A Re-examination of Two Linear Pentapeptides Claimed To Be Serine Protease Mimics

Anthony M. Vandersteen and Kim D. Janda*

Contribution from The Scripps Research Institute, Departments of Molecular Biology and Chemistry, The Skaggs Institute for Chemical Biology, 10550 North Torrey Pines Road, La Jolla, California 92037

Received May 14, 1996[⊗]

Abstract: The synthesis, characterization, and kinetic study of two linear pentapeptides L-threonyl-L-alanyl-L-seryl-L-histidyl-L-aspartic acid (TASHD) and L-seryl-γ-aminobutyryl-L-histidyl-γ-aminobutyryl-L-aspartic acid (Ser-Gaba-His-Gaba-Asp) that were previously reported by J. C. Sheehan *et al.* (Cruickshank, P.; Sheehan, J. C. *J. Am. Chem. Soc.* **1963**, *86*, 2070−2071 and Sheehan, J. C.; Bennett, G. B.; Schneider, J. A. *J. Am. Chem. Soc.* **1966**, *88*, 3455−3456) to be esterolytic catalysts modeling the active site of chymotrypsin is described. In contrast to these two reports, we observed no catalysis of ester hydrolysis for substrates *p*-nitrophenyl acetate (*p*-NPA), *N*-methoxycarbonyl-L-and-D-phenylalanine-*p*-nitrophenyl esters using the original conditions reported. In order to probe our conflicting results, accurate measurement of second order rate constants for the hydrolysis of each of these three *p*-nitrophenyl esters was carried out. The second order rate constants for these hydrolysis reactions were an order of magnitude lower than those originally reported for peptide Ser-Gaba-His-Gaba-Asp and three times lower than those reported for peptide TASHD. A very small difference in the rates of hydrolysis of *N*-methoxycarbonyl-L-and-D-phenylalanine-*p*-nitrophenyl esters in the presence of peptides TASHD and Ser-Gaba-His-Gaba-Asp was observed. Kinetic studies of the hydrolysis of *p*-NPA in the presence of peptide TASHD using a variety of concentrations of substrate and peptide are reported. A series of measurements of initial rates of hydrolysis of *p*-NPA in the presence of peptide TASHD provided no evidence for saturation kinetics. Treatment of peptide TASHD with the serine-protease inhibitor diisopropylfluorophosphate (DFP) produced no retardation in the initial rates of hydrolysis of *p*-NPA. The second order rate constants for the two peptides fell on the Brønsted line for a variety of substituted imidazoles from the literature. Our interpretation of these findings is that the peptides are behaving as simple imidazole catalysts for the hydrolysis of *p*-NPA and that there is no evidence for the involvement of the serine residue side chain during catalysis or any substrate binding by these peptides.

Introduction

The serine-protease family of enzymes is integral to the physiological processes of food digestion, cleavage of signal peptides, and control of blood pressure and blood clotting.[1] They constitute one of the most intensively studied families of enzymes, with a large number of biochemical and chemical reports providing a better understanding of their mechanism and the characteristics of enzyme catalysis in general.[2−4]

The catalytic triad of chymotrypsin (histidine, serine, and aspartic acid), has been incorporated into linear peptides,[5−8] cyclic peptides,[9−11] polymers of amino acids,[12−14] and small organic compound models.[15,16] Three reports by J. C. Sheehan and co-workers were among the first attempts to mimic the active site of an enzyme using a chemically synthesized model peptide.[7,8,10]

The discovery of novel pharmaceutical lead compounds, synthetic receptors, and solid state materials has recently been reported using "combinatorial" approaches.[17,18] We and others[19,20] have been interested in applying combinatorial chemistry to the area of *de novo* peptide catalyst design or what we term "combizymes". As a starting point for such studies we decided to reinvestigate the two linear pentapeptides: L-threonyl-L-alanyl-L-seryl-L-histidyl-L-aspartic acid (TASHD)[7,8] and L-seryl-γ-aminobutyryl-L-histidyl-γ-aminobutyryl-L-aspartic acid (Ser-Gaba-His-Gaba-Asp).[7] These peptides had been reported by Sheehan *et al.* to catalyze the hydrolysis of *p*-nitrophenyl acetate (*p*-NPA) with second order rate constants 3 to 25 times greater

* To whom correspondence should be addressed.

[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1996.

(1) Branden, C.; Tooze, J. *Introduction to protein structure*; Garland Publishing Inc.: 1991.
(2) Jencks, W. P. *Catalysis in chemistry and enzymology*; Dover Publications Inc.: 1987.
(3) Fersht, A. *Enzyme structure and mechanism*; W. H. Freeman and Co.: 1985.
(4) Kraut, J. *Ann. Rev. Biochem.* **1977**, *46*, 331−358.
(5) Vorherr, T.; Altmann, K.-H.; Mutter, M. *Helv. Chim. Acta* **1986**, *69*, 410−414.
(6) Photaki, I.; Sakarellou-Daitsiotou, M. *J. Chem. Soc., Perkin Trans. I* **1976**, 589−591.
(7) Sheehan, J. C.; Bennett, G. B.; Schneider, J. A. *J. Am. Chem. Soc.* **1966**, *88*, 3455−3456.
(8) Cruickshank, P.; Sheehan, J. C. *J. Am. Chem. Soc.* **1963**, *86*, 2070−2071.
(9) Kopple, K. D.; Nitecki, D. E. *J. Am. Chem. Soc.* **1962**, *84*, 4457−4464.
(10) Sheehan, J. C.; Mcgregor, D. N. *J. Am. Chem. Soc.* **1962**, *84*, 3000−3005.
(11) Schultz, R. M.; Huff, J. P.; Anagnostaras, P.; Olsher, U.; Blout, E. R. *Int. J. Peptide Protein Res.* **1982**, *19*, 454−469.
(12) Katchalski, E.; Fasman, G. D.; Simons, E.; Blout, E. R.; Gurd, F. R. N.; Koltun, W. L. *Arch. Biochem. Biophysics* **1960**, *88*, 361−365.
(13) Nishi, N. *Peptide Chemistry* **1978**, 151−156.
(14) Mutter, M. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 639−653.
(15) Mallick, I. M.; D'Souza, V. T.; Yamaguchi, M.; Lee, J.; Chalabi, P.; Gadwood, R. C.; Bender, M. L. *J. Am. Chem. Soc.* **1984**, *106*, 7252−7254.
(16) Cram, D. J.; Lam, P. Y.-S.; Ho, S. P. *J. Am. Chem. Soc.* **1986**, *108*, 839−841.
(17) Janda, K. D. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 10779−10785.
(18) Hsieh-Wilson, L. C.; Xiang, X.-D.; Schultz, P. G. *Acc. Chem. Res.* **1996**, *29*, 164−170.
(19) Menger, F. M.; Eliseev, A. V.; Migulin, V. A. *J. Org. Chem.* **1995**, *60*, 6666−6667.
(20) Perez-Paya, E.; Houghten, R. A.; Blondelle, S. E. *Peptide Research* **1994**, *7*, 286−288.

S0002-7863(96)01614-9 CCC: $12.00

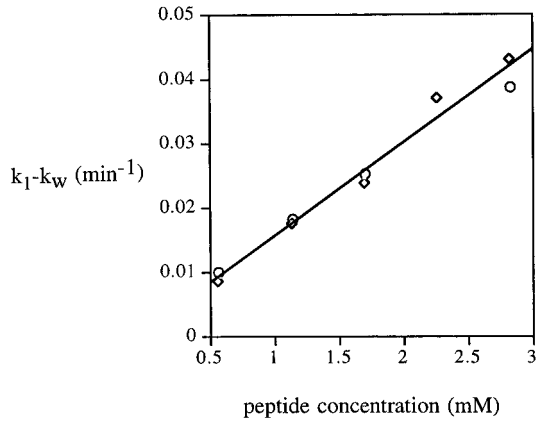

Figure 1. A plot of $k_1 - k_w$ (min^{-1}) vs peptide concentration (mM) for peptide TASHD and substrate *p*-NPA. Reactions were carried out in 3.2% by volume of 1,4-dioxane in 0.2 M phosphate buffer at pH 7.7, 25.5° ± 0.5 °C using a *p*-NPA concentration of 28.2 μM and varying peptide TASHD concentrations. The plot shows two measurements of $k_1 - k_w$ at each concentration of peptide and the line is fitted to the average of the two values.

Table 1. Second Order Rate Constants (L mol^{-1} s^{-1})[a] for the Hydrolysis of *p*-Nitrophenyl Acetate

catalyst	k_2 (L mol^{-1} s^{-1})
peptide TASHD	0.29
peptide Ser-Gaba-His-Gaba-Asp	0.14

[a] Second order rate constants were measured by varying peptide concentrations between 0.57 and 2.83 mM with a substrate concentration of 28.2 μM in 3.2% (by volume) of dioxane in buffer (0.2 M phosphate pH 7.8) at 28.0 °C ± 0.5 °C.

than imidazole and L-histidine, respectively, to show catalytic stereoselectivity and to be subject to inhibition by the serine protease inhibitor DFP. The conclusion from these findings was that polyfunctional effects associated with enzyme active sites might be operative in these two peptides. We investigated each of these claims and also sought to determine if any peptide−substrate complex and hence saturation kinetics could be observed.

Results

Kinetic studies for the hydrolysis of *p*-NPA and *N*-methoxycarbonyl-L-and-D-phenylalanine-*p*-nitrophenyl esters in the presence of peptide TASHD were carried out using the conditions originally reported by Sheehan *et al.*[8] No rate acceleration was observed under these conditions. A second set of conditions reported by Sheehan *et al.*[7,10] was used to measure the catalysis produced by peptides TASHD and Ser-Gaba-His-Gaba-Asp; the small rate acceleration over background which was observed could not be accurately quantified under these conditions.

In a second series of experiments a higher concentration of peptide was used. A plot of $k_1 - k_w$ (min^{-1}) vs peptide TASHD concentration (mM) for the hydrolysis of *p*-nitrophenyl acetate is shown in Figure 1. The second order rate constants measured for the hydrolysis of *p*-NPA in the presence of TASHD and Ser-Gaba-His-Gaba-Asp are reported in Table 1. The second order rate constants measured for the hydrolysis of *N*-methoxycarbonyl-L-and-D-phenylalanine-*p*-nitrophenyl esters in the presence of TASHD and Ser-Gaba-His-Gaba-Asp are reported in Table 2.

Attempts to observe saturation type kinetics with peptide TASHD and *p*-NPA substrate using conditions previously reported by Lowe *et al.*[21] in their study of the cysteine protease analog of peptide TASHD, L-threonyl-L-alanyl-L-cysteinyl-L-histidyl-L-aspartic acid (TACHD) proved fruitless. We observed a linear increase in the initial rates of reaction with increasing substrate concentration using a number of different concentrations of peptide. A plot of the initial rates of hydrolysis of *p*-NPA in the presence of 500 μM peptide TASHD and the background rates is shown in Figure 3.

The effect of a serine specific protease inhibitor diisopropylfluorophosphate (DFP) on the reaction rate was measured. Despite a large molar excess of DFP we saw no decrease in the initial rates of reaction relative to the control experiments.

Table 2. Second Order Rate Constants (L mol^{-1} s^{-1})[a] for the Hydrolysis of *N*-Methoxycarbonyl-L-and-D-phenylalanine-*p*-nitrophenyl Esters

catalyst	k_2 (L mol^{-1} s^{-1}) L-substrate	k_2 (L mol^{-1} s^{-1}) D-substrate
peptide TASHD	0.33	0.23
peptide Ser-Gaba-His-Gaba-Asp	0.25	0.12

[a] Second order rate constants were measured by varying peptide concentrations between 0.57 and 2.83 mM with a substrate concentration of 28.2 μM in 3.2% (by volume) of dioxane in buffer (0.2 M phosphate pH 7.8) at 28.0 °C ± 0.5 °C.

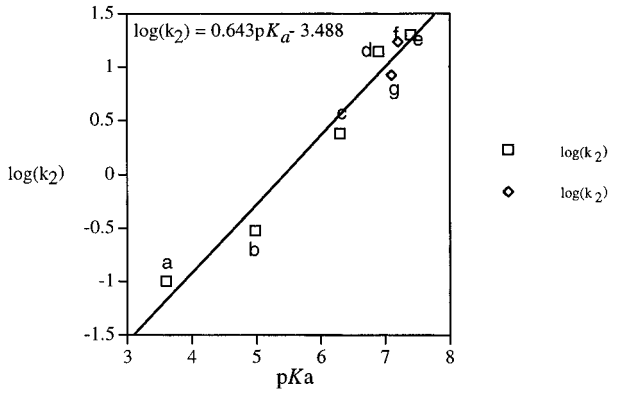

Figure 2. A Brønsted plot for a series of substituted imidazoles[23] and peptides (I) and (II) (a) 4(5)-bromoimidazole, (b) benzimidazole, (c) 4(5)-hydroxymethylimidazole, (d) imidazole, (e) 4(5)-methylimidazole, (f) peptide (I), and (g) peptide (II).

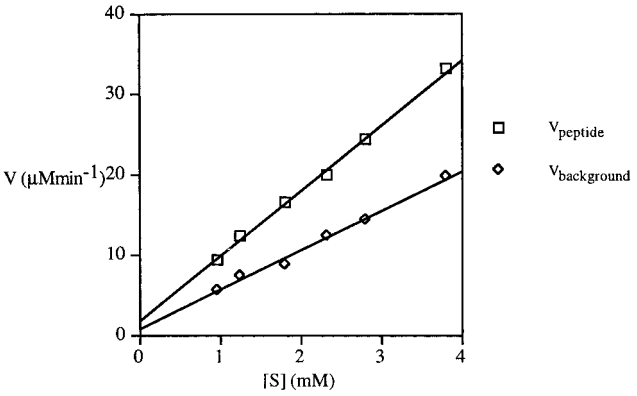

Figure 3. A plot of initial rate of reaction V (mM min^{-1}) vs *p*-NPA concentration [S] (mM) for peptide TASHD. Reactions were carried out in 5% by volume of 1,4-dioxane in 0.1 M phosphate buffer at pH 7.6, 35.0° ± 0.5 °C using a peptide concentration of 500 μM and varying *p*-NPA concentrations. The plot shows the mean value of two measurements of the initial rate at each substrate concentration.

Discussion

The original data reported by Sheehan *et al.*[7,10] is summarized in Tables 3 and 4. It is evident after comparing the data in

(21) Photaki, I.; Bardakos, V.; Lake, A. W.; Lowe, G. *J. Chem. Soc. (C)* **1968**, 1860−1864.

Table 3. Reported Second Order Rate Constants (L mol^{-1} s^{-1}) for the Hydrolysis of *p*-Nitrophenyl Acetate[7,8,24]

catalyst	k_2 (L mol^{-1} s^{-1})
peptide TASHD	1.53
peptide Ser-Gaba-His-Gaba-Asp	2.45
peptide TASHD + DFP	0.80
imidazole	0.33 (ref 7) 0.58 (ref 24)

Table 4. Reported Second Order Rate Constants (L mol^{-1} s^{-1}) for the Hydrolysis of *N*-Methoxycarbonyl-L-and-D-phenylalanine-*p*-nitrophenyl Esters[7]

catalyst	k_2 (L mol^{-1} s^{-1}) L-substrate	k_2 (L mol^{-1} s^{-1}) D-substrate
peptide TASHD	1.03	0.53
peptide Ser-Gaba-His-Gaba-Asp	2.58	1.87
imidazole	1.00	1.00

Tables 1 and 3 that the second order rate constants for the hydrolysis of *p*-NPA are significantly lower than those originally reported and are of a similar magnitude as those reported for other histidine-containing peptide enzyme models.[6,11,13] In order to probe what interactions may be taking place between the peptide and substrate during the reaction we constructed a Brønsted plot using available second order rate constants for the hydrolysis of *p*-NPA. A discussion of general base catalysis and its relationship to enzymatic hydrolysis has been reported for a variety of bases[22] and substituted imidazoles.[23] Detailed studies of the catalysis of ester hydrolysis for a variety of substrates by imidazole has also been reported.[24,25] A Brønsted plot for the second order rate constants of the hydrolysis of *p*-NPA by a variety of four (5)-substituted imidazoles[23] together with peptides TASHD and Ser-Gaba-His-Gaba-Asp is shown in Figure 2. It is clear that the second order rate constants for these peptides fall closely on the Brønsted line of gradient 0.64. Based on this plot we contend that peptides TASHD and Ser-Gaba-His-Gaba-Asp are acting as substituted imidazoles under these experimental conditions and are not exhibiting any enhanced catalytic acceleration connected with the presence of the "catalytic triad" of chymotrypsin.

With respect to the hydrolysis of *N*-methoxycarbonyl-L-and-D-phenylalanine-*p*-nitrophenyl esters in the presence of peptides TASHD and Ser-Gaba-His-Gaba-Asp, we observed significantly lower second order rate constants than reported by Sheehan *et al*.[7] (Tables 2 and 4).

The difference between the second order rate constants for the L and D substrates is five times smaller than originally reported. Such an insignificant difference in rate constants led us to conclude that these peptide enzyme models were not displaying significant stereoselectivity, especially in comparison to the exquisite stereoselectivity displayed by enzymes.[3]

Sheehan and co-workers originally observed a 48% decrease in the catalytic coefficient after treatment of the peptide (TASHD) with DFP.[7] Despite a large molar excess of DFP we saw no decrease in the initial rates of reaction relative to the control experiments and thus concluded that the serine residue was not involved in the catalytic mechanism.

Conclusion

The initial rates of reaction in the presence of peptide TASHD and the results of the DFP experiment coupled with the above Brønsted analysis for both TASHD and Ser-Gaba-His-Gaba-Asp (*vide supra*) has led us to the conclusion that the histidine residue of these peptides is acting as a simple chemical catalyst (either nucleophilic or general base catalysis of the magnitude expected for imidazoles of similar pK_a). This can be contrasted with the role of histidine in activating the serine hydroxyl group for nucleophilic catalysis in the active site of the serine protease enzymes. In addition the small difference in second order rate constants for the hydrolysis of *N*-methoxycarbonyl-L-and-D-phenylalanine-*p*-nitrophenyl esters by peptides (TASHD) and (Ser-Gaba-His-Gaba-Asp) does not provide evidence for stereoselective substrate recognition by the peptides.

None of the characteristics of enzyme catalysis, as seen with chymotrypsin such as substrate recognition/binding, formation of an acyl-enzyme intermediate or rate acceleration/transition state stabilization beyond that produced by substituted imidazoles is produced by these two peptides.

Experimental Section

Materials. Fully protected amino acid building blocks and derivatized Wang resins were purchased from Novabiochem. *N*,*N*-Dimethylformamide, *N*-methylpyrollidone, isopropyl alcohol, and methylene chloride were used as supplied by Baxter. Diisopropylcarbodiimide, 1-hydroxybenzotriazole, piperidine, ethane dithiol, and trifluoroacetic acid were used as supplied by the Aldrich Chemical Company. *p*-Nitrophenyl acetate was purchased from the Sigma chemical company and was used without further purification, 1,4-dioxane of the purest grade available was used during kinetic runs without futher purification. Volumetric standard solutions of 0.1 N KOH and pH calibration standard solutions of pH 4 and 7 were purchased from Fisher scientific.

Methods. Peptides TASHD and Ser-Gaba-His-Gaba-Asp were synthesized by manual solid-phase synthesis using N-terminal fluorenylmethoxycarbonyl (FMOC) protection and acid labile side chain protection according to published methods.[26] The peptides were purified by preparative reversed phase HPLC and were fully characterized. Physical data for peptide TASHD matched Sheehan's data closely; however, we measured a different melting point and optical rotation from the reported values for peptide Ser-Gaba-His-Gaba-Asp. It should be noted that the original synthetic work was carried out using the water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride coupling reagent[27] which has recently been reported to lead to significant racemization during amino acid coupling reactions.[28] TASHD was purified by reversed phase HPLC using a Waters prep LC4000 and Waters 1000 prepak column module eluting with 99.9% H_2O 0.1% TFA. Peptide Ser-Gaba-His-Gaba-Asp was purified using a Rainin HPXL and a Vydac C18 90Å semipreparative column eluting with a gradient 0−40% acetonitrile in 0.1%TFA/water. Analytical HPLC was carried out on a Hitachi L6200A instrument with a reversed phase Vydac C18 column. Detection of the peptides during preparative and analytical HPLC was carried out at 210 nm.

All pH titrations were carried out according to reported methods[29] in duplicate with a Corning general purpose electrode and an Aldrich combination ultrathin long-stem electrode calibrated between pH 4 and 7 on an Orion pH/ISE meter. The pK_a of the histidine residue for each peptide was determined by direct potentiometric titration of a 0.01 N aqueous peptide solution with standardized 0.1 N solution of potassium hydroxide at 25 ± 0.5 °C with stirring by bubbling nitrogen gas through the solution. The pK_a value was determined as the point of inflection in the region of imidazole ionization.[12] No pK_a values were reported in the original papers.[7,8]

Melting points were measured on a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured with

(22) Bender, M. L.; Turnquest, B. W. *J. Am. Chem. Soc.* **1957**, *79*, 1656−1662.
(23) Bruice, T. C.; Schmir, G. L. *J. Am. Chem. Soc.* **1957**, *79*, 1663−1667.
(24) Kirsch, J. F.; Jencks, W. P. *J. Am. Chem. Soc.* **1964**, *86*, 833−837.
(25) Jencks, W. P.; Carriuolo, J. *J. Am. Chem. Soc.* **1961**, 1743−1750.
(26) Atherton, E.; Sheppard, R. C. *Solid phase peptide synthesis, a practical approach*; IRL Press, 1989.
(27) Sheehan, J. C.; Preston, J.; Cruickshank, P. A. *J. Am. Chem. Soc.* **1965**, *87*, 2492−2493.
(28) Ho, G.-J.; Emerson, K. M.; Mathre, D. J.; Shuman, R. F.; Grabowski, E. J. J. *J. Org. Chem.* **1995**, *60*, 3569−3570.
(29) Albert, A.; Serjeant, E. P. *The determination of ionization constants, a laboratory manual*, 3rd ed; Chapman and Hall.

a Perkin-Elmer 241 polarimeter. ^{1}H-NMR spectra were recorded on a Bruker AM300 spectrometer with tetramethylsilane as an internal standard, and coupling constants are reported in Hertz. High resolution fast atom bombardment (FAB) and electrospray ionization (ESI) mass spectra were recorded by the Scripps Research Institute mass spectrometry facility.

Kinetic Experiments. The progress of the reactions were measured by the release of the *p*-nitrophenolate ion at 405 nm in a quartz ELISA plate with a Molecular Devices Spectramax 500 plate reader.[30] A stock peptide solution was prepared by dissolving 4−5 mg of peptide in 0.1 M phosphate buffer. A typical reaction volume was 200 μL. The aqueous buffered peptide solution was transferred into an ELISA well and prewarmed for at least 15 min. A solution of the substrate ester in 1,4-dioxane was added, and the reaction monitoring was started immediately using the SoftMax Pro software program. Values of initial rates of reaction in OD min^{-1} were converted into μM min^{-1} of *p*-nitrophenol by determining the conversion factor using standardized *p*-nitrophenol solutions. The DFP solutions were prepared using recommended safety procedures in a fume-hood. These experiments were carried out in a disposable plastic ELISA plate.

The reactions using *p*-NPA, *N*-methoxycarbonyl-L-and-D-phenylalanine-*p*-nitrophenyl esters (concentration 28.2 μM) were followed over at least five half-lives, and the pseudo-first order rate constants k_1 were calculated by the semi-log method.[2,7,12] The second order rate constant k_2 (L mol $^{-1}$ min^{-1}) was calculated according to eq 1.[12]

$$k_2 = (k_1 - k_w)/c \qquad (1)$$

where k_1(min^{-1}) = measured pseudo-first order rate constant in the presence of the catalyst, k_w (min^{-1}) is the background rate under identical conditions in the absence of catalyst, and c (mol L^{-1}) is the molar concentration of uncharged histidine residues.

Determination of values for the pseudo-first order rate constants at five different concentrations of each peptide between 0.57 and 2.83 mM in duplicate ensured reproducibility.[2] The concentration of uncharged histidine residues in solution was calculated using the Henderson−Hasselbalch eq.[2]

Enzyme saturation kinetics are normally investigated by measuring initial rates of reaction using a large excess of substrate over catalyst. Lowe *et al*.[21] had observed saturation kinetics during the hydrolysis of *p*-NPA in the presence of the peptide TACHD with a K_m = 1.18 ± 0.06 mM and a k_{cat} = 21 ± 0.2 × 10^{-3} s^{-1}. A larger percentage of dioxane and an increased temperature was required to ensure that the substrate remained in solution. Thus reactions were carried out in 5% (by volume) of dioxane in buffer (0.1 M phosphate pH 7.61) at 35.0 ± 0.5 °C with a peptide concentration of 50 μM and seven substrate concentrations ranging from 0.31 to 28.00 mM.[21]

These experiements were carried out with peptide TASHD in duplicate, and the data were analyzed by an initial rates analysis.[2,21] A slight rate enhancement was observed using these conditions (initial rate of appearance of *p*-nitrophenolate in the presence of peptide TASHD (μmol/min^{-1})/background rate (μmol/min^{-1}) ≈ 1.1 ± 0.1); however, it was too small to permit accurate determination of any kinetic parameters, due to random experimental error.

Initial rates were next determined by increasing peptide concentrations from between 100 and 500 μM at a fixed substrate concentration of 1.24 mM. These conditions allowed reaction rates of both the peptide and background to be accurately determined. The relationship seen between peptide concentration and the initial rates of *p*-NPA hydrolysis was linear.

In a further set of experiments, initial rates of *p*-NPA hydrolysis were measured at peptide concentrations of 100 and 500 μM and *p*-NPA concentrations between 1 and 4 mM. A linear relationship was observed between the initial rates of reaction and substrate concentration. A plot of initial rate (μmol min^{-1}) *vs* [*p*-NPA] for 500 μM peptide TASHD and the background rate is shown in Figure 3.

Inhibition studies with DFP were carried out by incubation of solutions of peptide TASHD (343 μM in sodium phosphate buffer 0.15 M pH 7.61) with solutions of DFP (in acetonitrile with concentrations ranging from 0.38 to 6.1 mM) for 15 min at room temperature. The substrate *p*-NPA in dioxane (2.2 mM) was added and the initial rates of reaction were observed at 25° ± 0.5 °C. The background rate without added DFP or peptide was 5.31 μmol min^{-1}, the rate with peptide was 10.77 μmol min^{-1} and the rate with added peptide and DFP was 9.80 μmol min^{-1}.

Analytical Data. Thr-Ala-Ser-His-Asp (TASHD).[8] δ[^{1}H 1:9 D_2O:H_2O v/v, internal reference: 1,4 dioxane] 1.3 (3H, d, J = 7.2), 1.4 (3H, d, J = 8.1), 2.9 (2H, d, J = 7.2), 3.2−3.4 (2H, m), 3.8 (2H, d, J = 4.5), 3.9 (1H, d, J = 7.2), 4.2 (1H, t, J = 7.2), 4.4 (2H,m), 7.3 (1H, s), 8.45−8.6 (4H,m), 8.55 (1H, s) 8.85 (2H, d, J = 5.3) Analytical HPLC R_t = 5.6 min (eluent water/0.1% TFA). *m*/*z* FAB ms = 530.2208 [$C_{20}H_{31}N_7O_{10}$ requires 530.2211], $\alpha_D(H_2O)$ = −4.3° [lit. −8.9°], T_m = 170 °C [lit. 177 °C], pK_a = 7.2 ± 0.1.

Amino acid analysis was carried out by the Scripps Research Institute Core Facility and gave the following ratios of amino acids: Asp/His (1): 0.99, 1.02 Ser (1): 0.98, 1.11 Ala (1): 1.0, 0.97 Thr (1): 0.94, 1.01.

Ser-Gaba-His-Gaba-Asp.[7] δ[^{1}H 1:9 D_2O:H_2O v/v, internal reference: 1,4 dioxane] 0.8 (8H, q, J = 7.9,9.5), 1.65−1.9 (4H, m) 2.9 (2H, m), 3.1−3.3 (2H, m), 3.9−4.2 (2H, m), 4.15 (1H, t, J = 4.7), 4.2 (2H, m), 7.4 (1H, s), 8.4 (1H, d, J = 7.9) , 8.5 (1H,d, J = 7.9), 8.58 (1H, s), 8.75 (2H, t, J = 3.9). Analytical HPLC R_t = 14.8 min (0 to 40% acetonitrile: 0.1% TFA/water over 40 min) *m*/*z* FAB ms = 550.2253 [$C_{21}H_{33}N_7O_9Na$ requires 550.2237], α_D (H_2O) = −33.5° [lit. +25.6°], T_m = 165−168 °C [lit. 280 °C dec], pK_a = 7.1 ± 0.1.

N-methoxycarbonyl-L-and-D-phenylalanine-*p*-nitrophenyl esters were synthesized according to the reported procedure.[31]

L isomer δ[^{1}H DMSO-d_6] 3.2 (2H, t, J = 8.1) 3.7 (3H, s), 4.8 (1H, m), 5.25 (1H, d, J = 9.7), 6.9−7.3 (7H, m), 8.2 (2H, m). *m*/*z* FAB ms = 345.1099 [$C_{17}H_{16}N_2O_6$ requires 345.1087], α_D (H_2O) = −8.9° [lit. −9.6°], T_m = 104 °C [lit. 103 °C]. D isomer *m*/*z* FAB ms = 345.1097 [$C_{17}H_{16}N_2O_6$ requires 345.1087], α_D (H_2O) = +10.9° [lit. +9.8°], T_m = 104 °C [lit. 103 °C].

Acknowledgment. We would like to thank Dr. Arnold C. Satterthwait and Dr. Edelmira Cabezas for assistance with the solid-phase synthetic methodology, Professor Jack F. Kirsch for critical reading of the manuscript, and Dr. Paul Wentworth, Jr. for preparation of the manuscript and assistance with the kinetic analysis. This work was supported in part by the Scripps Research Institute and The Skaggs Institute for Chemical Biology.

JA9616140

(30) Eisenthal, R.; Danson, M. J. *Enzyme Assays, a practical approach*; IRL Press: 1993.

(31) Elmore, D. T.; Smyth, J. J. *Biochem. J.* **1965**, *94*, 563−568.