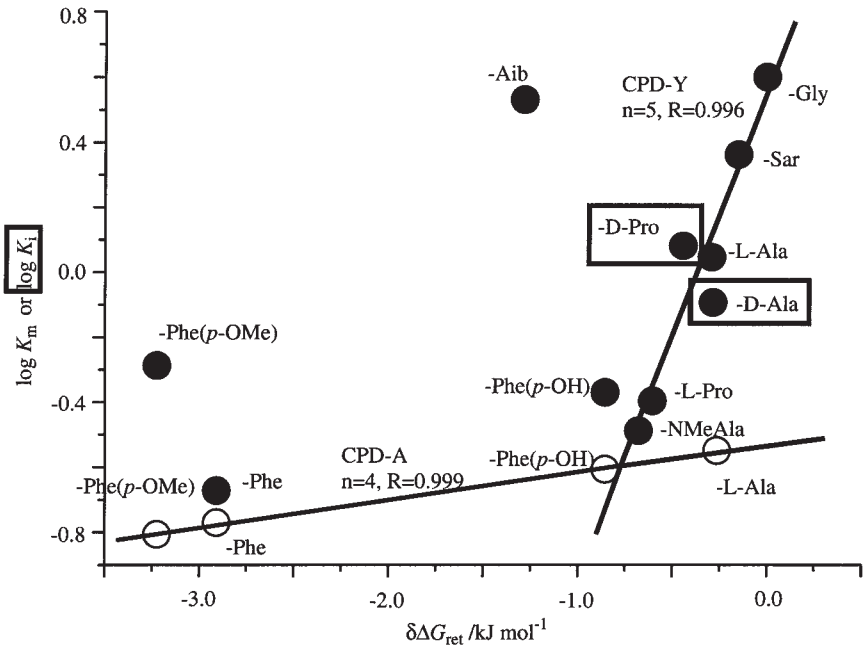


Fig. 1. A linear relationship between $\log K_m$ or $\log K_i$ and hydrophobic property ($\delta\Delta G_{ret}$) of the dipeptides. CPD-Y (●), CPD-A (○). Plots in the rectangles correspond to those of K_i.

Relationship between K_m and Hydrophobic Properties of Dipeptides. The order of the K_m values was Phe < NMeAla < L-Pro ≒ Phe(*p*-OH) < Phe(*p*-OMe) < L-Ala < Sar < Aib < Gly in the hydrolysis by CPD-Y and Phe(*p*-OMe) < Phe < Phe(*p*-OH) < L-Ala in the hydrolysis by CPD-A. Thus, the affinity of the Phe, NMeAla, and Pro substrates towards the CPD-Y enzyme is more favorable for making the E–S complex relative to that of Ala, Sar, Aib, and Gly substrates and the affinity of Phe(*p*-OMe), Phe, and Phe(*p*-OH) substrates towards CPD-A is more favorable than that of Ala. On the other hand, Z–Phe–D-Ala and Z–Phe–D-Pro caused the competitive inhibition of Z–Phe–L-Pro hydrolysis with the K_i values being in the order of Z–Phe–D-Ala > Z–Phe–D-Pro.

The hydrolysis of *N*-alkyl substituted substrates and Z–Phe–Aib by CPD-A was not observed, and the reaction of Z–Phe–Gly was too slow to measure.

The k_2 values of reactions involving CPD-Y [Phe(*p*-OH) > Phe ≒ L-Ala > Gly > Aib ≒ Phe(*p*-OMe) > L-Pro > Sar > NMeAla], show that *N*-unsubstituted amino acids were easily hydrolyzed, whereas those of CPD-A reactions [Phe(*p*-OMe) > Phe(*p*-OH) > Phe > L-Ala] indicated that aromatic amino acids were easily hydrolyzed.

Thus, we decided to elucidate the relationship between hydrophobicity and K_m or K_i for this case. There was found a linear free-energy relationship between $\log K_m$ and $\delta\Delta G_{ret}$, which reflects hydrophobic properties, as shown in Fig. 1. That is, $\log K_m = 1.62\delta\Delta G_{ret} + 0.51$ ($r = 0.997$, $n = 5$) for CPD-Y for dipeptide substrates [excluding those bearing amino acid moieties of the D-series, and those bearing Aib, Phe, Phe(*p*-OH), and Ph(*p*-OMe)], and $\log K_m = 0.0845\delta\Delta G_{ret} - 0.604$; $r = 0.999$, $n = 4$ for CPD-A.

Because peptides of the D-series were much less reactive as

substrates, their behavior as competitive inhibitors toward the hydrolysis of the L-series was evaluated by non-linear fitting of the Michaelis–Menten equation. Although the plots corresponding to the two D-series dipeptides were slightly deviated from the above linear line, as a whole, the K_i values of the D-substrates were found to be colinear with the other five K_m values according to the relationship, $\log K_m = 1.52\delta\Delta G_{ret} + 0.48$; $r = 0.946$, $n = 7$ (including D-Ala and D-Pro). Thus, the correlation between the hydrophobicity of the dipeptide substrates and K_m (along with K_i) elucidated in this study suggests that the hydrophobicity of the *C*-terminal amino acid is a major factor in governing the stability of the enzyme–substrate and enzyme–inhibitor complex. The large upper deviation of the plot of Z–Phe–Aib from the linear relationship implies that sterical factors resulting from the geminal α-methyl groups inhibit incorporation of the substrate. Namely, as illustrated in Fig. 2, the *pro-R* methyl group of Z–Phe–Aib apparently cannot fit into the hydrophobic region of the S1′ binding pocket of CPD-Y. For the same reason, neither can the α-side chains of D-amino acids

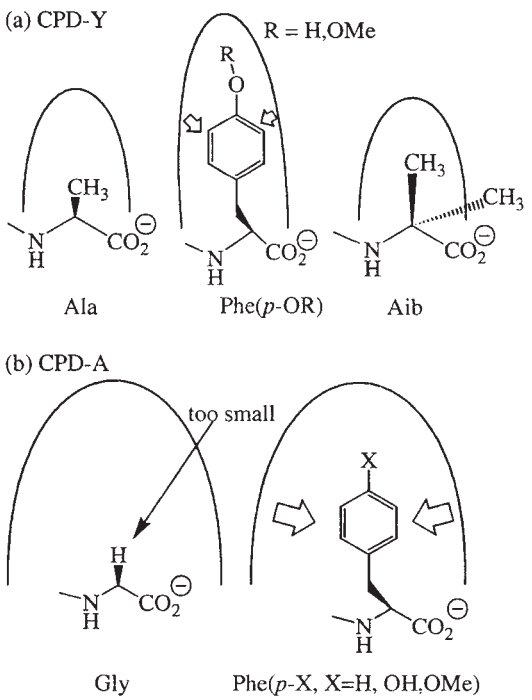


Fig. 2. Schematic illustration showing putative incorporation into S1′ pocket of (a) CPD-Y and (b) CPD-A.

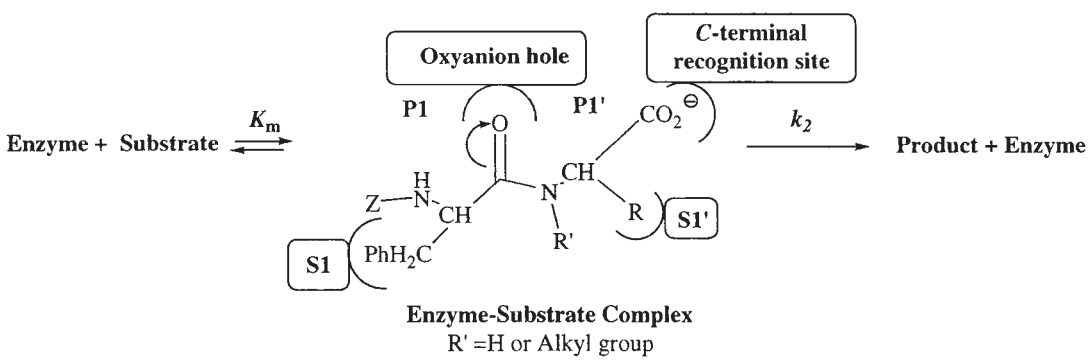
in conformations required for the ensuing hydrolysis reactions. Thus, positive hydrophobic interactions within the active site expected from chromatographic considerations is diminished. Judging from the extremely large upper side drift of the $\log K_m$ values from linearity for the Phe(*p*-X, X = H, OH, OMe) substrates, although they are of the L-series, the size of the pocket itself must be small. On the other hand, since the space within the S1′ binding pocket of CPD-A is presumably sufficiently large enough to incorporate these aromatic groups, the K_m values of the substrates bearing these substituents show a good correlation with that of L-Ala in relation with substrate hydrophobicity. Notwithstanding, the corresponding substituent of the Gly substrate (an H) is too small for the S1′ binding pocket of CPD-A to show the recognition required for effective hydrolysis by the enzyme.

According to the X-ray structures of CPD-Y[13] and CPD-A,[14] the active site or catalytic cavity consists of an oxyanion hole, a *C*-terminal recongition site, and two hydrophobic pockets (Scheme 3).

In CPD-Y, the hydrophobic S1′ binding site consists of the residues Thr 60, Phe 64, Glu 65, Tyr 256, Tyr 269, Leu 272, Met 398, and disulfide 56-298, while S1 consists of Tyr 147, Leu 178, Tyr 185, Tyr 188, Leu 245, Trp 312, Ile 340, and Cys 341, which makes it capable of accommodating hydrophobic side chains at the P1 (corresponding to the *N*-terminal amino acid moiety) and P1′ (corresponding to the *C*-terminal amino acid moiety) positions, respectively. Since P1 is fixed as Phe, it is likely that the observed K_m and K_i values are mostly dependent upon the hydrophobicity of the *C*-terminal amino acids at P1′.

The X-ray structures of the S1′ binding pockets of CPD-Y and CPD-A (Fig. 3) show the rim of the S1′ binding pocket of CPD-Y to be ca. 7–10 angstroms in width and ca. 5–7 angstroms in depth, while the rim of CPD-A to be ca. 10–12 angstroms in width and ca. 9–11 angstroms in depth. Therefore, the S1′ binding pocket of CPD-A is larger than that of CPD-Y, which coincides with the relation between K_m (or K_i) and hydrophobicity, discussed above.

The hydrophobicity values being those relative to Z–Phe–Gly, the *y*-intercept A of the linear regression of Fig. 1 corresponds to $\log K_m$ or $\log K_i$ observed or predicted for Z–Phe–Gly, and the slope B indicates the sensitivity of substrate hydrophobicity towards the binding pocket in the corresponding enzyme (Table 2), since the exterior of the enzyme in the present reaction system is surrounded by aqueous medium. From the



Scheme 3. Schematic Michaelis–Menten complex in enzymatic hydrolysis.

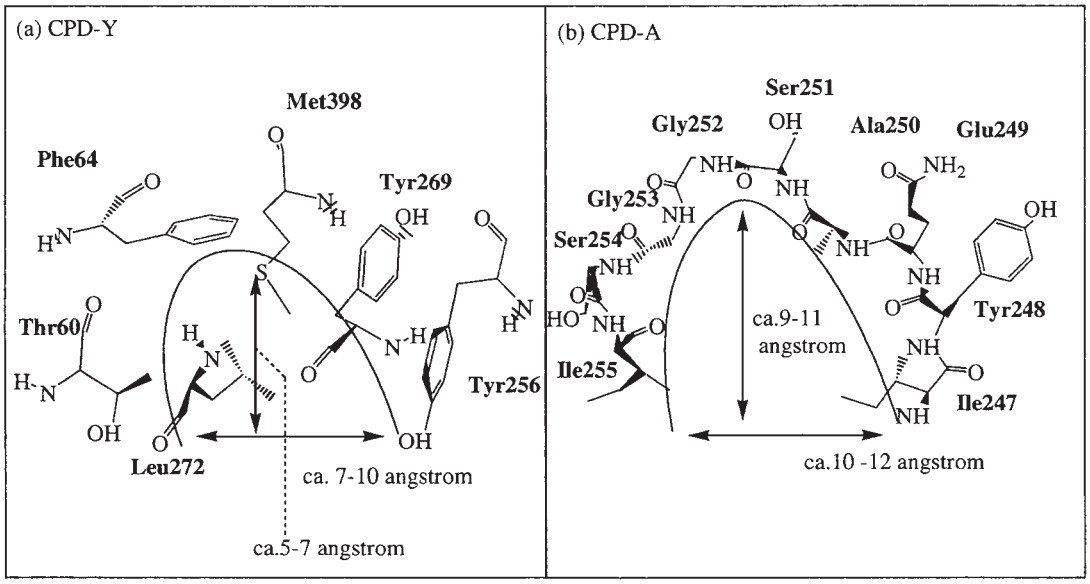


Fig. 3. The S1′ binding pocket of (a) CPD-Y [PDB CODE: 1YSC][13] and (b) CPD-A [PDB CODE: 5CPA][14,15] according to their respective X-ray structures.

Table 2. The Results of Linear Regression of Fig. 1

$Y = A + BX$	CPD-Y				CPD-A	
Dipeptides	Gly, Sar, L-Ala, L-Pro, NMeAla		Gly, Sar, D-Ala, L-Ala, D-Pro, L-Pro, NMeAla		Ala, Phe(*p*-OH), Phe(*p*-OMe), Phe	
	Value	Error	Value	Error	Value	Error
A	0.509	0.032	0.476	0.097	−0.604	0.005
B	1.62	0.07	1.52	0.23	0.0845	0.0023

Table 3. The Residues Comprising the S1′ Pocket[a,b]

CPD-Y[c]	Thr60	**Phe64**	Tyr256	Tyr269	**Leu272**	**Met398**			
Log *P*[d] (octanol/water)	−2.91	−1.63	−2.42	−2.42	−1.61	−1.84			
CPD-A[e]	**Ile247**	Tyr248	Glu249	Ala250	Ser251	Gly252	Gly253	Ser254	**Ile255**
Log *P*[d] (octanol/water)	−1.72	−2.42	−4.19	−2.89	−3.17	−3.25	−3.25	−3.17	−1.72

a) The residues highlighted in boldface type shows that the hydrophobicity value (log *P*) is more than −2. b) Underlined residue shows the Log *P* value lies between −2 and −3. c) Ref. 13. d) Ref. 11. e) Ref. 15.

data of Table 2, the value B of CPD-Y is found to be about 19-times as large as than that of CPD-A. In other words, the S1′ binding pocket of CPD-Y is about 19-times more hydrophobic than that of CPD-A.

The residues comprising the S1′ binding pocket of CPD-Y and CPD-A are given in Table 3. The S1′ binding pocket (consisting of Phe64, Leu272, and Met398) of CPD-Y is comprised of more hydrophobic residues than that (consisting of Ile247 and Ile255) of CPD-A. Thus, according to the structure, the S1′ binding pocket of CPD-Y is expected to be more hydrophobic than that of CPD-A; this assumption is consistent with a comparison between CPD-Y and CPD-A of the slope of the linear-regression lines (vide infra). Therefore, the experimental results presented here imply that in cases where there are substrates or inhibitors that have an obvious linear relationship between their hydrophobicity and K_m values (i.e., Gly, Sar, D-Ala, L-Ala, D-Pro, L-Pro, NMeAla for CPD-Y and Ala, Phe(*p*-OH), Phe(*p*-OMe), Phe for CPD-A in this case), a comparison of the magnitude of the slope of K_m plotted against hydrophobicity might be used as a quantitative measure of the relative hydrophobicity of the binding pockets of different enzymes. The generality of this method awaits further experimentation on other enzymes.

Experimental

Instruments. A Shimadzu UV-240 UV–visible recording spectrophotometer was used for kinetic measurements.

General Synthetic Method. The treatment of L-phenylalanine (Phe) with benzyloxycarbonyl chloride (Z–Cl) and L-alanine (Ala) with di-*t*-butyl dicarbonate (Boc_2O) afforded Z–Phe and Boc–Ala, respectively. Methylation of Boc–Ala with methyl iodide gave the *N*-methylalanine derivative (Boc–MeAla). The reaction of α-ami-

no acid (Xaa = D, L-Ala, Gly, NMeAla, D, L-Pro, Aib, Sar, Phe, and Tyr) with thionyl chloride, followed by MeOH, gave the corresponding methyl ester (HCl·Xaa–OMe). The coupling reactions were mediated by dicyclohexylcarbodiimide to give the corresponding *N*- and *O*-protected dipeptides (Z–Phe–Xaa–OMe). Methylation of Z–Phe–Tyr–OMe with methyl iodide gave Z–Phe–Tyr(*O*–Me)–OMe. Deprotection of the *C*-terminus of the dipeptides was carried out by treatment with an equivalent amount of 1 M NaOHaq for 0.5–3 h at room temperature to give the dipeptide substrates, Z–Phe–Xaa.

***N*-Benzyloxycarbonyl-L-phenylalanyl-2-methylalanine (Z–Phe–Aib).** mp 158 °C, (lit.[16] 161 °C), $[\alpha]_D^{20}$ −9.7, (*c* 0.10, MeOH), (lit.[16] $[\alpha]_D^{20}$ −9.5, *c* 0.1, MeOH), ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47–7.05 (10H, m), 6.48 (1H, s), 5.69 (1H, s), 5.08 (2H, s), 4.48 (1H, m), 3.05 (2H, m), 1.55 (3H, s), 1.42 (3H, s). FABMS *m/z* 383.1613. Calcd for $C_{21}H_{23}N_2O_5$ 383.1608.

***N*-Benzyloxycarbonyl-L-phenylalanyl-D-alanine (Z–Phe–D-Ala).** mp 155–156 °C, $[\alpha]_D^{20}$ −2.9, (*c* 0.10, MeOH), ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45–7.16 (10H, m), 6.97 (1H, d, $J = 6.5$ Hz), 6.01 (1H, d, $J = 8.5$ Hz), 5.03 (1H, d, $J = 12.5$ Hz), 4.95 (1H, d, $J = 12.5$ Hz), 4.66 (1H, d, $J = 7.5$ Hz), 4.45 (1H, q, $J = 6.5$ Hz), 3.00 (2H, d, $J = 6.0$ Hz), 1.19 (3H, d, $J = 6.5$ Hz). FABMS *m/z* 371.1612. Calcd for $C_{20}H_{23}N_2O_5$ 371.1608.

***N*-Benzyloxycarbonyl-L-phenylalanyl-L-alanine (Z–Phe–L-Ala).** mp 160–162 °C (lit.[17] 165 °C), $[\alpha]_D^{20}$ −10.8, (*c* 0.0198, MeOH), (lit.[17] $[\alpha]_D^{20}$ −11.0, *c* 2, alcohol), ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47–7.05 (10H, m), 6.47 (1H, s), 5.41 (1H, s), 5.08 (2H, s), 4.52 (1H, q, $J = 7.5$ Hz), 4.50 (1H, t, $J = 7.5$ Hz), 3.10 (1H, q, $J = 7.5$ Hz), 3.06 (1H, q, $J = 7.5$ Hz), 1.38 (3H, d, $J = 7.5$ Hz). FABMS *m/z* 369.1452. Calcd for $C_{20}H_{21}N_2O_5$ 369.1450.

***N*-Benzyloxycarbonyl-L-phenylalanyl-glycine (Z–Phe–Gly).** mp 144–147 °C, (lit.[18] 154 °C), $[\alpha]_D^{20}$ −9.9, (*c* 0.025, AcOH), (lit.[18] $[\alpha]_D^{18}$ −10.2, *c* 2.73, AcOH), ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47–7.05 (10H, m), 6.52 (1H, s), 5.39 (1H, s), 5.07 (2H, s), 4.54 (1H, dd, $J = 6.5$, 12.5 Hz), 4.06 (1H, d, $J = 17.9$ Hz), 3.92 (1H, d, $J = 17.9$ Hz), 3.96–3.92 (1H, m), 3.08 (2H, m). Found: C, 63.94; H, 5.57; N, 7.92%. Calcd for $C_{19}H_{20}N_2O_5$: C, 64.04; H, 5.66; N; 7.68%.

***N*-Benzyloxycarbonyl-L-phenylalanyl-*N*-methyl-L-alanine (Z–Phe–NMeAla).** mp 39–40 °C, $[\alpha]_D^{20}$ −33.2, (*c* 0.100, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33–7.16 (10H, m), 6.23 (1H, d, $J = 9.0$ Hz), 5.17 (1H, q, $J = 7.5$ Hz), 5.12 (1H, d, $J = 8.5$ Hz), 5.09 (1H, d, $J = 8.5$ Hz), 4.95 (1H, quintet, $J = 7.4$ Hz), 3.09–2.94 (2H, m), 2.86 (3H, s), 1.39 (3H, d, $J = 7.5$ Hz). Found: C, 65.47; H, 6.46; N, 7.19%. Calcd for $C_{21}H_{24}N_2O_5$: C, 65.63; H, 6.25; N, 7.29%.

***N*-Benzyloxycarbonyl-L-phenylalanyl-D-proline (Z–Phe–D-Pro).** mp 125–126 °C, $[\alpha]_D^{20}$ −38.5, (*c* 0.102, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33–7.19 (10H, m), 5.95 (1H, d, $J = 8.5$ Hz), 5.10 (1H, d, $J = 12.5$ Hz), 5.02 (1H, d, $J = 12.5$ Hz), 4.75–4.72 (1H, m), 4.35–4.32 (1H, m), 3.54 (1H, s), 3.15–3.06 (1H, m), 2.99–2.98 (1H, m), 2.65–2.60 (1H, m), 2.06–2.01 (1H, m), 1.86–1.73 (2H, m), 1.54–1.49 (1H, m). FABMS *m/z* 397.1783. Calcd for $C_{22}H_{25}N_2O_5$ 397.1763.

***N*-Benzyloxycarbonyl-L-phenylalanyl-L-proline (Z–Phe–L-Pro).** mp 107–108 °C, (lit.[19] 106.5 °C), $[\alpha]_D^{20}$ −43.7, (*c* 0.130, MeOH), ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37–7.12 (10H, m), 5.57 (1H, d, $J = 9.0$ Hz), 5.11 (1H, d, $J = 12.5$ Hz), 5.06 (1H, d, $J = 12.5$ Hz), 4.69 (1H, dd, $J = 8.0$, 15.1 Hz), 4.58 (1H, d, $J = 5.5$ Hz), 3.51 (1H, dd, $J = 8.5$, 15.9 Hz), 3.06–3.03 (2H, m), 2.85–2.80 (1H, m), 2.34–2.30 (1H, m), 2.01–1.93 (1H, m), 1.89–1.83 (1H, m), 1.78–1.71 (1H, m). Found: C, 66.64; H, 6.13; N 6.90%. Calcd for $C_{22}H_{24}N_2O_5$: C, 66.65; H, 6.10; N, 7.06%.

***N*-Benzyloxycarbonyl-L-phenylalanyl-sarcosine (Z–Phe–Sar).** mp 61–63 °C, (lit.[20] 46–50 °C), $[\alpha]_D^{20}$ −9.4, (*c* 0.40, EtOH), (lit.[20] $[\alpha]_D^{20}$ −9.5, *c* 4.0, EtOH), ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34–7.15 (10H, m), 5.91 (1H, d, $J = 9.0$ Hz), 5.08 (1H, d, $J = 12.5$ Hz), 5.02 (1H, d, $J = 12.5$ Hz), 4.95 (1H, q, $J = 8.2$ Hz), 4.20 (1H, d, $J = 17.5$ Hz), 3.94 (1H, d, $J = 17.5$ Hz), 3.07–2.94 (2H, m), 2.88 (3H, s). Found: C, 64.71; H, 6.24; N, 7.45%. Calcd for $C_{20}H_{22}N_2O_5$: C, 64.85; H, 5.99; N, 7.56%. FABMS *m/z* 371.1639. Calcd for $C_{20}H_{23}N_2O_5$ 371.1608.

***N*-Benzyloxycarbonyl-L-phenylalanyl-L-phenylalanine (Z–Phe–Phe).** mp 155–156 °C, (lit.[21] 158–160 °C), $[\alpha]_D^{20}$ −6.89, (*c* 0.100, MeOH), ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34–7.00 (15H, m), 6.51 (1H, d, $J = 6.4$ Hz), 5.41 (1H, d, $J = 7.1$ Hz), 5.05 (2H, s), 4.78–4.74 (1H, m), 4.46 (1H, m), 3.15–3.11 (1H, m), 3.04–2.94 (3H, m). FABMS *m/z* 445.1766. Calcd for $C_{26}H_{25}N_2O_5$ 445.1765.

***N*-Benzyloxycarbonyl-L-phenylalanyl-L-tyrosine [Z–Phe–Phe(*p*-OH)].** mp 180–182 °C, (lit.[22] 182–185 °C), $[\alpha]_D^{20}$ −2.56, (*c* 0.100, MeOH), ^{1}H NMR (500 MHz, CD_3OD) δ 7.31–7.15 (10H, m), 7.01 (2H, d, $J = 8.6$ Hz), 6.68 (2H, d, $J = 8.2$ Hz), 5.02 (1H, d, $J = 12.5$ Hz), 4.96 (1H, d, $J = 12.8$ Hz), 4.59–4.57 (1H, m), 4.38–4.35 (1H, m), 3.10–3.06 (2H, m), 2.93–2.89 (1H, m), 2.79–2.74 (1H, m). FABMS *m/z* 461.1709. Calcd for $C_{26}H_{25}N_2O_6$ 461.1714.

***N*-Benzyloxycarbonyl-L-phenylalanyl-*O*-methyl-L-tyrosine [Z–Phe–Phe(*p*-OMe)].** mp 120–121 °C, $[\alpha]_D^{20}$ −4.04, (*c* 0.120, MeOH), ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38–7.17 (8H, m), 7.12 (2H, d, $J = 7.3$ Hz), 6.97 (2H, d, $J = 8.2$ Hz), 6.72 (2H, d, $J = 8.5$ Hz), 5.52 (2H, d, $J = 7.1$ Hz), 5.06 (1H, d, $J = 12.5$ Hz), 5.00 (1H, d, $J = 11.9$ Hz), 4.65–4.61 (1H, m), 4.41–4.36 (1H, m), 3.71 (3H, s), 3.11–3.01 (2H, m), 2.96–2.87 (2H, m). FABMS *m/z* 475.1870. Calcd for $C_{27}H_{27}N_2O_6$ 475.1892.

Enzyme CPD-A and CPD-Y. CPD-Y prepared by Oriental Yeast Co. Ltd., and CPD-A prepared by ICN Biomedicals, Inc., were purchased from Wako Pure Chemical Industries, and were used without purification.

Kinetic Measurements. A spectrophotometer was used for kinetic measurements. Kinetics were measured by following the decrease in the absorbance at 230–240 nm. The reaction conditions were kept constant over all of the kinetic measurements: temperature, 310 K; concentration of the buffer, 50 mM; and pH, 6.5.

As a typical procedure for these experiments, carboxypeptidase-Y (4.68 mg, 7.8×10^{-8} mol) was dissolved into Tris–HCl buffer (1 mL), and carboxypeptidase-A (1.46 mg, 4.2×10^{-8} mol) was dissolved into Tris–HCl buffer (3 mL); the solutions were then diluted appropriately. A stock solution of the substrate was prepared as ca. 1 mM solutions by dissolving the substrate into Tris–HCl buffer and diluting appropriately. The solution of the substrate (3 mL) was placed in a thermostated compartment of the spectrophotometer and incubated for 5 min. In inhibition studies, a mixture of a Z–Phe–L-Pro solution as substrate (2 mL) and a Z–Phe–D-Xaa (Xaa = Pro or Ala) solution as inhibitor (1 mL) was placed in a thermostated compartment of the spectrophotometer and incubated for 5 min. After 20 μL of the enzyme solution was added, the mixture was shaken to make it homogeneous. The absolute absorbance of this solution was then measured. One set of measurements was composed of data from 5 different substrate concentrations, and the initial velocity of each substrate concentration was calculated as the average of 3–5 runs. The dipeptides and the hydrolysis products of dipeptide Z–Phe–Xaa by carboxypeptidase-Y and carboxypeptidase-A were checked by

reversed-phase HPLC; no diastereomer was detected. The mobile phase (0.5 mL/min) consisted of acetonitrile–water (3:7) containing 60 mM AcONa buffer (pH 6.0).

Measurements of Hydrophobicity. The free energy of partition was derived from the following equation: $\delta\Delta G_{ret} = -RT\ln(K_{Xaa}/K_{Gly}) = -RT[\ln(V_e - V_o)_{Xaa} - \ln(V_e - V_o)_{Gly}]$, where K_{Xaa}/K_{Gly} is the relative partition coefficient to that of Z–Phe–Gly, V_e is the elution volume, and V_o is the column volume in HPLC. Analytical conditions for HPLC: all chromatographic runs were carried out on a JASCO 980 unit equipped with a variable-wavelength UV detector at 254 nm. The column was a 250 × 4.6 mm ODS reversed-phase column (Wakosil II 5C18HG). The mobile phase (0.5 mL/min) consisted of acetonitrile–water (3:7) containing 60 mM AcONa buffer (pH 6.0).

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