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Studies on Chymotrypsin-Like Catalysis by Synthetic Peptides

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ABSTRACT

The synthetic peptide Chymohelizyme-1 (CHZ-1) exhibits esterase activity against carbobenzoxytyrosine *p*-nitrophenyl ester (ZTONP), carbobenzoxyalanine *p*-nitrophenyl ester (ZAONP), and *t*-butyloxycarbonyltyrosine *p*-nitrophenyl ester (BocTONP). However, earlier reports of catalytic activity against less labile esters and amides have proven to be incorrect. The major reason for the errors appears to have been the omission of certain controls in the previous work. Although the catalytic triad does not appear to be functioning as designed, the catalytic activity of CHZ-1 does depend on the integrity of its primary structure. The pH dependence of hydrolysis of ZTONP points to general-base catalysis, whereas a preference for hydrophobic substrates suggest that the structure of CHZ-1 is performing some other role in assisting catalysis.

Index Entries: Chymohelizyme; enzyme, synthetic; peptide synthesis.

Abbreviations: ADH, alcohol dehydrogenase; ATEE, acetyltyrosine ethyl ester; BocTONP, *t*-butyloxycarbonyltyrosine *p*-nitrophenyl ester; BTEE, benzoyltyrosine ethyl ester; CHES, 2-[*N*-cyclohexylamino]-ethanesulfonic acid; CHZ-1, Chymohelizyme-1 (blocking group on histidine α-amino group is acetyl); CHZ-1A, chymohelizyme-1A (blocking group on histidine α-amino group is *t*-butylacetyl); HPLC, high-performance liquid chromatography; NAD^+, nicotinamide adenine dinucleotide; ONP, *p*-Nitrophenyl ester; PMSF, phenylmethylsulfonyl fluoride; SBTI; soybean trypsin inhibitor; TFA, trifluoroacetic acid; TPCK, tosylphenylalanine chloromethyl ketone; ZAONP, benzyloxycarbonylalanine *p*-nitrophenyl ester; ZTONP, benzyloxycarbonyltyrosine *p*-nitrophenyl ester.

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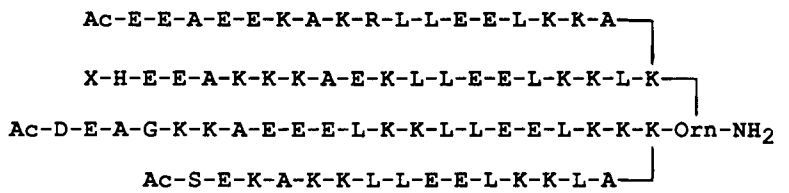

Fig. 1. Primary structures of CHZ-1 and CHZ-1A. One-letter amino acid codes: A = alanine, D = aspartic acid, E = glutamic acid, G = glycine, H = histidine, K = lysine, L = leucine, R = arginine, S = serine. Orn = ornithine. Ac = acetyl. X at end of histidine chain denotes acetyl (in CHZ-1) or *tert*-butylacetyl (in CHZ-1A).

INTRODUCTION

Goals of peptide chemists and enzymologists converge in the attempt to design and synthesize peptides with properties similar to those of natural enzymes. Success in designing and synthesizing a four-helix peptide with chymotrypsin-like behavior has recently been reported (*1*). The molecule CHZ-1 (primary structure, Fig. 1) was reported to exhibit enzyme-like esterase catalysis, including hydrolysis of the chymotrypsin substrates acetyltyrosine ethyl ester (ATEE), benzoyltyrosine ethyl ester (BTEE), and carbozenzoxytyrosine *p*-nitrophenyl ester (ZTONP); saturation kinetics; inactivation by phenylmethylsulfonyl flurode (PMSF) and reversible competitive inhibition by indole and *p*-cresol; a bell-shaped pH-activity profile, like that of many enzymes; reversible inactivation by heating; and specificity for chymotrypsin substrates over trypsin substrates (e.g., for BTEE over benzoylarginine ethyl ester). Subsequently, an improved design was synthesized, termed CHZ-1A (Fig. 1); it showed an improvement in k_{cat} of fivefold over the original design. The studies reported here were undertaken to examine the catalytic activity of these peptides in more detail.

EXPERIMENTAL

Materials

Methylbenzhydrylamine polystyrene resin having low crosslinking and substitution was purchased from Colorado Biotechnology Assoc., Casper, WY. Boc-Lys(Npys) and other Npys amino acid derivatives were synthesized as described (*2*). Other amino acid derivatives were from commercial sources. Substrates (acylamino acid esters), NAD^+, alcohol dehydrogenase (ADH), phenylboronic acid, proflavin, Tris, 2-[*N*-cyclohexylamino] ethanesulfonic acid (CHES), α-chymotrypsin, N_α-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and phenylmethylsulfonyl

fluoride (PMSF) were purchased from Sigma. Trypsin was purchased from US Biochemical Corp. Soybean trypsin inhibitor (type I-S) was a gift from Clark Bublitz.

Peptide Synthesis and Purification

Solid-phase synthesis of the peptides was carried out as previously reported in summary (*1*) and in detail (*3*). During synthesis, a portion of the peptide resin was removed after assembly of the first two chains (the aspartate and serine chains); the peptide from this resin was cleaved, purified, and studied. It is called "two-chain" peptide. The histidine chain was next assembled. Before coupling the histidine, a portion of resin was removed. In this material, the "histidine" chain was terminated by acetylation without coupling histidine; the fourth chain was then added in the same manner as for the intact molecule. This resin yielded the "des-histidine" analog of CHZ-1. For synthesis of CHZ-1A, the histidine chain was terminated with a *t*-butylacetyl group, rather than the acetyl group of CHZ-1, in order to give a more bulky and hydrophobic residue at this position. The fourth chain (the "glutamic chain") was assembled in the same way onto all peptide resins. Coupling reactions were mediated by several reagents, and were monitored extensively during syntheses using qualitative and quantitative Kaiser tests (*4*). Overall progress of synthesis was monitored by amino acid analysis of peptide resins. Chatropic salts (*5*) were added to the coupling mixture to promote coupling in many cases. When Npys-protecting groups were present, sodium perchlorate was used for this purpose, since the Npys group was not fully stable to potassium thiocyanate. Chaotropic salt was not included in the coupling mixture for amino acids having side-chain functional groups; in these cases, the resin was washed with the chaotropic salt solution before addition of the preactivated amino acid (*5*). A coupling solvent of *N*-methylpyrrolidine-dimethyl sulfoxide (4:1) was also used in many cases to promote coupling.

Peptide resins were cleaved by normal or "low–high" HF cleavage methods (*5*). Several different scavengers were tried for protection of the peptides during cleavage. Purification procedures used included ultrafiltration, gel chromatography and HPLC (reversed-phase, ion-exchange, and size-exclusion). Thin-layer chromatography, paper electrophoresis, gel electrophoresis, amino acid analysis, and laser desorption mass spectrometry were used to assess purity of product fractions.

Assay Methods

Hydrolysis of p-Nitrophenyl Esters

Hydrolysis of ZTONP and other *p*-nitrophenyl esters at 45°C was measured at 274 and 400 nm using a Hewlett-Packard diode-array spectrophotometer. The molar ΔA_{400} for complete hydrolysis of ZTONP was

determined to be 16,900 in this system at this temperature. Absorbance at the isosbestic point (316 nm for ZTONP) was used as a control against substrate precipitation or other effects not owing to hydrolysis. The limit of solubility of ZTONP was found to be ~40 μM (or ~30 μM in the presence of 7.5 μM CHZ-1). In a typical assay, CHZ-1 at 7.5 μM was incubated for 5 min at 45°C with Tris (final concentration 50 mM, pH 8.5, adjusted to I = 0.05 with NaCl). ZTONP was added in a volume of 20 μL to a final concentration of 14.5 μM. Data from 50–110s were taken for analysis.

To facilitate comparison with the kinetic parameters of chymotryspin, helizyme assays were also performed at pH 7.0, using the method of Dupaix et al. (*6*). Chymotrypsin assays against ZTONP and ZAONP as substrates were also performed under these conditions. Hydrolysis of other *p*-nitrophenyl esters (acetyltyrosine *p*-nitrophenyl ester, *t*-butyloxycarbonyltyrosine *p*-nitrophenyl ester, allyloxycarbonyltyrosine *p*-nitrophenyl ester, carbobenzoxylysine *p*-nitrophenyl ester, and carbobenzoxyasparagine *p*-nitrophenyl ester) was measured at pH values varying from pH 7.0 to 8.5 as convenient (*see* legend to Table 1).

Bimolecular or "second-order" rate constants were calculated either by linear regression to substrate-dependence data (chymotrypsin, CHZ-1, spontaneous hydrolysis by H_2O), or when there were two few data for this approach, by taking the arithmetic mean of the available data (des-*His* CHZ-1 and two-chain material). Observed rates were converted to bimolecular constants by means of the equation:

$$K_{BM} = (\Delta A_{400}/\text{min}) / \{(A_{\mu[\text{PNP}]}) * ([\text{C}]) * ([\text{S}])\} \quad (1)$$

where K_{BM} is the calculated bimolecular rate constant in units $(\mu M)^{-1}$ $(\text{min})^{-1}$, $A_{\mu(\text{PNP})}$ is the micromolar absorbance of *p*-nitrophenol (0.0169 at pH 8.5, 45°C; 0.0079 at pH 7.0, 45°C), [C] is the concentration of the catalyst, and [S] is the concentration of substrate.

Reactions at higher pH values were too rapid to measure using manual mixing. An Applied Photophysics SF.17MV stopped-flow apparatus was used for pH-dependence measurements. Data from 2–6 s were taken for analysis. Tris buffers were used from pH 8.3 to 9.2, and CHES buffers from pH 9.3 to 10.6.

ATEE Hydrolysis

Three methods were used to follow hydrolysis of ATEE: HPLC product analysis, titrimetric assay, and ADH-coupled assay.

HPLC Product Analysis

CHZ-1 and CHZ-1A were both assayed at 4.9 and 9.8 μM for hydrolytic activity against ATEE (10.3 mM) at 40°C, pH 8.5. Aliquots (19 μL) were diluted with 75 μL HPLC starting buffer, quenched with 6 μL 1M HCl, and stored on ice. HPLC product analysis was performed with a Vydac C-18 column, using isocratic elution at 20% CH_3CN, 0.1% TFA.

Table 1
Biomolecular Rate Constants for Hydrolysis of ZTONP, ZAONP, and BocTONP

	pH 7.0		pH 8.5			
Catalyst	ZTONP[a]	ZAONP[a]	ZTONP[a]	ZAONP[a]	ZTONP[b]	BocTONP[b]
CHZ-1	$3.8 \pm 1.3 \times 10^{-4}$	$1.3 \pm 0.3 \times 10^{-4}$	$5.8 \pm 0.9 \times 10^{-3}$	$2.8 \pm 0.6 \times 10^{-3}$	$2.1 \pm 0.3 \times 10^{-3}$	$1.7 \pm 0.5 \times 10^{-3}$
Des-*His* CHZ-1			$6.3 \pm 0.7 \times 10^{-3}$			
Two-chain[c]			$1.5 \pm 0.4 \times 10^{-3}$			
Chymotrypsin	$1.4 \pm 0.2 \times 10^{5}$	$2.1 \pm 0.2 \times 10^{3}$				
H_2O[d]	$1.3 \pm 0.4 \times 10^{-10}$	$1.2 \pm 0.1 \times 10^{-10}$	$1.6 \pm 0.1 \times 10^{-8}$	$1.2 \pm 0.1 \times 10^{-8}$	$5.9 \pm 0.2 \times 10^{-10}$	$5.4 \pm 0.6 \times 10^{-10}$

Units of rate constants are $(\mu M)^{-1}$ $(min)^{-1}$.

[a] Conditions: Tris (50 m*M*) adjusted to indicated pH with HCl, adjusted to $I_C = 0.05$ with KCl. Substrate dissolved in isopropanol; final isopropanol concentration 0.7%.

[b] Conditions: Tris (50 m*M*) adjusted to pH 8.5 with HCl, adjusted to $I_C = 0.05$ with KCl. Substrate dissolved in dimethylformamide; final dimethylformamide concentration 5%.

[c] Two-chain material consists of serine- and aspartate-containing chains (*see* Fig. 1).

[d] H_2O bimolecular rate constant is calculated by dividing the observed rate by the product of the substrate concentration and the calculated water concentration.

Titrimetric Assays

As previously reported (*1*), ATEE was dissolved in 1 mL isopropanol and diluted to 10 mL of the appropriate concentration (1–10 m*M*) with 50 m*M* NaCl. The pH was then adjusted to 8.3 with 10 m*M* NaOH. CHZ-1 was added in 0.1 mL of 50% isopropanol, 25 m*M* in NaCl to a final CHZ-1 concentration of 0.73 μM. The titration was performed manually at room temperature, using a pH meter. Any initial decline in pH was compensated by addition of 1 m*M* NaOH.

Alcohol Dehydrogenase-Coupled Assay

The assay for ethanol production (coupled to reduction of NAD^+ by alcohol dehydrogenase) was performed according to Mroz and Lechene (*7*). As performed here, the sensitivity was adequate to detect the amount of ethanol that would be expected if the synthetic peptides had 0.02% of native chymotrypsin activity.

BTEE Hydrolysis

BTEE hydrolysis was followed photometrically at 256 nm (*8*), using the conditions previously reported (*1*). Buffer (60 m*M* Tris, 50 m*M* NaCl, pH 8.5; 2.2 mL) was mixed with H_2O and isopropanol to yield a final isopropanol concentration of 15.4% in a 2.9-mL reaction volume. Substrate, dissolved in 0.2 mL isopropanol, was added to a final concentration of 0.3–2 m*M*. This mixture was incubated at 45°C for 5 min, after which CHZ-1 or CHZ-1A (0.2 mg) was added in 0.2 mL of H_2O or isopropanol/H_2O (1:1). Data from 20–100 s were used. A control in which substrate was not present and a modified experiment in which the solutions of substrate and synthetic peptide contained equal concentrations of isopropanol were also carried out. Hydrolysis of BTEE was also followed by HPLC product analysis, as described above for ATEE.

Trypsinolysis

CHZ-1 (2 mg, 113 μM final concentration) was digested at room temperature in 2 mL buffer (25 m*M* Tris, pH 8.5, adjusted to I = 0.025 with KCl) by 2.8 n*M* trypsin. Aliquots (230–250 μL) of the digestion reaction were removed immediately after addition of trypsin and at various times thereafter. These aliquots were boiled for 60 s and then plunged into an ice bath. Soybean trypsin inhibitor (SBTI) (1 μg in 5 μL, type I-S, approximately twofold excess over trypsin present) was then added to each aliquot. A 200-μL portion was then removed for assay against ZTONP as substrate in a reaction exactly as described above except that the assay vessel also contained 0.7 μM soybean trypsin inhibitor (control procedures showed that this amount of SBTI completely eliminated trypsin activity against ZTONP, but had no effect on CHZ-1 catalysis). Another 30 μL of each aliquot were mixed with 1.5 μL of 1*M* HCl and stored at 4°C for HPLC. For analysis of the products of trypsinolysis by HPLC, a linear

95-min gradient from H_2O (0.1% TFA) to 100% CH_3CN (0.08% TFA) on a Vydac C-4 column was used.

RESULTS

The crosslinked polystyrene synthesis resin used for the initial synthesis of CHZ-1 proved to have remarkably good properties. Difficulties in obtaining complete reactions in further synthesis prompted an extensive study of crosslinking and substitution in resins for solid-phase synthesis (*9*) in order that subsequent synthetic work could proceed. The purification methods used in the original work were found not to yield a homogeneous peptide. The synthetic product was shown by HPLC analysis and gel electrophoresis to be somewhat heterogeneous, both before and after chromatography on G-50, reverse-phase HPLC on C-4, and size-exclusion HPLC. ZTONP-hydrolytic assay of the various fractions with HPLC showed that later-eluting fractions were slightly more active than earlier fractions. The amino acid compositions of these fractions were nearly indistinguishable, however.

Different fractions from HPLC purification of CHZ-1A, the later synthesis that incorporated a *t*-butylacetyl blocking group at the end of the histidine chain in place of the original acetyl group, differed greatly in catalytic activity against the ZTONP substrate. Unfortunately, the amounts of highly active material were very small compared with the less active fractions. Since CHZ-1 was the molecule studied in the original paper, it was used for the majority of the studies reported here. Here also, fractions from HPLC purification were too small to be of use in experiments requiring many measurements. Therefore, CHZ-1 was used after cleavage and G-50 chromatography without further purification. All CHZ-1 came from the original synthesis, and fractions of material from the original cleavage and purification of CHZ-1 did not differ significantly in any properties from material obtained in later isolations. In the case of des-*His* CHZ-1, the peptide was desalted and purified by G-50 chromatography; the central portion of the G-50 peak was used for assays of catalysis.

Kinetics of Substrate Hydrolysis

ZTONP Assays

Hydrolysis of ZTONP showed linear dependence on substrate concentration up to 30 μM ZTONP (Fig. 2). At concentrations of 40 μM and above, the data were complicated by slow precipitation of the substrate. Under the conditions used in the previous work (145 μM ZTONP, 15.4% isopropanol, pH 8.5), precipitation can begin in 3–5 min, and the presence of CHZ-1 or CHZ-1A promotes this precipitation. Working in the 4–30 μM

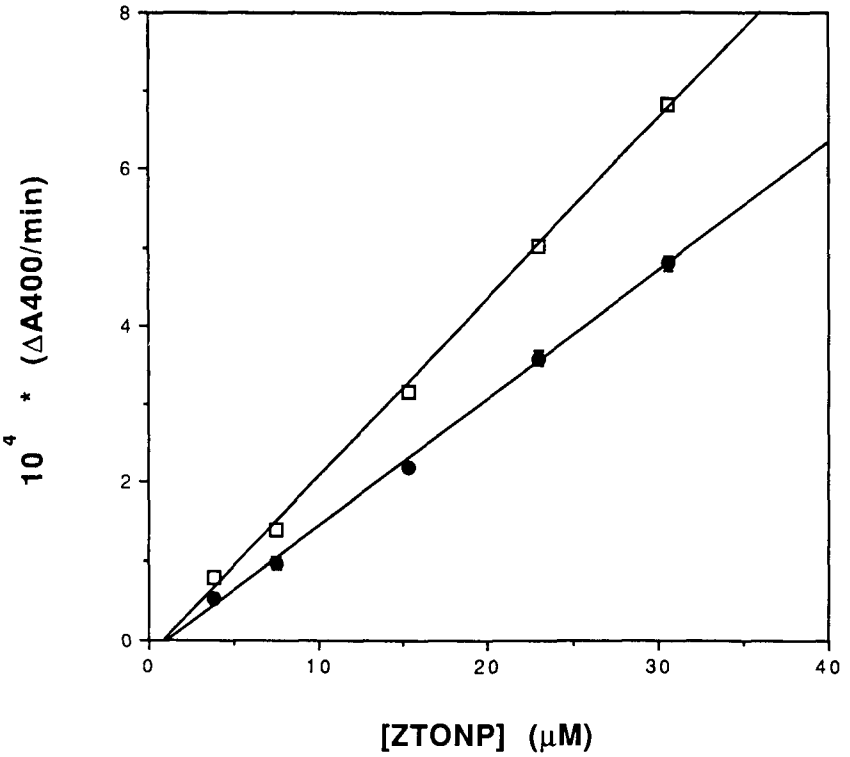

Fig. 2. Substrate dependence of spontaneous and CHZ-1-catalyzed hydrolysis of ZTONP. Reactions were at pH 8.5, 45°C, Ic = 0.05. ●, Spontaneous hydrolysis; □ CHZ-1 catalyzed hydrolysis. CHZ-1 rates are not corrected for blank.

range of substrate, a consistent bimolecular rate constant for hydrolysis was obtained, but K_m and V_{max} could not be measured because no saturation was evident in this range. Saturation could not be observed with ZAONP or BocTONP.

In this work, catalytic activity of CHZ-1 was not inhibited by PMSF, TPCK, indole, *p*-cresol, proflavin, phenylboronic acid, or boiling. However, digestion by a small concentration of trypsin (2.8 n*M*) destroyed both the catalytic activity (assayed against ZTONP) and the primary structure of CHZ-1 (as judged by reduction of the area of the HPLC peak) (Fig. 3). Under the conditions used, the $t_{½}$ of loss of activity was 135 ± 20 min., whereas the loss of overall structure appeared to take place much more slowly.

The catalytic activity of CHZ-1 against ZTONP was found to increase monotonically with pH (Fig. 4). Linear regression to the data yielded a Bronsted b value of 1.10 ± 0.10 for CHZ-1 catalysis of ZTONP hydrolysis. The comparable value for spontaneous hydrolysis was measured as 1.03 ± 0.34.

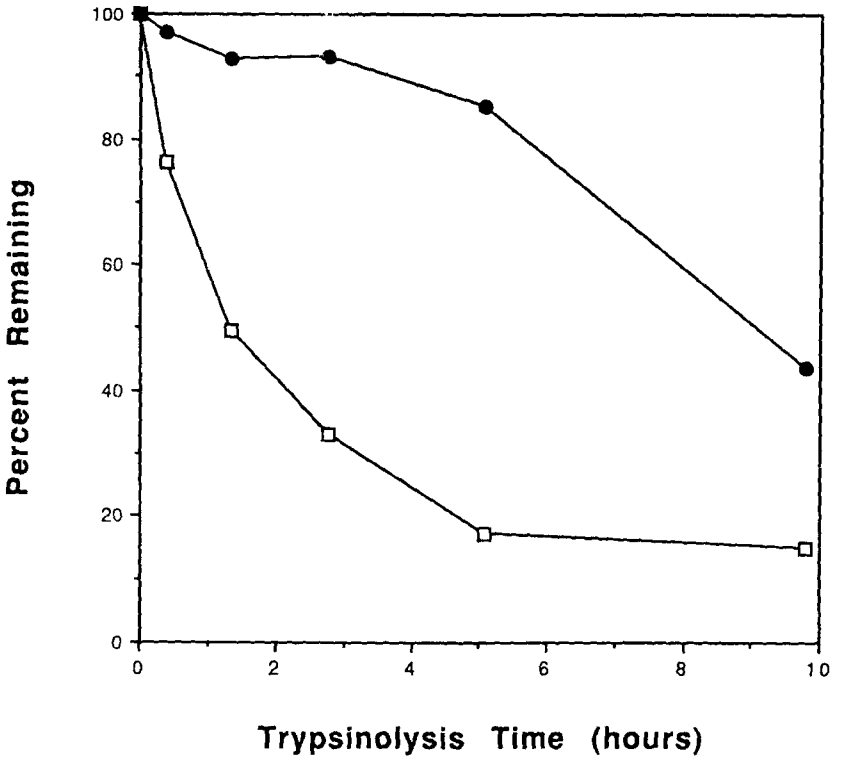

Fig. 3. Trypsinolysis of CHZ-1. Digestion was at 24°C, pH 8.5, Ic = 0.025. Assays for CHZ-1 activity were at 45°C, pH 8.5, Ic = 0.05. ●, Area of original HPLC peak of CHZ-1; □, catalytic activity against ZTONP.

The rate of hydrolysis of ZTONP catalyzed by des-*His*-CHZ-1 was indistinguishable from that of the CHZ-1 molecule, as indicated in Table 1. The "two-chain peptide" (serine and aspartate chains present) was also active against ZTONP, although at a lower level (Table 1). Data on CHZ-1-catalyzed hydrolysis of ZAONP and BocTONP, as well as bimolecular rate constants for hydrolysis with both chymotrypsin and H_2O as catalysts, are also presented in Table 1.

BTEE Assays

Absorbance of CHZ-1 at 256 nm was found to undergo a variable, solvent-dependent change with time; an increase in isopropanol concentration generally caused an increase in A_{256}. Under the standard assay conditions, where the isopropanol concentration was increased by addition of the isopropanol solution of substrate, this slow rise was much greater than that resulting from the background rate of hydrolysis of the substrate, but the rate was not quantitatively reproducible. Controls and assay runs in which both peptide and substrate were previously dissolved

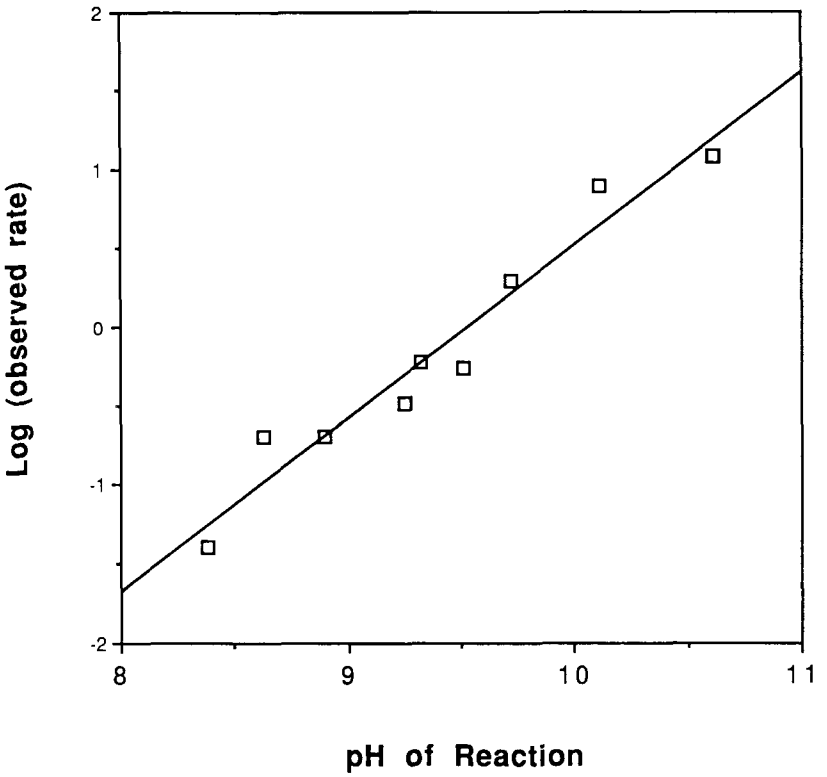

Fig. 4. Bronsted plot of ZTONP hydrolysis catalyzed by CHZ-1. Observed rates are in units ($10^4 \times \Delta A_{400}/s$) at 45°C, Ic = 0.05.

in and equilibrated with 15% isopropanol showed no change in A_{256} on mixing. Under these conditions, neither CHZ-1 nor CHZ-1A caused an increase over background in the signal at 256 nm. In the assay for BTEE hydrolysis coupled with ADH-NAD^+, no ethanol production by CHZ-1 was observed.

Assay of ATEE Hydrolysis by HPLC Product Analysis

Significant hydrolysis of ATEE above the background rate could not be demonstrated in several attempts, either with material cleaved and purified earlier, or with newly cleaved CHZ-1 and CHZ-1A.

Hydrolysis of ATEE: Titrimetric and Enzyme-linked Assays

Attempts to replicate the previously reported generation of acid by incubation of ATEE with CHZ-1 were not successful. Experiments with CHZ-1A also yielded negative results. Production of ethanol from ATEE in an ADH-coupled assay system similar to the one described above for BTEE also yielded negative results.

DISCUSSION

Extensive kinetic studies in the course of the current work have failed to confirm many of the original claims of enzyme-like catalysis by these peptides, particularly catalysis of hydrolysis of stable esters. Hydrolysis of the reactive nitrophenyl esters ZTONP, ZAONP, and BocTONP was catalyzed in a manner that depended on substrate concentration, catalyst concentration, temperature, and pH. However, this activity failed several tests of chymotrypsin-like character. In the studies reported here, the catalysis was not affected by any of the inhibitors tried, including PMSF, TPCK, indole, proflavin, phenylboronic acid, and *p*-cresol. Some of the previously reported results with the BTEE and ZTONP substrates can be explained by the presence of replicatible artifacts that were not originally recognized as such. A variant of CHZ-1 lacking the histidine of the intended catalytic triad showed the same catalytic rate constant for ZTONP hydrolysis as the original molecule, and the "two-chain" peptide showed significant, although slower, catalysis. The activity of these variants indicates that the chymotrypsin-like catalytic triad of the original design was not functioning as intended. These two variants contain many basic groups in the lysine side chains, and one or more of these may be assisting in catalysis. However, the fact that digestion of CHZ-1 by trypsin abolished the catalytic activity far more rapidly than it degraded the peptide structure suggests that the catalysis depends at least on the integrity of the primary structure of CHZ-1.

The peptide also exhibited greater catalytic activity against the tyrosine derivative ZTONP than against the alanine derivative ZAONP. Although spontaneous hydrolysis of ZTONP is slightly greater than that of ZAONP, the enhancement of the bimolecular rate constant for CHZ-1 (ZTONP/ZAONP = 2.92 at pH 7.0; 2.07 at pH 8.5) is greater than that for spontaneous hydrolysis (ZTONP/ZAONP = 1.08 at pH 7.0; 1.33 at pH 8.5), especially at the lower pH. If CHZ-1 were acting solely as a general base, then OH^- would be the actual catalytic species, and the ratio ZTONP/ZAONP should be the same as for the spontaneous reaction.

Other aliphatic acetyl derivates of tyrosine nitrophenyl ester were synthesized and tested as substrates. Acetyltyrosine ONP and allyloxycarbonyltyrosine ONP were not substrates for CHZ-1 (data not shown), whereas t-butyloxycarbonyltyrosine ONP was hydrolyzed (Table 1). The acylating group in the benzyloxycarbonyl (ZTONP) and the *t*-butyloxycarbonyl derivates is bulky and very hydrophobic. Thus, although the hydrophobic binding pocket of CHZ-1 is evidently not functioning precisely as intended by the designers, the aromatic or hydrophobic nature of the substrate is important in the catalysis.

The extended chemical work aimed at purification of the synthetic peptides showed that the original product was far more heterogeneous

than initially realized. Efforts to monitor the success of the synthesis during peptide assembly evidently gave misleading information. The very high redundancy of amino acid composition in the design of CHZ-1 made it very difficult to follow purification by amino acid analysis or NMR spectroscopy. Many of the problems in synthesis have been identified and corrected, and current design work is aimed at overcoming deficiencies now apparent in the original design. With these experiences in the background, future work should yield much improved enzyme-like catalysts.

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DISCUSSION

J. Stewart

McCafferty: In claiming you have catalytic activity, have you shown turnover?

Stewart: Yes, there is indeed turnover.

McCafferty: Did you use substrates other than the very activated *p*-nitrophenyl esters?

Stewart: In some of our early experiments, we had an indication that we did have catalysis of simple ethyl esters, but Dr. Corey was unable to repeat this observation. This is somewhat confusing, and I do not have a very clear answer to this. We had some problems with the people working on the project, and I really am not so sure of this any more.

Gololobov: Is there any inhibition of your activity by classical serine protease inhibitors?

Stewart: Yes, we had some indication that indole is an inhibitor. In the solid-phase synthesis, we use anisole as a scavenger to prevent unwanted side reactions. Anisole is a kind of a chymotrypsin inhibitor. In our initial work, this caused some problems, because, not really being enzymologists, we were not aware of this and anisole inhibited the peptide. Again, this observation was not repeatable by the later investigator.

Gololobov: I was referring to covalent modification of serine, for example by diisopropylfluorophosphate.

Stewart: We have not demonstrated that.

Stollar: Are the rates you see comparable to enzymes in which histidine has been eliminated by mutation? I think there is still a small catalytic rate there.

Stewart: That is correct. Trypsin has been mutated to eliminate the histidine. Although there is a tremendous loss, some significant activity remains. Our loss of activity by removal of histidine is not as great as is seen with trypsin. Of course, our rate is not as good as that of trypsin to start with.

Webster: You showed a helical model of the four peptide units. Was that a computer model, or is that an actual description of the molecule in solution?

Stewart: The model is the structure we are trying to produce. The computer graphics program suggests that the synthetic sequence will minimize to that or a related structure. The evidence for a helical structure comes from circular dichroism studies, although that does not tell us that we have the bundle. There are obvious things that we can do to try to stabilize a bundle structure, such as crosslinking between the chains.

McCafferty: Could the molecule form a helical structure from the four strands, giving a single helical bundle, rather than the four separate helices in each strand?

Stewart: I cannot visualize that structure or see how it would form. We have evidence for dimerization. The dimer seemed to have much less or no catalytic activity. One suggestion is that the hydrophobic sides stick together in some kind of linear array rather than the individual four-helix bundles.

McCafferty: You have a lot of acidic side chains. Is there any possibility that what you are seeing is acid-catalyzed hydrolysis?

Stewart: If there is any sort of nonenzymic hydrolysis, it would appear to be general base-catalysis. We originally thought we had a pH optimum at 8.5. With the activated esters, the rate appears to go up with increasing pH, even correcting for spontaneous hydrolysis. This does not necessarily mean that the system is not acting as we think it should, because the only reason rates fall off at high pH in the case of enzymes

is the destruction of the active site conformation. Until the pH approaches 10 where the lysines would be uncharged, we would not expect to have a similar problem with our peptide.

Tramontano: What are the K_m values that you find?

Stewart: One of the reasons that we have not so far determined accurate K_m values is that we are not confident that our material is pure. The peptide has sticky properties, and we have had a lot of trouble purifying it. Unfortunately, we do not have very good estimates at the moment.

Deyev: Your structure is not very complicated, but it is not very simple. Did you try to improve the activity by denaturation and renaturation?

Stewart: We can indeed denature it with heat. It loses activity and then it becomes active again when you bring the temperature back down. We have also tried to make some structural modifications when we saw that we did not have full helical composition at normal pH in water. We added hydrophobic groups instead of acetyl groups at the N terminus in an effort to increase the stability. Unfortunately, we do not yet have any real data on the modified peptide, but we have many ideas how to increase the stability. Again, the later work showed some problems in this area.

Green: You mentioned that the ethyl ester data were ambiguous. Did you try any other substrates, like phenyl esters or aromatic esters? You also mentioned that there was turnover. What was the actual number of turnovers?

Stewart: We have not tried very many other kinds of esters because of limited time and people. Sorry, I do not have the turnover number in my mind, but quite a number of turnovers were observed.

Svedas: You presented some data showing some substrate specificity. Do you have some more data characterizing specificity and binding of different amino acid derivatives, let us say inhibition of the activity by different compounds?

Stewart: There seemed to be inhibition by indole and anisole. These organic molecules can presumably fit into the substrate binding pocket. Unfortunately, I have not had a chance to do a large range of studies. Bulky, hydrophobic acylated tyrosine derivatives were the preferred substrates.

McCafferty: The data on activity after your material was degraded with trypsin are interesting. It looked like the activity did not go down to zero. There was 10–20% remaining activity, which suggests about 1000-fold increase over background reaction rate. Is there some way to account for that?

Stewart: It is well known that histidine peptides can catalyze nitrophenyl ester hydrolysis. Several people have tried to put together the three amino acids in various arrangements in linear sequences or sequences designed to make turns, and these structures show some catalytic activity. You would expect some catalytic activity to remain even after trypsin digestion just because of the histidine peptides.