Kinetic Evidence for the Hydrophobic Atmosphere of the Catalytic Cavity in Carboxypeptidase Y Based on a Linear Relationship between Michaelis–Menten Constant K_m and Hydrophobicity of the Substrates

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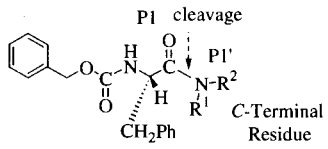
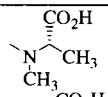
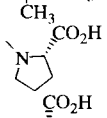
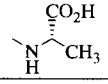
In the hydrolysis of several dipeptides by carboxypeptidase-Y (CPD-Y), there was found a good linear relationship (r = 0.999) between Michaelis–Menten constant K_m and hydrophobicity of the substrates evaluated as relative elution volume in reversed-phase HPLC.

Carboxypeptidase-Y (CPD-Y) from bakers yeast is a serine exopeptidase which catalyzes the hydrolysis of the carboxyl-terminal peptide bond of proteins. It has been established that the enzyme has a Ser-His-Asp catalytic triad as found in the trypsin and chymotrypsin families of endopeptidases.[1] From the X-ray structure of CPD-Y,[2] it has been speculated that the active site or catalytic cavity in CPD-Y consists of a binding pocket in which the amino acid residues Ser 146, Asp 338, and His 397 constitute the catalytic triad or charge relay system. Near the residues are two hydrophobic pockets (S1 and S1') which could accommodate hydrophobic side chains at the P1 and P1' positions. A fascinating feature of CPD-Y is that it readily hydrolyses peptide bonds bearing proline as the *N*-moiety whereas α-chymotrypsin can not. Implications are that the presence of the hydrophobic binding pockets is the cause of such phenomena. If this were so, hydrophobicity would be expected to be reflected in kinetic parameter K_m. With this idea in mind we have reinvestigated this process by undertaking kinetic examinations of the hydrolysis of two benzyloxycarbonyl dipeptides (Z-Phe-NMeAla and Z-Phe-Sar) of high hydrophobicity along with three previous probed benzyloxycarbonyl dipeptides (Z-Phe-Gly, Z-Phe-Ala, and Z-Phe-Pro).[3,4]

Scheme 1.



C-Terminal Residue	$\overset{R^1}{N}$-R^2	*C*-Terminal Residue	$\overset{R^1}{N}$-R^2
N-MeAlanine (NMeAla)		Sarcosine (Sar)	
Proline (Pro)		Glycine (Gly)	
Alanine (Ala)			

All kinetic measurements were performed in solutions of pH 6.5 at 37 °C.[5] The progress of the hydrolysis reactions was determined spectrophotometrically at 230–240 nm. Kinetic parameters as shown in Scheme 2 for the hydrolysis of these dipeptides by CPD-Y are summarized in Table 1. The reported values are the average of two runs.

Scheme 2.

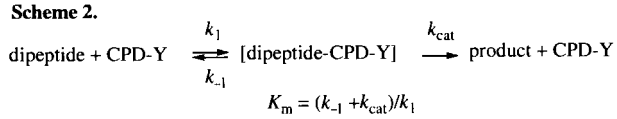


Table 1. Kinetic parameters for hydrolysis of dipeptides by CPD-Y

dipeptide	ΔG_{ret}[a] /kJ mol^{-1}	K_m /mmol	k_{cat} /s^{-1}	k_{cat}/K_m /mmol^{-1}s^{-1}
Z-Phe-NMeAla	-0.677	0.271±0.050	0.668±0.092	2.47±0.49
Z-Phe-Pro	-0.604	0.327±0.042	43.7±4.5	133±21
Z-Phe-Ala	-0.293	0.957±0.187	191.6±36.3	200±55
Z-Phe-Sar	-0.152	2.00±0.344	26.2±4.47	13.1± 1.66
Z-Phe-Gly	0.000	3.36±0.26	173.7± 13.4	51.7± 5.7

[a]ΔG_{ret} was calculated from the retention time in HPLC (ODS reversed-phase column).[6]

The order of the catalytic rate constants (k_{cat}) in the present work in regards with the *C*-terminal residue was Ala>Gly>Pro>Sar>NMeAla, while the order of overall reactivity (k_{cat}/K_m) was Ala>Pro>Gly>Sar>NMeAla. The K_m values for the Pro and NMeAla substrates were smaller than those of the *N*-unsubstituted amide substrates (Ala and Gly).

In general, K_m values are related to various types of interactions involved in enzyme–substrate complex (Michaelis complex) formations (covalent bonds, electrostatic interactions, hydrogen bonds, and hydrophobic interactions, etc.).[7] The K_m values obtained in this study seemed to point to the importance of hydrophobic interactions. Thus, we decided to examine whether a quantitative correlation exists.

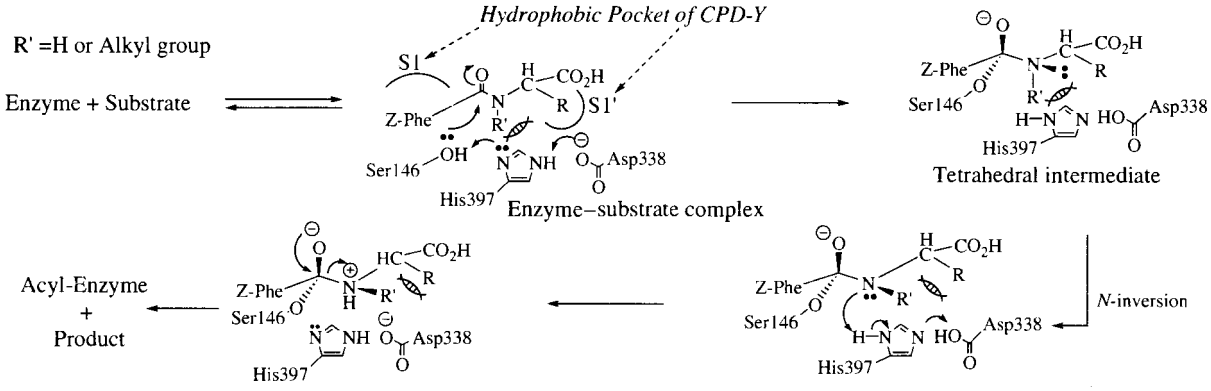
V. Pliska and his co-workers demonstrated that the hydrophobic parameters π for α-amino acids are highly correlated to the R_f values of thin-layer chromatography on silica gel and cellulose plates.[8a] The hydrophobicities for the dipeptides were evaluated as their affinity towards the stationary phase in reversed-phase HPLC relative to the Gly containing dipeptide (ΔG_{ret}),[9] which is related to elution volume (calculated from the column volume, flow rate, and retention time). In order to confirm the validity of using ΔG_{ret}, we first examined the relationship between ΔG_{ret} and π for Gly, Ala, and Pro and observed complete linearity ($\Delta G_{ret} = -0.784\pi + 6.65 \times 10^{-3}$, r = 0.999).[8b,c]

In the hydrolysis of the dipeptides by CPD-Y, there was found a linear relationship between relative K_m and ΔG_{ret}, as shown in Figure 1.

$$\log(K_m / K_m^{Gly}) = 1.64\Delta G_{ret} - 0.0082,\ r = 0.999$$

where K_m^{Gly} is the Michaelis–Menten constant in the hydrolysis

Scheme 3.



of Z-Phe-Gly. The slope represents the sensitivity of K_m to hydrophobic atmosphere. The high correlation between hydrophobicity of the dipeptide substrates and K_m elucidated in this study suggests that the hydrophobicity of the *C*-terminal amino acid is a major factor governing the stability of the enzyme–substrate complex.

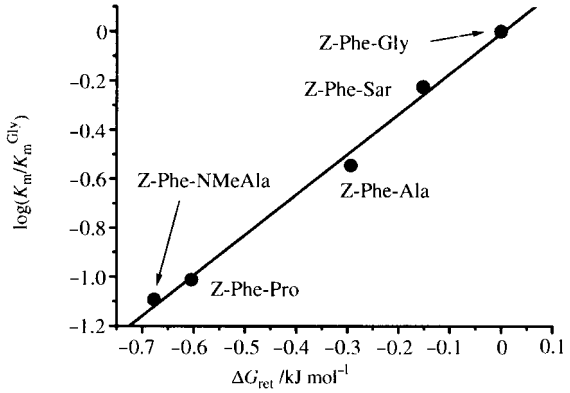


Figure 1. A linear relationship between relative K_m and hydrophobic property (ΔG_{ret}) of the dipeptides.

While the Pro and Sar residues were resistant to α-chymotrypsin cleavage, they were easily cleaved by CPD-Y enzyme catalysis. This distinct difference can be understood by assuming that the rotational barrier of the *C*-terminal residue in the active cavity is relatively low as compared with that in α-chymotrypsin.[10] The mechanism of the amide bond cleavage by CPD-Y proceeds via the formation of a tetrahedral intermediate which collapses to an acyl-enzyme intermediate at Ser 146. The role of His 397 and Asp 338 residues is thought to be to maintain the Ser 146 residue in a state capable of reacting with the incoming peptide chain, and also to stabilize the transition state leading to the tetrahedral intermediate formed during catalysis (Scheme 3). On the basis of the principle of microscopic reversibility and the stereoelectronic control theory,[11] the higher reactivity of the Pro substrate as compared with the other *N*,*N*-disubstituted substrates (Sar and NMeAla) can be interpreted in terms of the difference in steric demands upon conformational change of the P1' residues during the transition state.

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References and Notes

1 Isolation: H. Zuber, *Nature (London)*, **201**, 613 (1964). Large scale purification and mechanistic study; R. Hayashi, Y. Bai, and T. Hata, *J. Biol. Chem.*, **250**, 5221 (1975). Recent review: G. Jung, H. Ueno, and R. Hayashi, *J. Biochem.*, **126**, 1 (1999).

2 J. A. Endrizzi, K. Breddam, and S. J. Remington, *Biochemistry*, **33**, 11106 (1994).

3 R. Hayashi, Y. Bai, and T. Hata, *J. Biochem.*, **77**, 69 (1975).

4 General synthetic method. 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). Reaction of L-α-amino acid (Xaa = Ala, Gly, NMeAla, Pro, and Sar) 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). Deprotection of the *C*-terminus of the dipeptides was carried out by treatment with NaOH to give the dipeptide substrates Z-Phe-Xaa. All the dipeptides have been fully characterized by ^{1}H NMR and possess satisfactory combustion analyses.

5 CPD-Y prepared by Oriental Yeast Co. Ltd., was purchased from Wako Pure Chemical Industries and was used without purification.

6 The free energy of partition was derived from the following equation:
$\Delta G_{ret} = -RT \ln(K_{Xaa}/K_{Gly}) = -RT\,[\ln(Ve-Vo)_{Xaa} - \ln(Ve-Vo)_{Gly}]$;
K_{Xaa}/K_{Gly}: partition coefficient relative to that of Z-Phe-Gly, *Ve*: elution volume, *Vo*: column volume in HPLC. Conditions for HPLC analysis: all chromatographic runs were made with JASCO PU-980 equipped with variable-wavelength UV detector JASCO UV-970 and detected 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).

7 W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York (1969), Chap. 8, p. 393; W. Kauzman, *Adv. Protein Chem.*, **14**, 1 (1959).

8 a) V. Pliska, M. Schmidt, and J.-L. Fauchere, *J. Chromatogr.*, **216**, 79 (1981). b) M. Charton, "Progress in Physical Organic Chemistry," ed. by R. W. Taft, John Wiley & Sons, New York (1990), Vol. 18, p. 177. c) π values of Ala, Gly, and Pro are 0.40, 0.00, and 0.77, respectively.

9 C. Hansch, *Acc. Chem. Res.*, **2**, 232 (1969); R. F. Rekker, "Hydrophobic Fragmental Constant," Elsevier, Amsterdam (1977); K. Kiehs, C. Hansch, and L. Moore, *Biochemistry*, **5**, 2602 (1966); C. Hansch, K. Kiehs, and G. L. Lawrence, *J. Am. Chem. Soc.*, **87**, 5770 (1965); C. Hansch, E. W. Deutsch, and R. N. Smith, *J. Am. Chem. Soc.*, **87**, 2738 (1965); R. Xu, M.-K. Sim, and M.-L. Go, *Chem. Pharm. Bull.*, **46**, 231 (1998).

10 S. A. Bizzozero and B. O. Zweifel, *FEBS Lett.*, **59**, 105 (1975).

11 H. Dugas, "Bioorganic Chemistry, a chemical approach to enzyme action," 3rd ed., Springer, New York (1996), Chap. 4, p. 222.