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A reinvestigation of a synthetic peptide (TrPepz) designed to mimic trypsin

(protein design/serine proteases/synthetic peptides)

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ABSTRACT **Recently, a 29-residue cyclic peptide was synthesized (TrPepz) that was reported to possess nearly the same catalytic activity and specificity as the pancreatic serine protease, trypsin, for hydrolysis of a small ester substrate, *N*-tosyl-L-arginine methyl ester (TAME), and small and large peptides [Atassi, M. Z. & Manshouri, T. (1993) *Proc. Natl. Acad Sci. USA* 90, 8282–8286]. To study these results we have resynthesized TrPepz and a related cyclic peptide reported to possess some trypsin-like activity. The authenticity of each peptide was confirmed by mass spectrometry, peptide sequencing, compositional analysis, and ^{1}H NMR spectroscopy. However, neither peptide exhibited any detectable esterase activity or amidase activity under a variety of conditions tested. Molecular modeling studies indicated it was possible for TrPepz to be nearly superimposed upon the active site of trypsin. However, NMR experiments showed the structure of the cyclic peptide to be disordered. Thus, we were unable to confirm the results of Atassi and Manshouri. Our results are consistent with the view that serine protease activity depends not only on the presence of catalytic groups but also on their precise and stable alignment.**

Serine proteases are some of the most well-studied, practically useful, and ubiquitous enzymes in biology (for reviews, see refs. 1–3). These proteases are characterized by a catalytic triad, Ser-His-Asp, and additional determinants that accelerate the rate of amide or ester bond hydrolysis by factors of 10^8 to 10^{10} over the spontaneous rate in neutral aqueous solution. Part of this enormous rate enhancement involves nucleophilic attack upon the carbonyl of the scissile bond by the hydroxyl of the catalytic Ser. The Ser hydroxyl is activated by transfer of its proton to the catalytic His to form an imidazolium ion, which is stabilized by the catalytic Asp.

Studies of the structure and function of these enzymes indicate the nature and precise alignment of the active site is crucial for catalysis. For example, even though the serine proteases, subtilisin and trypsin, have completely different folds, their active sites can be virtually superimposed (for review, see ref. 3). Mutational analysis of the catalytic triad for subtilisin (4) and trypsin (5, 6) has shown that substitutions at any one of the residues in the triad result in 10^4- to 10^6-fold reductions in the catalytic turnover number (k_{cat}). These enzymes also have an oxyanion binding site in which hydrogen bond donors (main-chain amides from trypsin or a mixture of main-chain and side-chain donors in subtilisin) stabilize the tetrahedral oxyanion intermediate that is generated when the catalytic serine attacks the scissile peptide bond (3). Substitutions of one of the donor side chains (Asn-155) in subtilisin cause a 250- to 2500-fold reduction in k_{cat} depending upon the mutant (7, 8).

The chemical identities of the catalytic groups and their precise orientations are important for activity. Disorder or slight perturbations in the structure of the active site can have enormous effects on activity. For example trypsinogen, a precursor to trypsin, contains the same catalytic apparatus yet is $\approx 10^5$-fold less active; this huge difference in activity is accounted for by the fact that the catalytic triad in trypsinogen is less well-ordered than trypsin (for review, see ref. 9). In another example, k_{cat} is decreased by a factor of 10 when the nucleophilic hydroxyl of Ser-221 of subtilisin is shifted by just 0.3 Å, upon mutating a nearby residue, Pro-225, to Ala (10, 11).

The substrate specificity of proteases ranges widely and these specificities are determined by complementary contacts between the substrate side chains and binding sites on the protease. A number of protein-engineering studies have shown that it is possible to rationally or randomly alter the specificity of proteases (for examples, see refs. 12–14). However, this can be complicated. For example, attempts to convert the specificity of trypsin to chymotrypsin involved many mutations outside of those that make direct contact with the substrate (15).

Recently, Atassi and Manshouri (16) reported the extraordinary design of synthetic cyclic peptides ranging from 23 to 29 residues in length that mimic the specificity and catalytic efficiency of trypsin (TrPepz) or chymotrypsin (ChPepz), pancreatic serine proteases containing 223 and 241 residues, respectively. A cyclic disulfide peptide called TrPepz (Table 1) was designed to link the catalytic triad and substrate binding residues of trypsin using flexible glycine or diglycine spacers. In fact, more than one-third of all residues in this peptide were glycine. Aside from the cyclic nature of this peptide, there were no additional constraints on its structure.

Remarkably, the researchers report that the peptide had k_{cat} and K_m values for *N*-tosyl-L-arginine methyl ester (TAME) that were within a factor of three of the values reported by them for bovine trypsin (16). Moreover, the peptide could cleave several other peptides containing Lys or Arg residues as well as myoglobin to generate the same peptide fragments produced by digestion with trypsin. The design of TrPepz was achieved by changing 4 residues in the 29-residue ChPepz peptide. ChPepz is reported to possess an activity and specificity virtually identical to that of bovine chymotrypsin.

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Abbreviations: TAME, *N*-tosyl-L-arginine methyl ester; N-t-GPR-pna, *N*-tosyl-Gly-L-Pro-L-Arg-*p*-nitroanilide; FABms, fast atom bombardment mass spectrometry; TrPepz, peptide designed to mimic the active site of trypsin; ChPepz, peptide designed to mimic the active site of chymotrypsin.

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Table 1. Sequences of two cyclic peptides reported to possess trypsin-like activity (16)

Design 3:	CFGGSDGQGSDGGVSWGLGGDGAAHC
TrPepz:	CGYHFGGSDGQGSDGGVSWGLGGDGAAHC

Others have attempted to mimic proteases by generating catalytic antibodies (17) or placing catalytic triads on peptide (ref. 18; M. J. Corey, personal communication) or organic (19) scaffolds. However, none of these efforts comes close to matching the catalytic efficiency and k_{cat} values reported for TrPepz or ChPepz (16). Thus, the results of Atassi and Manshouri (16) are unprecedented and extraordinary.

In an effort to verify the kinetic results and further characterize these peptides, we requested a sample of TrPepz from M. Z. Atassi (Baylor College of Medicine, Houston). This was not provided to us, and so we synthesized and characterized two peptides that were reported to mimic trypsin (Table 1). However, unlike the results reported by Atassi and Manshouri (16), we found that neither peptide had any detectable esterase or amidase activity. We suggest the reason for this is that despite the cyclic nature of the peptides and the presence of appropriate functional groups, the peptides lack a stable conformation that mimics the active site of trypsin.

MATERIALS AND METHODS

N-Tosyl-Gly-L-Pro-L-Arg-*p*-nitroanilide (N-t-GPR-pna) was from Sigma. Bovine trypsin was obtained from Sigma or Worthington. TAME was from Sigma or Vega.

Peptide Synthesis. Peptides were synthesized on a PEG-polystyrene resin by standard fluoren-9-ylmethoxycarbonyl chemistry. The side-chain protecting groups were all *t*-butyl, except for Cys, Gln, and His, where we used trityl groups. The crude peptides were cleaved from the resin with trifluoroacetic acid in the presence of scavengers. The reduced peptide (≈50% pure by HPLC) was diluted to 0.3 mg/ml in aqueous solution (pH 8.0). To form the single disulfide bond, the dilute peptide solution was stirred at 25°C. Cyclization by air oxidation was monitored by HPLC because the reduced linear peptide had a longer retention time on a Vydac C_{18} column. Both monomeric cyclic peptides were purified by HPLC on a 1 × 50 cm Vydac C_{18} (300-Å pore size) column. The cyclic peptides were lyophilized and stored at 25°C. The peptides freely dissolved in aqueous buffers to a concentration of 1–1.5 mM in 30 mM Tris·HCl (pH 7.5). Peptide solutions were stored in frozen aliquots and used typically after one or two thaws.

Peptide Characterization. All fast atom bombardment mass spectrometry (FABms) spectra were collected on a JOEL HX110/110 tandem mass spectrometer operated in a two-sector mode. The mass range was scanned up to 3000 Da. The matrix was thiolglycerol at neutral pH. Reduction on the probe was accomplished by addition of 0.5 μl of concentrated ammonium hydroxide to the sample in thiolglycerol. Cesium iodide was used to calibrate the mass axis and gave a mass accuracy of ±0.2 Da. Electrospray ionization mass spectrometry was performed using a PE/Sciex API 3 mass spectrometer. Samples were dissolved in 10% (vol/vol) acetonitrile/1% acetic acid and infused directly into the mass spectrometer.

Enzyme Assays. The esterase activities of both peptides were measured by two investigators (J.A.W. and M.L.) separately by using the substrate TAME. The peptides were assayed in 1 or 3 ml of assay mixture containing 0.5–1 mM TAME (46 mM Tris·HCl, pH 8.0/11 mM $CaCl_2$ at 25°C) essentially as described by Atassi and Manshouri (16) or recommended by Worthington. Under these substrate conditions, bovine trypsin from Sigma and Worthington had specific activity values that were 46% and 53%, respectively, of the value reported for highly purified and recrystallized trypsin (20). The change in absorbance at 247 nm per min in our TAME assays with trypsin was typically 0.05–0.2 A_{247} unit/min (depending on substrate and enzyme concentration as recommended by Worthington). This was against a blank value that ranged from 0.0015 to 0.003 A_{247} unit/min for 60-min or 2-min assays, respectively. The use of 0.5–1 mM TAME is convenient for assay sensitivity and to compare our results directly with those of Atassi and Manshouri (16). However, it is noteworthy that the K_m value determined over this range of substrate concentrations is 100-fold too high because of extreme substrate activation (21). Assays using the *p*-nitroanilide substrate, N-t-GPR-pna, were run essentially as described for TAME except that the stock N-t-GPR-pna was dissolved in dimethyl sulfoxide. As a result the assay mixture contained 0.33% dimethyl sulfoxide at the substrate concentrations used (16 μM, which is ≈1.8 times the K_m for trypsin). Hydrolysis of N-t-GPR-pna was followed by monitoring the release of *p*-nitroanilide from the change in absorbance at 410 nm.

NMR Spectroscopy. NMR experiments were performed at 10°C on a solution of TrPepz at 7 mg/ml in 90% H_2O/10% ^2H_2O/50 mM potassium phosphate, pH 5.5, by using a Bruker AMX-500 NMR spectrometer. A two-dimensional total correlation spectroscopy spectrum (22, 23) with a spin-lock period of 90 ms was acquired using the "clean" DIPSI-2rc sequence (24) for isotropic mixing. Two-dimensional rotating frame nuclear Overhauser effect spectroscopy spectra (25) with mixing times of 250 ms and 450 ms were also acquired. All spectra were recorded in the phase-sensitive mode using time-proportional phase incrementation for quadrature detection in F_1 (26).

RESULTS AND DISCUSSION

We synthesized TrPepz and a related peptide we call design 3 (Table 1). Design 3 was the prototype peptide to TrPepz that was reported to be just 72-fold less active than TrPepz on TAME (16). The linear peptides were prepared using fluoren-9-ylmethoxycarbonyl chemistry and cyclized by air oxidation essentially as described (16). The peptides were purified by HPLC and by this criterion we estimate them to be >85% homogeneous (Fig. 1).

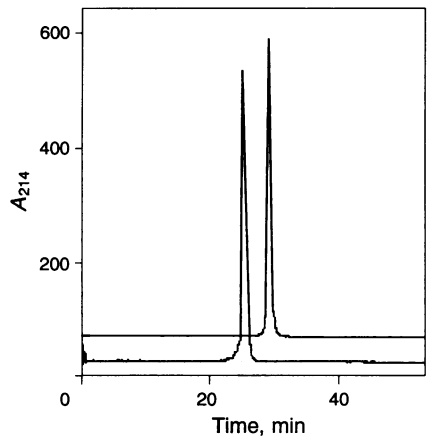

FIG. 1. HPLC profiles of purified cyclic peptides, design 3 (lower trace) and TrPepz (upper trace). Samples were dissolved in 0.1% trifluoroacetic acid and loaded onto a 1 × 50 cm Vydac C_{18} column (300-Å pore size). The flow rate was 9 ml/min. Cyclic peptides were eluted using a 10–50% acetonitrile gradient in 0.1% trifluoroacetic acid over 80 min at 25°C.

To establish precisely the molecular mass of the cyclized peptides, we analyzed them by FABms (Fig. 2). From these spectra the monoisotopic molecular weights of design 3 and TrPepz were within 0.1 Da of their expected masses (Table 2). When each peptide was reduced, the corresponding masses increased by 2 Da consistent with the reduction of the disulfide bond. The mass spectra (Fig. 2) also show small amounts (≈15%) of compounds that have masses that are intervals of 57 Da smaller than the parent cyclic peptides. These likely represent peptides in which a Gly residue is missing. The small amounts of these contaminants should have little significant effect on the activity measurements of the parent peptide. Additional small peaks in these spectra do not reflect impurities but noise in the spectra because the narrow slits used to give high resolution reduced the sensitivity of the analysis.

Similar mass values were obtained by electrospray mass spectrometry. Each cyclic peptide gave an average isotopic molecular weight within experimental error of that expected for the cyclic monomer (Design 3: expected, 2352.4; actual, 2352.0. TrPepz: expected, 2709.8; actual, 2709.0). These spectra (not shown) had a much higher signal-to-noise ratio than the FABms but give less precise molecular weight values (typically ±0.6 Da).

Neither cyclized peptide exhibited detectable reaction with the thiol titrant, 5,5′-dithiobis(2-nitrobenzoic acid) (data not shown). From the detection limit in the 5,5′-dithiobis(2-nitrobenzoic acid) analysis, we estimate that >98% of the thiols in each peptide were oxidized. Both cyclic peptides exhibited UV absorption spectra consistent with their Trp, Tyr, and Cys content [ε_{280} ($M^{-1}\cdot cm^{-1}$) for design 3: 5860, calculated; 5400, actual. ε_{280} for TrPepz: 7060, calculated; 7010, actual]. The amino acid analysis for each peptide was within experimental error (measured value is listed first followed by expected in parentheses). For design 3: Cys-x 1.84(2), Phe 0.97(1), Gly 9.72(10), Ser 2.32(3), Asp (3, reference standard), Glu plus Gln 0.97(1), Val 1.03(1), Leu 1.03(1), His 1.29(1), Ala 2.15(2). For TrPepz: Cys-x 1.85(2), Phe 0.99(1), Gly 11.18(11), Ser 2.28(3), Asp (3, reference standard), Glu plus Gln 1.00(1), Val 1.00(1), Leu 1.04(1), His 2.24(2), Tyr 0.96(1), Ala 2.10(2). Hydrolysis conditions were 6 M HCl, 165°C for 2 hr in the presence of 3,3′-dithiopropionic acid. Cys was converted to a mixed disulfide, called Cys-x. These hydrolysis conditions destroyed Trp and converted Gln to Glu. To be sure Gln was present, we sequenced the first 11 residues of TrPepz; this gave the

Table 2. Summary of monoisotopic molecular weights (^{12}C, ^{1}H, ^{14}N, ^{16}O, ^{32}S) determined by FABms shown in Fig. 2

	Molecular weight			
	Oxidized state		Reduced state	
Peptide	Expected	Found	Expected	Found
Design 3	2351.9	2352.0	2353.9	2354.0
TrPepz	2709.0	2709.0	2711.0	2711.2

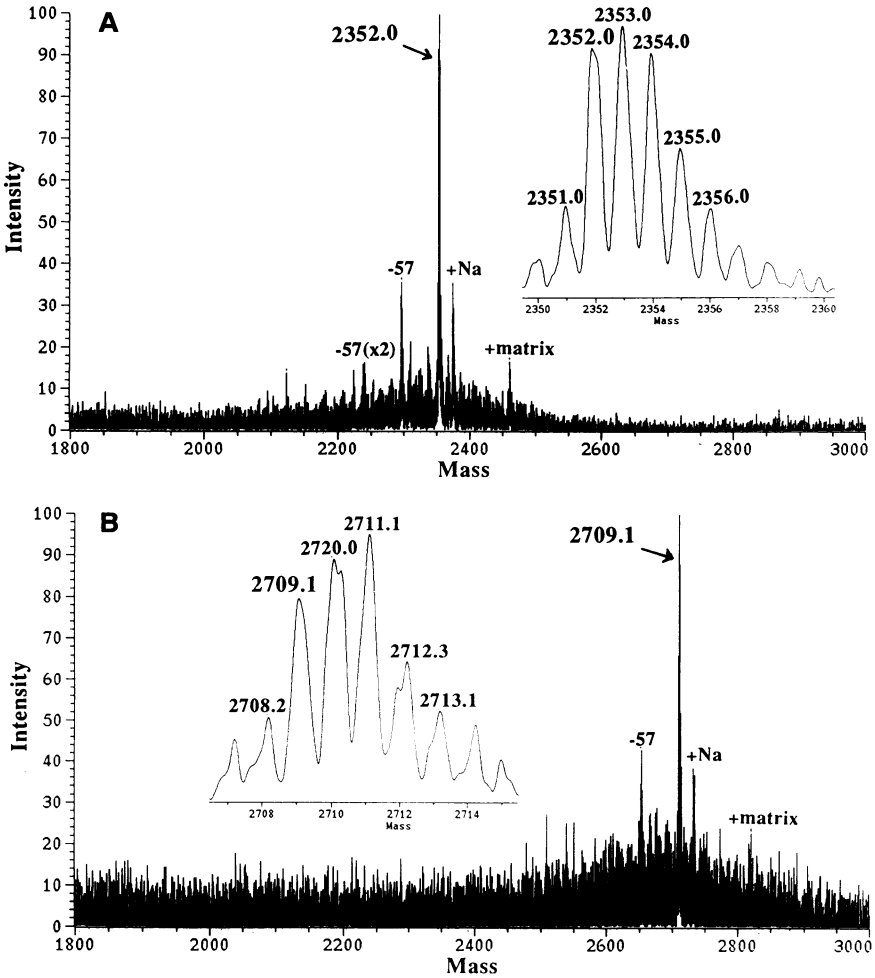

FIG. 2. FABms ionization spectra of design 3 (*A*) and TrPepz (*B*). The +Na and +matrix peaks represent the expected sodium salt and single thiolglycolate adducts of the parent peptides. Each protonated molecular ion ($[M+H]^+$) is expanded (see *Insets*) to show isotopic distribution. Monoisotopic masses are summarized in Table 2.

Table 3. Activity of bovine trypsin and cyclic peptides with the ester substrate (TAME) or the amide substrate (N-t-GPR-pna)

Sample	Concentration, M	Substrate	ΔA, unit/min	Relative activity (trypsin/peptide)
Trypsin*	3×10^{-8}	TAME (1.0 mM)	0.144	
Design 3	1.8×10^{-5}		0.003	>80,000
TrPepz	0.9×10^{-5}		0.003	>40,000
Blank	—		0.003	
Trypsin†	2.8×10^{-8}		0.116	
Design 3	1.9×10^{-5}		0.003	>80,000
TrPepz	0.9×10^{-5}		0.003	>40,000
Blank	—		0.003	
Trypsin†	2.8×10^{-8}	TAME (0.5 mM)	0.077	
Design 3	1.9×10^{-5}		0.002	>50,000
TrPepz	0.09×10^{-5}		0.002	>2,000
	0.9×10^{-5}		0.002	>20,000
	9.0×10^{-5}		0.002	>200,000
Blank	—		0.002	
Trypsin	3.0×10^{-10}	N-t-GPR-pna (16 μM)	0.007	
Design 3	3.0×10^{-5}		<0.001	>600,000
TrPepz	1.5×10^{-5}		<0.001	>300,000
Blank	—		<0.001	

Relative activity was the estimated relative activity of trypsin/peptide calculated from {(ΔA per min for trypsin/ΔA per min for peptide) × [peptide]/trypsin}. Because the ΔA of the peptide never exceeded the blank measurement, to give a maximal estimate of activity for the peptide, we gave the peptide activity a nominal value of 0.001, which is the minimal detectable change in absorbance per minute.

*From Worthington.

†From Sigma.

sequence XGYHFGGSDGQ– as expected. Analysis of the NMR spectra also confirmed the presence of Gln and Trp (see below). Thus, the peptides were monomeric and cyclized, and they exhibited the expected composition and sequence.

We assayed the peptides for esterase activity using TAME as described (16) and for amidase activity using N-t-GPR-pna (Table 3). Under these assay conditions (and several others tried), neither peptide exhibited measurable catalytic activity. After each peptide was assayed (and always failed to show activity), we added trypsin to be sure nothing in the peptide solution inhibited trypsin-like activity. We incubated TAME/peptide mixtures for up to 1 hr but still failed to detect measurable hydrolysis over the TAME blank.

Neither peptide exhibited detectable activity under the assay conditions even at 10^3- to 10^5-fold molar excess compared to trypsin (Table 3). To define an upper limit to the activities of the peptides, we assigned a nominal activity to the peptides that we took to be the minimum change in absorbance per minute that we were capable of detecting (0.001). From the ratios of specific activities, we estimated that trypsin was >80,000 and >600,000 times more active than design 3 on TAME and N-t-GPR-pna, respectively; trypsin was >200,000 and >300,000 times more active than TrPepz on TAME and N-t-GPR-pna, respectively. Of course the peptides could have been even less active or simply inactive. The basis for these enormous discrepancies between our results and those published by Atassi and Manshouri (16) is unclear.

Given the need for a precise arrangement of catalytic groups in the active site of serine proteases, it is reasonable to consider how feasible it would be for TrPepz to attain the same conformation as in trypsin. We applied template forcing (27, 28) to align the side-chain atoms of the cyclic peptide with the corresponding active-site residues of trypsin, using the program DISCOVER (Biosym Technologies, San Diego) with the consistent valence force field (29). As can be seen from Fig. 3, it is possible for the side-chain atoms of TrPepz to attain a configuration close to that of the native enzyme; the rms difference between the 69 aligned atoms is 0.16 Å. However, a substantial discrepancy exists between the optimal alignment of the catalytic triads and the oxyanion binding sites. In trypsin the oxyanion forms hydrogen bonds with the main-chain amides of Ser-195 and Gly-193. However, in TrPepz the trypsin-like oxyanion binding site and the catalytic triad cannot be aligned simultaneously because residues 8–14 in TrPepz run in the opposite direction from the corresponding residues in trypsin (residues 189–195) (16). Although this would indicate a flaw in the original TrPepz design, it may be feasible for the TrPepz peptide to attain an active conformation using an unorthodox oxyanion binding site. In the modeled conformation of TrPepz (Fig. 3), the oxyanion could form different hydrogen bonds, namely with main-chain amides of Asp-9 (equivalent to Asp-194 in trypsin) and Gln-11 (equivalent to Gln-192 in trypsin). Of course this exercise gives no information on whether this conformation is stable in solution or not. Moreover, this model for TrPepz

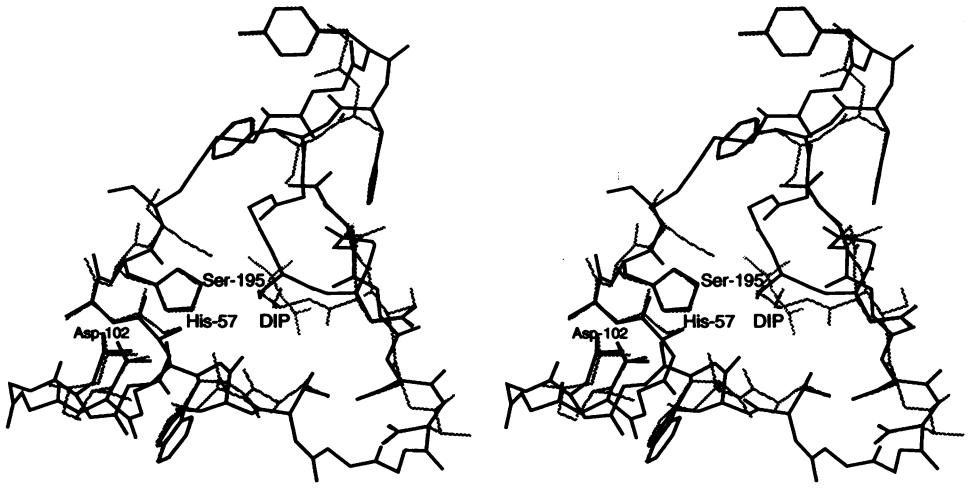

FIG. 3. Superposition of the TrPepz model (solid lines) on the corresponding residues from bovine trypsin (lighter lines). The model was generated by template forcing the side-chain heavy atoms of the cyclic peptide to the corresponding atoms of trypsin (Brookhaven Protein Data Bank designation 4PTP, deposited by J. L. Chambers, R. M. Stroud, and J. Finer-Moore). The labels indicate the catalytic triad residues of trypsin and the trypsin inhibitor diisopropyl phosphate (DIP). The rms deviation between the aligned side chain atoms is 0.16 Å.

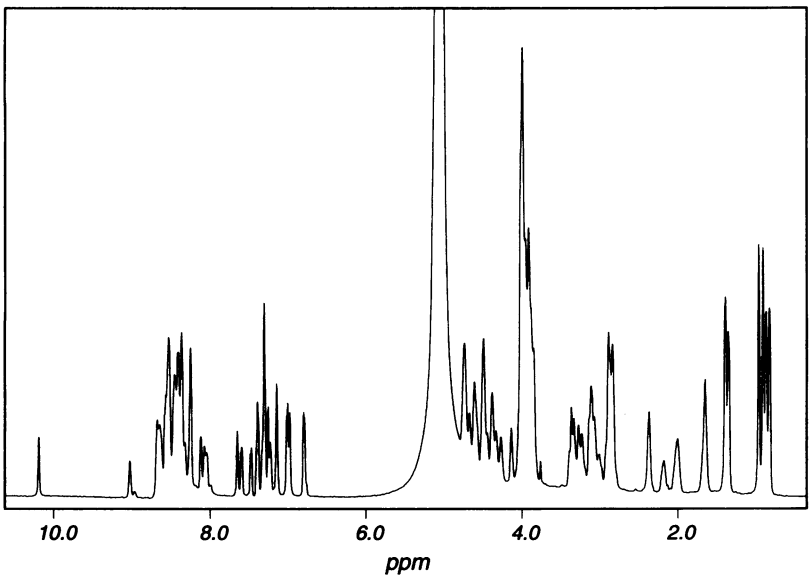

FIG. 4. One-dimensional ^1H NMR spectrum of TrPepz (≈7 mg/ml in 90% H_2O/10% ^2H_2O/50 mM potassium phosphate, pH 5.5) at 10°C.

does not contain an obvious hydrophobic core required to stabilize this structure. In trypsin much of the protein acts as a scaffold for the active site.

NMR solution studies were, therefore, undertaken to investigate the conformation of TrPepz in solution. Almost complete assignment (≈94%) was achieved, although some assignments were tentative due mainly to chemical shift degeneracy of the 11 Gly residues and 4 Ser residues in the peptide. The chemical-shift dispersion (Fig. 4) was found to be similar to that expected for a conformationally flexible peptide (30); note, for instance, the large peak at ≈3.9 ppm that corresponds to the degenerate Gly H^{α} and Ser H^{β} resonances. In addition, analysis of the 250-ms and 450-ms mixing-time rotating-frame nuclear Overhauser effect spectroscopy spectra revealed no evidence of a stable conformation in solution; no medium or long range nuclear Overhauser effect connectivities were identified in either spectrum, with the exception of two weak nuclear Overhauser effects observed between the aromatic protons of Tyr-3 and the C^{δ} protons of Phe-5. The observed lack of activity for TrPepz may, therefore, be correlated with a high degree of flexibility, as was seen from the x-ray structure for trypsinogen (9).

After our studies were nearly completed, we became aware of experiments conducted by Corey and Phillips (31). They obtained results in a completely independent synthesis and analysis of TrPepz that were virtually identical to our activity results. Neither group has been able to detect any esterase or amidase activity for TrPepz. Thus, we cannot confirm the unprecedented results reported by Atassi and Manshouri (16).

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