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Effect of Enzyme–Substrate Interactions Away from the Reaction Site on Carboxypeptidase A Catalysis

JOHN F. SEBASTIAN,[1] GUIQING LIANG, ANNISSA JABARIN, KAREN THOMAS, AND H. BONNIE WU

Department of Chemistry, Miami University, Oxford, Ohio 45056
E-mail: Sebastjf@miamiu.acs.muohio.edu

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The kinetics of 14 peptide substrates of carboxypeptidase A have been studied for the purpose of evaluating P_1–P_3/S_1–S_3 interactions. It was found that the amide group at P_1–P_2 is required for efficient catalysis. This observation is consistent with previously proposed hydrogen bonding interactions, based on crystallographic data, between the P_1 NH and Tyr-248 and between the P_2 carbonyl oxygen and Arg-71. In contrast, substitution of the benzamido amide group (at P_2–P_3) of *N*-benzoylglycylglycyl-L-phenylalanine by $-CH_2CH_2-$ resulted in more effective catalysis. In this case hydrophobic interactions are important in the ground state and in the transition state of the rate-determining step.

INTRODUCTION

Bovine carboxypeptidase A (CPA) is a digestive protease that catalyzes the hydrolysis of oligopeptides, polypeptides, and proteins at the peptide bond of C-terminal residues with aromatic and large aliphatic side chains (*1*). The active site of CPA binds the side chain and the carboxylate group of the C-terminal residue at an apolar pocket and at Arg-145, respectively. Five binding subsites on the enzyme, S_1' and S_1 through S_4, have been reported (*2*). Cleavage of the peptide bond occurs between P_1' and P_1 of the substrate. Asn-144 and Tyr-248 also have been implicated in the binding of the substrate's terminal carboxylate group to the enzyme. Groups believed to be involved in catalysis include Glu-270, Arg-127, and Zn^{2+}. Glu-270 acts as a general base, abstracting a proton from a zinc-bound water molecule (*1*). Arg-127 was shown to be a catalytic group by site-directed mutagenic studies on rat carboxypeptidase (*3*). Tyr-198's hydroxyl group appears to play a role in catalysis by stabilizing the transition state (*4*). The edge-to-face arrangement of Tyr-198 and Phe-279 constitutes a putative modifier binding site (*5*).

X-ray studies of bovine CPA complexed with the products of *N*-benzoyl-L-phenylalanyl-L-phenylalanine hydrolysis suggest that Arg-71 is hydrogen-bonded to the amide carbonyl oxygen of the *N*-benzoyl-L-phenylalanine product (*6*). The NH of that amide is hydrogen-bonded to the hydroxyl oxygen of Tyr-248. It has been proposed that these two hydrogen bonding interactions, away from the site of bond

[1] To whom correspondence should be addressed. Fax: (513) 529–5715.

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breaking/bond making, occur in an intact bound substrate as well (*7*). There is a hydrophobic cleft at the S_1 subsite that binds apolar side chains at P_1. X-ray structures of bound phosphonates (transition state analogs) show that phenyl rings at P_2 interact favorably in an edge-to-face fashion with Tyr-248 (*7*). Phenyl rings at P_3 reside in the S_3 subsite near the S_1 cleft, and interact with Tyr-248 and Tyr-198. In this paper, we report detailed kinetic studies of peptide substrates of bovine CPA that probe the nature of the interactions between the P_1, P_2, and P_3 substrate sites and the corresponding sites on the enzyme and specifically test the proposed hydrogen bonding interactions of the P_1–P_2 amide group with Arg-71 and Tyr-248.

MATERIALS AND METHODS

Preparation of Substrates

The reference substrate Benzoylglycyl-L-phenylalanine (BGP) was purchased from Sigma and used without further purification. The range of BGP concentration used in the kinetic study was 2.66×10^{-4} to 9.31×10^{-4} M. Glycyl-L-phenylalanyl-L-phenylalanine (GPP) and glycyl-L-seryl-L-phenylalanine (GSP) were also purchased from Sigma.

N-(*trans*-Styrylacetyl)-L-phenylalanine (SAP) was synthesized from *trans*-styrylacetyl chloride and L-phenylalanine (Sigma) as described by Haas (*8*). *trans*-Styrylacetyl chloride was synthesized from *trans*-styrylacetic acid (purchased from Aldrich) and thionyl chloride (purchased from Aldrich). About 2.7 ml of thionyl chloride and 5 g of *trans*-styrylacetic acid were placed in a 120-ml flask. The flask was heated in an 80°C water bath for 30 min. The black crystals of the styrylacetic acid chloride were dissolved in 19 ml of *p*-dioxane. Another flask was used to suspend 3.6 g of L-phenylalanine in 76 ml water. The suspension was stirred in an ice bath, followed by addition of 1 *N* NaOH and 3.2 g sodium carbonate. The *trans*-styrylacetyl chloride/dioxane solution was added to the phenylalanine solution in five portions over a 90-min period with continued stirring in the ice bath. After the second addition, 19 ml of diethyl ether was added. The mixture was then stirred in the ice bath for 4 to 5 h. The product precipitated as light yellow crystals after the addition of 1.5 ml of 85% phosphoric acid. Ethyl acetate and ether were used to wash the crystals. The product was recrystallized from anhydrous ethanol and used without further purification. The melting point was 179.2–181.0°C. ^{1}H NMR (MeOD, 200 MHz) δ: 7.4–7.2 (m, 10H, phenyl rings), 6.5 (d, 1H, CH), 6.2 (m, 1H, CH), 4.7 (dd, 1H, CH), 3.2–2.9 (m, 4H, CH_2). ^{13}C NMR (MeOD, 50 MHz) δ: 174.6, 173.6 (carbonyl carbons), 138.2, 134.6, 130.3, 129.4, 129.3, 128.4, 127.7, 127.2 (phenyl ring carbons), 138.4, 123.8 (−CH=CH−), 54.9 (CH), 40.8, 38.3 (CH_2). MS (FAB)*m/z* (relative intensity), 309 (23) M^+, 205 (22), 174 (20), 161 (100), 146 (54), 120 (39), 117 (91), 103 (16), 91 (72), 79 (24), 77 (15), 65 (22): calculated *m/z* 309.1378; measured *m/z*, 309.1359. *Anal.* Calcd for SAP:C, 73.82; H, 6.20; N, 4.53. Found: C, 73.05; H, 6.23; N, 4.47. The range of SAP concentration used in the kinetic study was 8.54×10^{-5} to 1.47×10^{-4} M.

Benzoyl-L-alanyl-L-phenylalanine (BAP) was synthesized by the method de-

scribed above by using L-alanyl-L-phenylalanine (purchased from Sigma) and benzoyl chloride (purchased from Sigma). The yield was 88%. ^{1}H NMR (MeOD, 200 MHz) δ: 7.8–7.2 (m, 10H, phenyl rings), 4.6 (m, 1H, CH), 3.2 (d, 2H, CH_2), 3.0 (m, 1H, CH), 1.4 (d, 3H, CH_3). ^{13}C NMR (MeOD, 200 MHz) δ: 174.8, 174.5, 170.0 (three carbonyl carbons), 138.3, 135.2, 132.9, 130.4, 129.5, 129.4, 128.6, 127.8 (phenyl ring carbons), 55.1 (CH), 50.8 (CH), 38,.4 (CH_2), 17.8 (CH_3). MS (FAB) *m/z* (relative intensity), 341 (15.3) $(M + H)^+$, 190 (15.2), 176 (19.5), 166 (35.2), 148 (19.8): calculated *m/z* $(BAP + H)^+$, 341.1496; measured *m/z*, 341.1513. The range of BAP concentration used in kinetic study was 2.52×10^{-5} to 1.26×10^{-4} M.

N-(4-Phenylbutanoyl)-L-phenylalanine (PBP) was prepared from 4-phenylbutyryl chloride and L-phenylalanine by the method described above. The 4-phenylbutyryl chloride was synthesized from 4-phenylbutyric acid (purchased from Sigma). The yield was 54.6%, mp 178–182°C. ^{1}H NMR (DMSO-d_6, 200 MHz) δ: 8.1 (d, 1H, NH) 7.3–7.1 (m, 10H, phenyl rings), 4.4 (m, 1H, CH), 3.0 (m, 2H, CH_2), 2.4 (t, 2H, CH_2), 2.0 (t, 2H, CH_2), 1.7 (m, 2H, CH_2). ^{13}C NMR (DMSO-d_6, 200 MHz) δ: 173.5, 172.2 (carbonyl carbons), 141.9, 138.0, 129.2, 128.42, 128.37, 128.2, 126.5, 125.8 (phenyl ring carbons), 53.6 (CH), 36.9 (CH_2), 34.7 (CH_2), 34.5 (CH_2), 27.2 (CH_2). MS (MALDI)*m/z* $(M + K^+)$: calculated, 350.48; measured, 350.4. The range of PBP concentration used in kinetic study was 6.58×10^{-5} to 2.39×10^{-4} M.

N-(4-Phenylbutanoyl)glycyl-L-phenylalanine (PBGP) was prepared from 4-phenylbutyryl chloride and Gly–L-Phe (purchased from Sigma) as described above. 4-Phenylbutyryl chloride was synthesized from 4-phenylbutyric acid. The crude product was recrystallized from ethanol–H_2O twice. The yield was 51.1%, mp 127.0–129.5°C. ^{1}H NMR (acetone-d_6, 200 MHz) δ: 7.3–7.1 (m, 10H, phenyl rings), 4.7 (m, 1H, CH), 3.8 (m, 2H, CH_2), 3.1 (m, 2H, CH_2), 2.6 (t, 2H, CH_2), 2.2 (t, 2H, CH_2), 1.9 (m, 2H, CH_2), ^{13}C NMR (acetone-d_6, 200 MHz) δ: 173.5, 172.7, 169.8 (carbonyl carbons), 142.9, 137.9, 130.2, 129.3, 129.1, 127.4, 126.5 (phenyl ring carbons), 54.1 (CH), 43.2 (CH_2), 38.0 (CH_2), 35.8 (CH_2), 35.7 (CH_2), 27.5 (CH_2). MS (MALDI) *m/z* $(M + K^+)$: calculated, 407.53; measured, 407.0. The range of PBGP concentration used in kinetic study was 3.44×10^{-5} to 1.29×10^{-4} M.

N-(2-Phenylacetyl)gylcyl-L-phenylalanine (PAGP) was prepared from phenylacetyl chloride (purchased from Aldrich) and Gly–L-Phe by the procedure described above. The crude product was recrystallized from ethanol–H_2O twice. The yield was 61.3%, mp 135–137°C. ^{1}H NMR (acetone-d_6, 200 MHz) δ: 7.3–7.2 (m, 10H, phenyl rings), 4.7 (m, 1H, CH), 3.8 (d, 2H, CH_2), 3.5 (s, 2H, CH_2), 3.0 (m, 2H, CH_2). ^{13}C NMR (acetone-d_6, 200 MHz) δ: 172.5, 171.5, 169.5 (carbonyl carbons), 138.0, 136.9, 130.2, 130.1, 129.2, 129.1, 127.4, 127.3 (phenyl ring carbons), 54.2 (CH), 43.4 (CH_2), 43.3 (CH_2), 38.1 (CH_2). MS (MALDI) *m/z* $(M + K^+)$: calculated, 379.48; measured, 378.9. The range of PAGP concentration used in the kinetic study was 3.30×10^{-5} to 5.50×10^{-4} M.

N-(3-Benzoylpropionyl)-L-phenylalanine (BPP) was synthesized from 3-benzoylpropionic acid (purchased from Aldrich) and L-phenylalanine methyl ester hydrochloride (purchased from Aldrich) in two steps by the method described by Morishita *et al.* (*9*).

1. *N*(3-Benzoylpropionyl)-L-phenylalanine methyl ester was synthesized from

3-benzoylpropionic acid and L-phenylalanine methyl ester hydrochloride. The yield was 77.8% [Lit. 69.2% (*9*)]. Recrystallization from hot ethanol–water twice gave white crystals. The yield was 69.8% [Lit. 60% (*9*)], mp 93.5–94.5°C [Lit 94.5–95°C (*9*)]. ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 8.0–7.1 (m, 10H, phenyl rings), 6.2 (d, 1H, NH), 4.9 (m, 1H, CH), 3.3 (m, 2H, CH_2), 3.1 (m, 2H, CH_2), 3.0 (m, 2H, CH_2). ^{13}C NMR ($CDCl_3$, 200 MHz) δ: 198.7, 172.0, 171.6 (carbonyl carbons), 136.5, 135.9, 133.3, 129.3, 128.6, 128.5, 128.1, 127.1 (phenyl ring carbons), 53.2, 53.3 (CH, OCH_3), 37.9, 33.8, 30.0 (CH_2). IR (KBr, Bio-Rad 3240-SPC) 3320, 1730, 1727, 1695, 1650 cm^{-1}.

2. *N*-(3-Benzoylpropionyl)-L-phenylalanine was synthesized from the hydrolysis of its methyl ester. The product was recrystallized from ethyl acetate–petroleum ether. The yield was 78.8% [Lit. 86.2% (*9*)], mp 115.5–116.0°C [Lit. 115°C (*9*)]. ^{1}H NMR (MeOD), 200 MHz) δ: 8.2 (d, 1H, NH), 8.0–7.1 (m, 10H, phenyl rings), 4.7 (m, 1H, CH), 3.3 (m, 2H, CH_2), 3.1 (m, 2H, CH_2), 2.9 (m, 2H, CH_2). ^{13}C NMR (MeOD, 200 MHz) δ: 200.4, 174.8, 174.7 (carbonyl carbons), 138.4, 138.0, 134.3, 130.4, 129.7, 129.4, 129.1, 127.8 (phenyl ring carbons), 55.0 (CH), 38.5, 34.9, 30.5 (CH_2). IR (KBr, Bio-Rad 3240-SPC); the −NH− band overlapped with the carboxyl −OH band at 3364 cm^{-1}, 1739, 1679, 1651 cm^{-1}. MS (FAB) *m/z* (relative intensity); 326 (25) $(M + H)^+$, 185 (100), 166 (40), 161 (96), 133 (16), 120 (30), 105 (18), 93 (98): calculated $(BPP + H)^+$, 326.1387; measured, 326.1401. The range of concentration of BPP used in the kinetic study was 7.89×10^{-5} to 4.93×10^{-4} M.

N-(4-Benzoylbutyryl)-L-phenylalanine (BBP) was synthesized by the method described by Morishita *et al.* (*9*) using 4-benzoylbutyric acid (purchased from Aldrich) and L-phenylalanine methyl ester hydrochloride as starting materials.

1. The yield for BBP methyl ester was 48.9%. ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 8.0–7.1 (m, 10H, phenyl rings), 6.1 (d, 1H, NH), 4.9 (m, 1H, CH), 3.7 (s, 3H, CH_3), 3.1 (m, 2H, CH_2), 3.0 (t, 2H, CH_2), 2.3 (t, 2H, CH_2), 2.0 (m, 2H, CH_2). ^{13}C NMR ($CDCl_3$, 200 MHz) δ: 199.7 (ph−C=O), 172.1 (two carbonyl carbons overlapped); 136.7, 135.8, 133.1, 129.2, 128.5, 128.0, 127.1 (phenyl ring carbons), 53.0 (CH), 52.3 (CH_3), 37.7 (CH_2), 37.2 (CH_2), 35.2 (CH_2), 19.9 (CH_2).

2. The yield for BBP was 59.6%, mp 115–116°C. FT-IR (KBr); the −NH− band overlapped with the carboxyl −OH band at 3290 cm^{-1}; 1718, 1684, 1652, 1543 cm^{-1}. ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 7.9–7.1 (m, 10H, phenyl rings), 6.3 (d, 1H, NH), 4.9 (m, 1H, CH), 3.2 (m, 2H, CH_2), 3.1 (t, 2H, CH_2), 2.3 (t, 2H, CH_2), 2.0 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$, 200 MHz) δ: 200.2 (ph−C=O), 174.5, 173.3 (two carbonyl carbons); 136.6, 135.8, 133.3, 129.3, 128.6, 128.1, 127.1 (phenyl ring carbons), 53.2 (CH), 37.3 (CH_2), 37.2 (CH_2), 35.2 (CH_2), 20.0 (CH_2). MS (MALDI) *m/z* $(M - H^+ + K^+)$: calculated, 378.49; measured, 378.3. The range of BBP concentration used in the kinetic study was 3.43×10^{-5} to 1.143×10^{-4} M.

N-(3-Benzoylpropanoyl)glycyl-L-phenylalanine (BPGP) was synthesized from 3-benzoylpropionic acid, glycine methyl ester hydrochloride (purchased from Aldrich), and L-phenylalanine methyl ester hydrochloride in four steps.

1. *N*-(3-Benzoylpropanoyl)glycine methyl ester was prepared from 3-benzoylpropionic acid and glycine methyl ester hydrochloride by the method described by Morishita *et al.* (*9*). The yield was 60.8%, mp 98.9–100.8°C. ^{1}H NMR ($CDCl_3$, 200

MHz) δ: 8.0–7.4 (m, 5H, phenyl ring), 6.4 (broad, 1H, NH), 4.1 (d, 2H, CH_2), 3.8 (s, 3H, CH_3), 3.4 (t, 2H, CH_2), 2.7 (t, 2H, CH_2). ^{13}C NMR ($CDCl_3$, 200 MHz) δ: 198.8, 172.3, 170.4 (carbonyl carbons), 136.5, 133.3, 128.6, 128.1 (phenyl ring carbon), 52.4 (CH_3), 41.4 (CH_2), 33.9 (CH_2), 29.9 (CH_2).

2. *N*-(3-Benzoylpropanoyl)glycine (BPG) was prepared via hydrolysis of its methyl ester. The yield was 65.1%. ^1H NMR (acetone-d_6, 200 MHz) δ: 8.1–7.5 (m, 5H, phenyl ring), 4.0 (m, 2H, CH_2), 3.3 (t, 2H, CH_2), 2.7 (t, 2H, CH_2). ^{13}C NMR (acetone-d_6, 200 MHz) δ: 199.1, 172.8, 171.4 (carbonyl carbons), 138.0. 133.7, 129.4, 129.2, 128.7, 126.9 (phenyl ring carbons), 41.4 (CH_2), 34.2 (CH_2), 30.0 (CH_2, overlapped in solvent peaks).

3. *N*-(3-Benzoylpropanoyl)glycyl-L-phenylalanine methyl ester was prepared from BPG and L-phenylalanine methyl ester hydrochloride as described by Auld and Vallee (*10*). The yield was 21.3%. ^1H NMR ($CDCl_3$, 200 MHz) δ: 8.0–7.1 (m, 10H, phenyl rings), 6.7 (d, 1H, NH), 6.4 (t, 1H, NH), 4.9 (m, 1H, CH), 4.0 (m, 2H, CH_2), 3.7 (s, 3H, CH_3), 3.4 (t, 2H, CH_2), 3.1 (t, 2H, CH_2), 2.6 (t, 2H, CH_2). ^{13}C NMR ($CDCl_3$, 200 MHz) δ: 199.2, 172.5, 171.8, 168.7 (carbonyl carbons), 136.3, 135.9, 133.4, 129.2, 128.65, 128.57, 128.1, 127.1 (phenyl ring carbons), 53.3 (CH), 52.3 (CH_3), 43.2 (CH_2), 37.8 (CH_2) 34.0 (CH_2), 30.1 (CH_2).

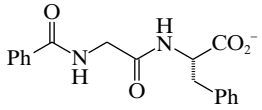
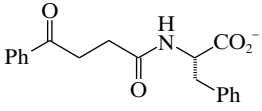
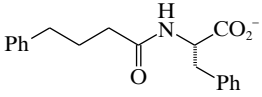
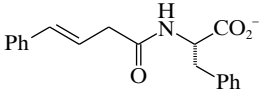
4. BPGP was prepared from the hydrolysis of its methyl ester as described by Morishita *et al.* (*9*). The yield was 46.5%, mp 154.0–155.4°C. ^1H NMR (MeOD, 200 MHz) δ: 7.9–7.1 (m, 10H, phenyl rings), 4.6 (m, 1H, CH), 3.7 (d, 2H, CH_2), 3.3 (t, 2H, CH_2), 3.1 (m, 2H, CH_2), 2.5 (t, 2H, CH_2). ^{13}C NMR (MeOD, 200 MHz) δ: 200.1, 175.6, 174.3, 171.7 (carbonyl carbons), 138.3, 137.9, 134.5, 130.3, 129.7, 129.4, 129.2, 127.8 (phenyl ring carbons), 55.1 (CH), 43.5 (CH_2), 38.4 (CH_2), 34.9 (CH_2), 30.7 (CH_2). MS (MALDI) *m/z* ($M + K^+$): calculated, 421.52; measured, 420.9. The range of BPGP concentration used in the kinetic study was 2.64×10^{-5} to 2.64×10^{-4} M.

N-Benzoylglycylglycyl-L-phenylalanine (BGGP) was prepared by the same method as described by Auld and Vallee (*10*). The yield was 86.5%, mp 221.4–221.9°C [Lit. 218–219°C (*10*)]. FT-IR (KBr): the —NH— band overlapped with the carboxyl —OH band at 3380 cm^{-1}; 1745, 1662, 1631, 1552 cm^{-1}. ^1H NMR (DMSO, 200 MHz) δ: 8.8–7.2 (m, 10H, phenyl rings), 4.5–3 (m, CH, CH_2). ^{13}C NMR (DMSO, 200 MHz) δ: 172.7, 169.2, 168.6, 166.6 (carbonyl carbons), 137.3, 133.8, 131.3, 129.90, 128.2, 128.1, 127.3, 126.4 (phenyl ring carbons), 53.5 (CH), 42.6 (CH_2), 41.5 (CH_2), 36.7 (CH_2). MS (MALDI) *m/z* ($M - H^+ + K^+$): calculated, 421.49; measured, 421.4. The range of BGGP concentration used in the kinetic study was 2.05×10^{-5} to 2.05×10^{-4} M.

Enzyme

CPAα (Cox) was purchased from Sigma. An aliquot was removed after the CPA toluene suspension was vortexed. The aliquot was centrifuged at 10,000 rpm for 15 min and the supernatant was removed immediately. The CPA crystals were washed twice with ice-cold double-distilled water and dissolved in ice-cold 3.0 M NaCl and diluted to 2 ml. The enzyme solution was stored at 4°C. The protein concentration

TABLE 1
Values of Kinetic Constants for the CPA-Catalyzed Hydrolysis of *N*-Acyl-L-phenylalanines

Substrate	k_{cat} (s^{-1})	$K_S \times 10^4$ (M)	ΔG (kcal/mol)[a]	$(k_{cat}/K_S) \times 10^{-4}$ (s^{-1} M^{-1})	ΔG_b,[b] (kcal/mol)
 *N*-Benzoylglycyl-L-phenylalanine (BGP)	139 ± 5	8.36 ± 0.18	0.0	16.6	0.0
 3-Benzoylpropanoyl-L-phenylalanine (BPP)	5.23 ± 0.18	2.34 ± 0.07	−0.75	2.24	1.2
 4-Phenylbutanoyl-L-phenylalanine (PBP)	0.709 ± 0.031	1.37 ± 0.01	−1.1	0.518	2.0
 *N*-(*trans*-Styrylacetyl)-L-phenylalanine (SAP)	0.224 ± 0.025	2.40 ± 0.02	−0.74	0.0933	3.1

[a] Example: $\Delta G = RT \ln(K_S^{BPP}/K_S^{BGP}) = -0.75$ kcal/mol.
[b] ΔG_b is the binding energy of the enzyme–transition state complexes (*13*, *14*). The apparent effect on the binding energy of the enzyme–substrate complex in the transition state due to a change in substrate structure is calculated as follows. (*13*, *14*). Example: BPP/BGP: $\Delta G_b = -RT \ln\{[k_{cat}^{BPP}/K_S^{BPP}]/[k_{cat}^{BGP}/K_S^{BGP}]\} = 1.2$ kcal/mol.

was determined spectrophotometrically. The molar absorptivity of CPA at 278 nm is 6.41×10^4 M^{-1} cm^{-1} (*11*). The protein concentration of CPA stock solution is about 2×10^{-4} M.

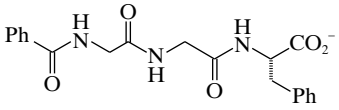
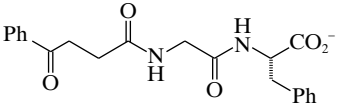
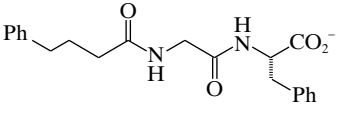
Buffer Solution

A 1.0 M NaCl/0.05 M Tris : pH 7.50 buffer at 25.0°C was used for the kinetic assay. The pH was measured with a Beckman Expandomatic pH meter. All solutions were stored at 4°C and allowed to warm to room temperature before use.

Kinetic Measurements

Kinetic data were collected on a Cary E1 spectrophotometer equipped with a thermostated cell compartment. The cell compartment was maintained at 25.0 ± 0.1°C with a water bath circulator.

TABLE 2
Values of Kinetic Constants for the CPA-Catalyzed Hydrolysis of *N*-Benzoylglycylglycyl-L-phenylalanine and Two Derivatives

Substrate	k_{cat} (s^{-1})	$K_S \times 10^4$ (M)	ΔG^a (kcal/mol)	$(k_{cat}/K_S) \times 10^{-4}$ (s^{-1} M^{-1})	ΔG_b,^{a} (kcal/mol)
 *N*-Benzoylglycylglycyl-L-phenylalanine (BGGP)	18.8 ± 0.8[b]	7.03 ± 0.08[b]	−0.10	2.67	1.1
 *N*-(3-Benzoylpropanoyl)glycyl-L-phenylalanine (BPGP)	141 ± 7	3.98 ± 0.06	−0.44	35.4	−0.4
 *N*-(4-Phenylbutanoyl)gylcyl-L-phenylalanine (PBGP)	134 ± 3	0.739 ± 0.019	−1.4	181	−1.4

[a] Defined in Table 1; reference substrate is BGP.
[b] In good agreement with previously reported values (*17*).

Data Analysis

The kinetic parameters K_M and V_{max} were obtained from a nonlinear least-squares fit of the Michaelis–Menten equation. For inhibition experiments, the form of the inhibition and K_i values were determined as described by Uhr *et al.* (*12*). The errors given are standard errors.

Molecular Modeling

Molecular models were generated from Protein Data Bank (PDB) files via CS Chem3D Pro, version 3.2.

RESULTS

The *N*-acyldipeptide *N*-benzoylglycyl-L-phenylalanine is the reference substrate in this study. According to the crystallographic studies of a variety of transition-state analogs, the P_1 NH of the benzamido group of this substrate in the transition-state complex should be hydrogen-bonded to the hydroxyl oxygen of Tyr-248 and the P_2 carbonyl oxygen of the amide hydrogen-bonded to Arg-71 (*7*). We have