Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8282–8286, September 1993
Biochemistry

Design of peptide enzymes (pepzymes): Surface-simulation synthetic peptides that mimic the chymotrypsin and trypsin active sites exhibit the activity and specificity of the respective enzyme

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Communicated by Howard L. Bachrach, April 19, 1993 (received for review January 26, 1993)

ABSTRACT **Two 29-residue peptides were prepared, one of which (ChPepz) was designed by surface-simulation synthesis to mimic the active site of α-chymotrypsin, and the other (TrPepz), which contained four substitutions relative to ChPepz, was fashioned after the active site of trypsin. Each peptide was cyclized by a disulfide bond. The ChPepz monomer effected hydrolysis of the ester group in *N*-benzoyl-L-tyrosine ethyl ester, an α-chymotrypsin substrate, with K_m and k_{cat} values that were comparable to those of α-chymotrypsin. ChPepz was completely inactivated by diisopropyl fluorophosphate (DIFP), L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK), or reduction of the disulfide bond. It had no catalytic activity on *N*-tosyl-L-arginine methyl ester, a trypsin substrate. On the other hand, TrPepz, which had no effect on *N*-benzoyl-L-tyrosine ethyl ester, hydrolyzed *N*-tosyl-L-arginine methyl ester with a K_m value that was essentially identical to that of trypsin, but its k_{cat} value was almost half that of trypsin. TrPepz was fully inactivated by reduction of the disulfide bond, by DIFP, or by phenylmethylsulfonyl fluoride but not by TPCK. It was also completely inhibited by soybean trypsin inhibitor, bovine pancreatic trypsin inhibitor, and human α_1-antitrypsin. ChPepz and TrPepz hydrolyzed proteins (myoglobin and casein) to give panels of peptides that were similar to those of the same protein obtained with the respective enzyme. However, TrPepz was more efficient than trypsin at hydrolyzing the C bonds of two or more consecutive lysine and/or arginine residues. Like its esterase activity, the proteolytic activity of ChPepz was inhibited by either DIFP or TPCK whereas that of TrPepz was inhibited by either DIFP or phenylmethylsulfonyl fluoride but not by TPCK. Finally, ChPepz and TrPepz were each more active at low temperature than the respective enzyme. This ability to construct fully functional peptide enzymes (pepzymes) of chosen specificities should find many practical applications.**

The critical role of enzymes in the catalysis of biological processes has, for decades, made enzymes the subject of the most intense studies. The duplication of the activities of enzymes by synthetic analogs has been a prime goal of biochemists. X-ray crystallography of enzymes and their complexes with substrate analogs or inhibitors has provided detailed information about the architecture and contact residues of the active sites of many enzymes. In 1976, Atassi and coworkers (1, 2) devised the technique of "surface-simulation" synthesis, in which the spatially adjacent residues constituting a protein binding site are directly linked via peptide bonds with appropriate spacing and directionality. A peptide is thus generated that does not exist in the protein but mimics the conformation and disposition of the residues of the binding site. Surface-simulation synthesis (for review, see ref. 3) has been employed to mimic protein antigenic sites (1, 2, 4–8), the combining sites of antibodies (9–11), and the substrate-binding site of trypsin (12). Although the peptide that mimicked the latter substrate-binding site displayed all the expected binding activities of trypsin with substrate analogs and inhibitors, it had no enzymatic activity. After several revisions of surface-simulation synthesis, we have now produced a number of catalytically efficient peptide enzymes (named herein pepzymes) that exhibit the enzymatic activity and specificity of the correlate enzyme. This paper reports our studies with two pepzymes that possess the activities and specificities of α-chymotrypsin (αCT) and trypsin.

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MATERIALS AND METHODS

Materials. Myoglobin (Mb) was the major chromatographic component from crystallized sperm-whale Mb as described (13). Sources of other reagents were as follows: *N*-benzoyl-L-tyrosine ethyl ester (BTEE) and *N*-tosyl-L-arginine methyl ester (TAME) (Aldrich); αCT, L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK)-trypsin, bovine pancreatic trypsin inhibitor, and soybean trypsin inhibitor (Worthington); bovine milk β-casein [which contained ≈10% (wt/vol) α-casein], diisopropyl fluorophosphate (DIFP), phenylmethylsulfonyl fluoride (PMSF), and human α_1-antitrypsin (Sigma); peptide synthesis reagents and N^{α}-fluoren-9-ylmethoxycarbonyl amino acid derivatives (Vega Biotechnologies).

Peptide Synthesis. The rationale for the design of the peptides and their structures is given in the *Discussion*. The peptides were prepared on a Coupler 2200 peptide synthesizer (Vega Biotechnologies), starting with a benzyloxybenzyl alcohol resin to which 9-fluorenylmethylcarbonyl-*S*-*tert*-butyl cysteine had been coupled. The methods for synthesis and cleavage from the resin have been described elsewhere in detail (14).*

Cyclization of the Peptide and Purification of the Monomer. A portion (50 mg) of the synthetic product was dissolved in 2 ml of 8.0 M urea containing 5% (vol/vol) 2-mercaptoethanol, preadjusted to pH 8.5 with triethylamine. The solution was stirred gently on a magnetic stirrer for 3 hr at room temperature, after which it was applied on a column (90 × 1.6 cm) of Sephadex G-15 and eluted with 0.025 M acetic acid to remove the urea and mercaptoethanol. The fractions containing the peptide were pooled and diluted with 3 liters of 0.025 M acetic acid and the pH was adjusted to 8.0 on the pH meter by the addition of triethylamine. The solution was

Abbreviations: BTEE, *N*-benzoyl-L-tyrosine ethyl ester; αCT, α-chymotrypsin; ChPepz, peptide designed to mimic the active site of α-CT; DIFP, diisopropyl fluorophosphate; PMSF, phenylmethylsulfonyl fluoride; TAME, *N*-tosyl-L-arginine methyl ester; TPCK, L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone; TrPepz, peptide designed to mimic the active site of trypsin; Mb, myoglobin.

*The precise methods used for synthesizing the peptides and other experimental details will be provided by the authors upon request.

stirred magnetically at room temperature for 4 days and then freeze-dried. The dry peptide was dissolved in 1 ml of 0.025 M acetic acid and subjected to ascending chromatography on two columns (90 × 2.5 cm each) of Sephadex G-25 fine, connected in series, and eluted with 0.025 M acetic acid. The fractions (2 ml) were monitored by A_{280}. The oligomers, eluting as a single peak at 438 ml, were saved for reduction and recyclization to obtain more monomer. The tubes containing the single peak of monomeric species (eluting at 523 ml) were pooled and freeze-dried [yields: peptide designed to mimic the active site of αCT (ChPepz) 26.7%; peptide designed to mimic the active site of trypsin (TrPepz) 33.2%]. The monomer was further purified by HPLC on a 5-μm C_{18} column (10 mm i.d. × 25 cm) using a gradient of 0.05% acetic acid·triethylamine (pH 5.5) and acetonitrile in 0.05% acetic acid, 9:1 (vol/vol). The fractions were monitored by A_{256} nm and by their hydrolytic activity toward BTEE (for ChPepz) or TAME (for TrPepz). The active fraction was freeze-dried and reapplied on the same column using a gradient of 0.1% acetic acid and acetonitrile in 0.1% acetic acid, 9:1 (vol/vol). The catalytically active fractions (yields: ChPepz, 12.1%; TrPepz, 11.5%, of the monomer) were homogeneous by high-voltage paper electrophoresis and by analytical HPLC. Their amino acid compositions were (in residues/mol of peptide, expected values are in parentheses) as follows: ChPepz: Asp 1.95 (2), Ser 3.98 (4), Gly 11.08 (11), Ala 1.91 (2), Cys 2.08 (2), Val 0.98 (1), Met 1.03 (1), Ile 0.96 (1), Phe 1.99 (2), His 1.96 (2), and Trp 0.98 (1). TrPepz: Asp 3.10 (3), Ser 2.96 (3), Glu 1.08 (1), Gly 11.12 (11), Ala 2.09 (2), Cys 1.96 (2), Val 0.96 (1), Leu 1.02 (1), Tyr 1.03 (1), Phe 1.01 (1), His 2.03 (2), and Trp 0.96 (1).

Measurements of Catalytic Activity. *(i) Chymotryptic activity.* Chymotryptic activity was determined at 25°C by hydrolysis of BTEE in 0.08 M Tris·HCl (pH 7.8) containing 0.01 M $CaCl_2$ (15), using 7.26×10^{-4} μmol of αCT or 4.49×10^{-4} μmol of ChPepz and various substrate concentrations (from 3 to 6 mM) in a total reaction volume of 1.0 ml. The change of A_{256} was monitored on a recording spectrophotometer against a reference cuvette containing 1 ml of the same concentration of BTEE, but without αCT or ChPepz. Controls included trypsin, TrPepz (which are inactive against BTEE), and several linear and cyclic peptides from our own peptide library.

(ii) Tryptic activity. This was measured at 25°C in 0.046 M Tris·HCl (pH 8.0), containing 0.0115 M $CaCl_2$ (15) using 8.4×10^{-4} μmol of trypsin or 1.66×10^{-3} μmol of TrPepz, which were allowed to hydrolyze various concentrations of TAME in a reaction volume of 1.0 ml. Hydrolysis was monitored on a recording spectrophotometer by change in A_{247} against a reference cuvette containing the same concentration of TAME, but without trypsin or TrPepz. αCT and ChPepz (which do not hydrolyze TAME) and several linear and cyclic peptides from our own library were used as controls.

(iii) Temperature effects. Measurements were also carried out at different temperatures (from 10°C to 48°C) as described above.

Kinetic constants for hydrolysis of BTEE and TAME were determined from the linear plots of 1/initial velocity (V_i in μmol/min) versus 1/substrate concentration as described (16).

Inhibition of Enzymatic Activity. The effects of inhibitors or disulfide bond reduction on activities were determined as described above except that the enzyme (αCT, 6.04×10^{-5} μmol; trypsin, 2.1×10^{-5} μmol) or pepzyme (ChPepz, 7.06 μg = 2.65×10^{-3} μmol; TrPepz, 4.55 μg = 1.69×10^{-3} μmol) was premixed (3 hr, 25°C) with a 10 molar excess of inhibitor (or dithiothreitol) prior to addition to the substrate (BTEE, 200 μg = 0.638 μmol; TAME, 240 μg = 0.634 μmol) in a final reaction volume of 1 ml. Activities were monitored spectrophotometrically as above and were compared to uninhibited controls.

Hydrolysis of Proteins by Enzymes or Pepzymes. Hydrolyses were done at 37°C on aliquots (200 μl) containing 1 mg of Mb (5.6×10^{-2} μmol) or casein (4.2×10^{-2} μmol) in 0.1 M triethylamine·acetic acid (pH 8.0) with 51 μg of αCT (2.06×10^{-3} μmol) and 2.9 μg of ChPepz (1.1×10^{-3} μmol) for 3.5 hr or with 49 μg of trypsin (2.06×10^{-3} μmol) and 5.0 μg of TrpPepz (1.87×10^{-3} μmol) for 8 hr. The samples were then acidified (to pH 3.0) with 0.1 M HCl, freeze-dried, and redissolved in 100 μl of H_2O at pH 3.0. The entire sample was applied as a single spot to Whatman 3MM paper and subjected to ascending chromatography in the first dimension, in 1-butanol/acetic acid/water, 4:1:5 (vol/vol), followed by high-voltage electrophoresis (3000 V, 55 min) and, in the second dimension, in pyridine/acetic acid/water, 1:10:289 (vol/vol), pH 3.65, as described (17). The papers were dried, steamed, stained with 0.2% ninhydrin in ethanol, allowed to develop at room temperature, and photographed 48 hr after staining.

RESULTS

Hydrolysis of Ester Substrates by αCT and ChPepz. Like αCT, the action of ChPepz on BTEE caused hydrolysis of the ester bond. Lineweaver–Burk plots (Fig. 1*a*) of experiments at different substrate concentrations showed saturation of the pepzyme by substrate and the kinetic constants of the hydrolysis of BTEE by αCT and by ChPepz were comparable (Table 1). The values of K_m (a measure of substrate affinity) for ChPepz and αCT were almost identical, and the k_{cat} value for ChPepz was only slightly lower than that of αCT. The specificity constants (k_{cat}/K_m) for BTEE with αCT and ChPepz were also quite comparable (Table 1). ChPepz was completely inactivated by the αCT inhibitors TPCK and DIFP and also by reduction of the disulfide bond (time-course

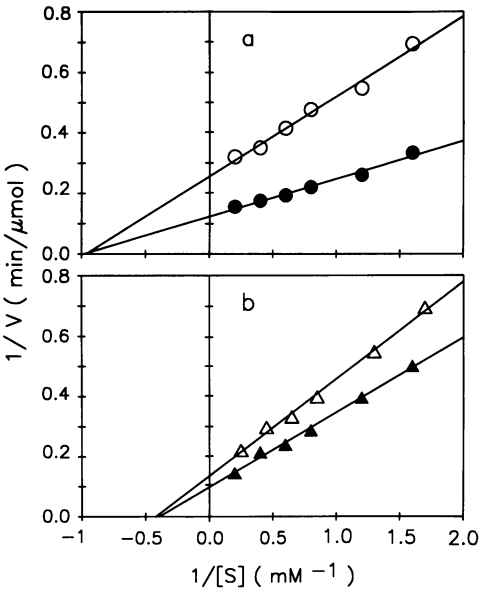

FIG. 1. Lineweaver–Burk plots for hydrolysis of the following compounds. (*a*) BTEE by ChPepz (○) and αCT (●). (*b*) TAME by TrPepz (△) and trypsin (▲). The assays in *a* employed 4.49×10^{-4} μmol of ChPepz and 7.25×10^{-4} μmol of αCT and were carried out at pH 7.8 and 25°C; those in *b* had 1.66×10^{-3} μmol of TrPepz and 8.40×10^{-4} μmol of trypsin and were done at pH 8.0 and 25°C. Measurements were done in three to six replicates at different substrate concentrations as described in the text. The V_{max} values obtained from these plots were as follows: (*a*) ChPepz, 3.966 μmol/min; αCT, 8.058 μmol/min. (*b*) TrPepz, 8.505 μmol/min; trypsin, 11.167 μmol/min. The K_m and k_{cat} values are given in Table 1.

Table 1. Kinetic constants for hydrolysis of BTEE by αCT and ChPepz and of TAME by trypsin and TrPepz

Substrate	Enzyme	K_m, M × 10^{-3}	k_{cat}, sec^{-1}	k_{cat}/K_m, $M^{-1} \cdot sec^{-1}$
BTEE	ChPepz	1.11 ± 0.15	147 ± 8.5	1.32 × 10^5
	αCT	1.07 ± 0.16	185 ± 10.3	1.72 × 10^5
TAME	TrPepz	2.42 ± 0.09	85 ± 2.6	3.5 × 10^4
	Trypsin	2.56 ± 0.16	221 ± 9.7	8.6 × 10^4

Values of the constants for ChPepz and αCT were obtained at pH 7.8 and 25°C and those for TrPepz and trypsin were derived at pH 8.0 and 25°C. Note that BTEE is not hydrolyzed by trypsin or TrPepz and TAME is not hydrolyzed by αCT or ChPepz.

spectrophotometric scans of these reactions at 256 nm traced on the zero-hydrolysis baseline). ChPepz did not hydrolyze TAME (which is a trypsin substrate) and, as mentioned below, BTEE was not hydrolyzed by TrPepz or by control cyclic and linear peptides that are not related to αCT. At 10°C, ChPepz was more active than αCT (percent activity relative to that of αCT at 35°C: αCT, 6.8%; ChPepz, 12.6%). At 48°C (where αCT was totally inactive) ChPepz continued to exhibit some catalytic activity (≈3.5% relative to the αCT activity at 35°C).

Activity of TrPepz on Ester Substrates. The activity of TrPepz on TAME was very much like that of trypsin. Lineweaver–Burk plots (Fig. 1*b*) of reactions at different TAME concentrations showed that the affinity of TrPepz for the substrate (K_m) was similar to that of trypsin. It hydrolyzed TAME at a rate that was ≈40% relative to that obtained with the enzyme itself (Table 1). The activity of TrPepz on TAME was not affected by TPCK, but it was completely lost by reduction of the disulfide bond or by premixing with DIFP, PMSF, soybean trypsin inhibitor, bovine pancreatic trypsin inhibitor, and human α_1-antitrypsin (these reactions traced on the zero-hydrolysis baseline in the spectrophotometric scans at 247 nm). TrPepz had no effect on BTEE and, as mentioned above, TAME was not hydrolyzed by ChPepz or by control unrelated (to trypsin) cyclic or linear peptides. TrPepz was more active than trypsin at 10°C (percent activity relative to that of trypsin at 35°C; trypsin, 4.5%; TrPepz, 7.4%). In the range 18°C–48°C, the activity of TrPepz was about half that of trypsin.

Proteolytic Activity of ChPepz. The action of ChPepz on Mb and casein resulted in the hydrolysis of each protein into peptides whose fingerprints were identical to those obtained by αCT hydrolysis of the respective protein (Fig. 2). The ChPepz hydrolysis of proteins was quite efficient, being achieved in a time frame that was enzyme-like, and this activity was completely inhibited by TPCK.

Proteolytic Activity of TrPepz. The action (3 hr) of TrPepz on the peptide QLEPSTSSAVPLIGKG [TrPepz/substrate, 1:60 (mol/mol)] resulted in >96% hydrolysis of the Lys-Gly bond (monitored by the release of free glycine). Hydrolysis of the lysozyme sequences AAMKRHGLDN, DNYRGYSLG, and AKKIVSDG (hydrolysis sites are underlined) was monitored by amino acid analysis and/or high-voltage paper electrophoresis. Complete cleavage by TrPepz at the "C-peptide" (by convention rightward) bond of the underlined lysine and arginine residues was achieved in ≈3 hr. No other products were obtained. The peptide pattern obtained by the action of TrPepz on Mb or casein was essentially the same as the pattern of the respective protein from tryptic hydrolysis (Fig. 3). However, TrPepz was in fact more efficient at hydrolyzing Lys-Lys, Lys-Lys-Lys, Arg-Lys, and Lys-Arg bonds as evident from the apparent higher yields of lysine and arginine in hydrolyses by TrPepz, as compared to those by trypsin. Finally, the proteolytic action of TrPepz, like that of trypsin, was completely inhibited by either DIFP or PMSF whereas TPCK had no effect.

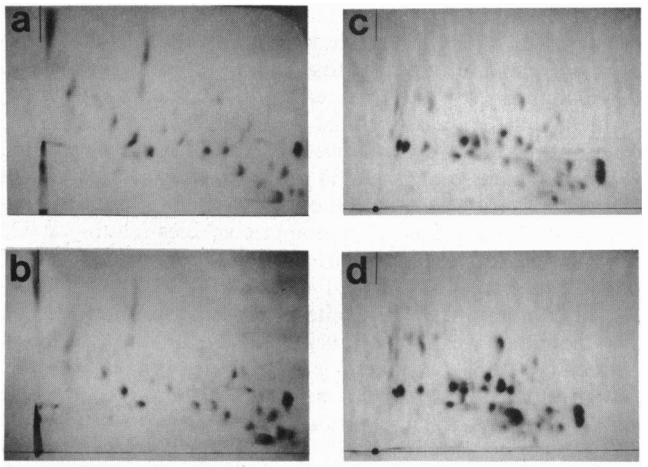

FIG. 2. Maps of the peptides obtained from Mb and casein by hydrolysis with αCT or ChPepz. (*a*) Mb peptides obtained with αCT. (*b*) Mb peptides obtained with ChPepz. (*c*) Casein peptides obtained with αCT. (*d*) Casein peptides obtained with ChPepz. Peptide maps were done by chromatography in the ascending dimension followed by high-voltage paper electrophoresis (from left to right).

DISCUSSION

For almost a century, enzymes have occupied center stage in biochemistry and biology. After developing a method for peptide synthesis in 1907, Emil Fisher hoped to synthesize the first "artificial ferment" (enzyme) (18). Subsequently, major advances in the knowledge of protein structure and in solid-phase peptide chemistry and automation have enabled the synthesis of whole enzymes (19–22). These enzymes, however, were relatively small proteins (e.g., ribonuclease and human immunodeficiency virus protease) and their yields were extremely low, due to the inherent limitations of solid-phase peptide synthesis. But, clearly, to make a whole protein is not a viable approach for the construction of small enzymes in practical amounts and is not at all useful for larger enzymes. Quite recently, a construct was reported that consisted of four helical peptides in a bundle that contained at its amino end serine, histidine, and aspartic acid in a spatial arrangement similar to that in chymotrypsin (23). This 73-residue assembly bound ester substrates of αCT and hydro-

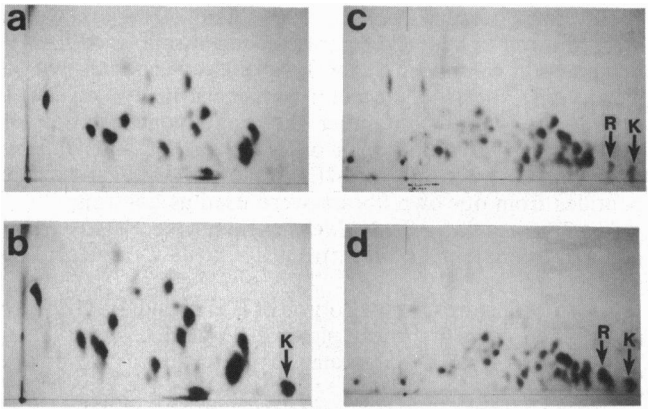

FIG. 3. Fingerprints of the Mb and casein peptides obtained by hydrolysis with trypsin or TrPepz. Mb peptides were obtained by hydrolysis with trypsin (*a*) or TrPepz (*b*). Casein peptides were obtained with trypsin (*c*) or TrPepz (*d*). Peptide maps were done by paper chromatography in the ascending dimension, followed by high-voltage paper electrophoresis (from left to right). Note the apparent higher amounts of free lysine (K) and arginine (R) (indicated by arrows) produced in the TrPepz hydrolyses (*b* and *d*) in comparison to those in the trypsin hydrolysates (for details, see the text).

lyzed acetyltyrosine ethyl ester for ≈100 turnovers, but at a rate of catalysis that was only 0.02% that of αCT. Also, monoclonal antibodies with catalytic activity have been prepared by using transition-state intermediates of the substrate as the immunizing antigen (24–28). These contributions may potentially have important applications but, realistically to date, no protein or peptide designed *de novo* has functioned like a true enzyme (e.g., hydrolyzing protein substrate in a specific manner at preselected peptide bonds and with a useful rate of catalysis).

As stated in the Introduction, a surface-simulation synthetic peptide was constructed (12) that possessed the expected binding activities of trypsin with substrates and inhibitors but had no significant catalytic activity. Nevertheless, the ability to produce substrate binding encouraged us to improve the designs to achieve an enzymically active peptide. We made 10 design changes. The following cyclic peptide designs were active (shown here for the trypsin active site): design 1, CDSGGVSWGGLGDGAAHGGFHYC; design 2, DSGQCDSGGVSWGGLGDGAAHGGFHYC; design 3, CFGGSDGQGSDGGVSWGLGGDGAAHC (active site residues are underlined; others are spacers). Under the conditions described above, the rate of hydrolysis of TAME by the final TrPepz design (Fig. 4) was better than designs 1–3 by 250, 123, and 72 times, respectively. The peptide maps of 144-hr Mb or casein hydrolysates by designs 2 and 3 compared well to the respective tryptic hydrolysates. The cyclic structures of designs 1–3 were essential for activity. Similar results were obtained with the corresponding analogs of the αCT active site.

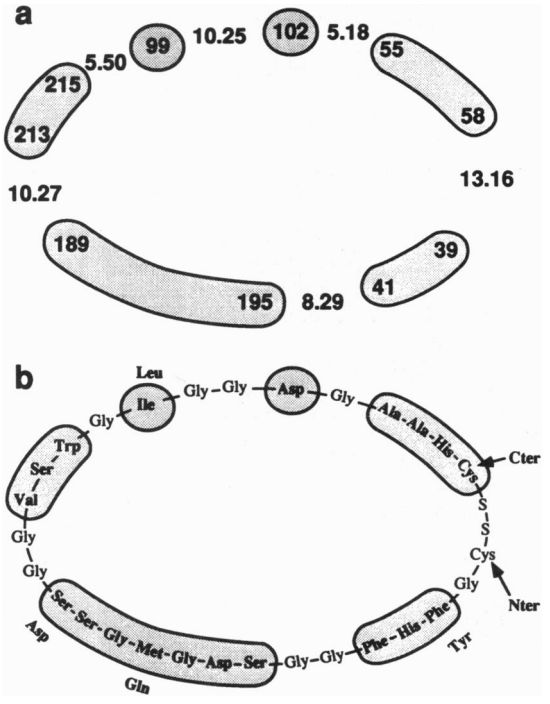

FIG. 4. Design of the surface-simulation synthetic peptides mimicking the active sites of αCT (ChPepz) and trypsin (TrPepz). (*a*) Contact residues of the active site (shaded areas) and the C^{α}-to-C^{α} distances (in Å) separating the appropriate residues. Residue numbers are based on bovine chymotrypsinogen sequence. (*b*) Surface-simulation synthetic peptides designed to mimic the active sites. The residues in the shaded areas are the active site residues of bovine αCT and those linking these shaded areas are glycine spacers used to achieve appropriate distances of separation between the respective residues and regions of the site. Cter is the C-terminal cysteine and Nter denotes the N-terminal cysteine of the peptides that are cyclized by the disulfide between the two cysteine residues. The outer sequence (which differs from ChPepz in four positions only: Phe-39 → Tyr, Ile-99 → Leu, Ser-189 → Asp, and Met-192 → Gln) represents the structure of TrPepz, which mimics the active sites of trypsin.

The essential residues of the active sites of αCT and trypsin are shown in Fig. 4*a*, together with their position in the sequence (using bovine chymotrypsinogen sequence numbers) and the distances (in Å) separating them. These have been implicated as essential active site residues by chemical and crystallographic evidence, which has been reviewed (12). The sequence of αCT was obtained from ref. 29 and the x-ray coordinates are known to 1.68-Å resolution (30). The bovine trypsin sequence and x-ray coordinates were from refs. 31 and 32, respectively. To determine the appropriate lengths of spacers to be used for linking the active site residues, we calculated the average (of 15) C^{α}-to-C^{α} distances in single peptide bonds and two and three consecutive peptide bonds. These distances were as follows: one peptide bond (C^{α}-to-C^{α}), 3.80 ± 0.20 Å; two peptide bonds (distance between C_1^{α} and C_3^{α} in C_1^{α}-C_2^{α}-C_3^{α}), 6.20 ± 1.00 Å; three peptide bonds (distance between C_1^{α} and C_4^{α} in C_1^{α}-C_2^{α}-C_3^{α}-C_4^{α}), 7.94 ± 2.07 Å. Therefore, the distances separating the contact residues (Fig. 4*a*) could be well accommodated by the glycine spacers shown in Fig. 4*b*. Glycine was found (3) to be most suited for use in spacers, probably because of its flexibility and absence of interfering side chains. The cyclic design, which is crucial for activity, requires closure. We measured several disulfide bonds in proteins and found that the C^{α}-to-C^{α} distance in Cys-S-S-Cys is 5.5 ± 0.4 Å (range, 5.11–5.93 Å). Closure of the peptide could be effected anywhere an appropriate space occurs, provided the bond angles in the disulfide bridge do not interfere or induce undue distortion in the orientation of active site residues. The best design was obtained by closure between residues 58 and 39, where the C^{α}-to-C^{α} distance is 13.16 Å. This is satisfied by a Gly-Cys spacer that, with the disulfide bond, would give an effective separation of 11.7 ± 1.4 Å.

The peptide ChPepz, which was designed to mimic the active site of αCT, behaved functionally very much like the enzyme itself. The kinetic constants for hydrolysis BTEE by ChPepz and by αCT were comparable (Table 1). The affinity of ChPepz for the substrate, from the K_m value, was very similar to that of the whole enzyme. The k_{cat} values indicated that ChPepz effected hydrolysis at a rate that was in the same order of magnitude as, and only slightly lower than, that of the enzyme. Our k_{cat} value (185 sec^{-1}) for BTEE hydrolysis by αCT is similar to the value of 193 sec^{-1} reported (33) at pH 7.9 and 25°C. The specificity constant (k_{cat}/K_m) of ChPepz was also comparable to that of αCT, indicating that BTEE functioned equally well as a substrate for both ChPepz and αCT. The similarity of the kinetic constants of αCT and ChPepz and the inhibition of the ChPepz activity by DIFP (a serine esterase inhibitor) and by TPCK (an αCT inhibitor) suggest that the catalytic process by ChPepz employs the same mechanism as αCT. ChPepz was inactive when rendered acyclic. The inability of ChPepz to hydrolyze TAME, a trypsin substrate, further confirmed that it had an αCT specificity. But the most compelling performance of ChPepz was its ability to hydrolyze proteins producing, from a given protein, peptides that were essentially the same as those produced by αCT.

To further confirm catalytic activity, an analog was synthesized in which four residues were substituted to obtain a peptide (TrPepz) that would mimic the active site of trypsin. Trypsin does not hydrolyze BTEE but hydrolyzes TAME. TrPepz behaved precisely like trypsin, exhibiting an almost identical affinity (K_m) for the substrate, and its k_{cat} and k_{cat}/K_m values were about half the corresponding values of the enzyme (Table 1). The values of trypsin K_m (2.56×10^{-3} M) and k_{cat} (221 sec^{-1}) found here were in agreement with the

reported values of 2.76×10^{-3} M (34) and 187 sec^{-1} (35), respectively, at pH 8.0 and 25°C. The activity of TrPepz was completely inhibited by DIFP, PMSF, bovine pancreatic trypsin inhibitor, soybean trypsin inhibitor, and human α_1-antitrypsin, all known to be inhibitors of trypsin. Like ChPepz, the cyclic structure of TrPepz was essential for activity. The most striking finding was the exquisite specificity of TrPepz for cleavage of the C-peptide bonds of arginine and lysine residues in peptides and proteins. Its action on Mb or casein yielded peptide fragments that were similar to those obtained by hydrolysis with trypsin itself. In fact, TrPepz appeared to be more efficient than trypsin at hydrolyzing Lys-Lys, Lys-Arg, Arg-Lys, and Lys-Lys-Lys bonds. Thus, the substitution of four residues in the ChPepz design caused an unequivocal functional conversion from a chymotryptic to a tryptic activity.

The lower rate of catalysis by ChPepz and TrPepz (relative to their respective enzyme) is probably caused by the flexibility of the peptide as it searches, through an equilibrium of conformational states and induced fit, for a catalytically productive conformation. Also, the reverse sequence of residues 189–195 may have caused reduction of the rate of catalysis, although an analog with D-amino acids in this segment showed similar kinetics. The virtual loss of catalytic activity when the peptides are rendered acyclic is most probably due to the inability of the open-chain structure to achieve such a conformation. This would explain why the first-generation open-chain peptide (12) exhibited binding but did not possess measurable catalytic activity. Finally, pepzymes could be useful at temperatures where enzymes are not efficient catalysts. Clearly, pepzymes should have enormous biological, clinical, therapeutic, industrial, and other applications.

Could pepzymes have been forerunners of protein enzymes? Because of their relative simplicity and small size, could they have conceivably formed in the early stages of millions of years of organic evolution by random polymerization of amino acids in the primordial soup? If so, this could fill a gap in the evolutionary transition from organic molecules to biologically active molecules and would explain how the initial biological processes might have been catalyzed. But as unicellular and more complex organisms evolved, such molecules could have disappeared because of evolutionary pressure for regulation, stability, and survival in cell and body fluids.

M.Z.A. wishes to thank the Welch Foundation for the award of the Robert A. Welch Chair of Chemistry.

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